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Auxin's origin: do PILS hold the key?

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Abstract:

Auxin is a key regulator of many developmental processes in land plants and plays a strikingly similar role in the phylogenetically distant brown seaweeds. Emerging evidence shows that the PIN and PIN-like (PILS) auxin transporter families have preceded the evolution of the canonical auxin response pathway. A wide conservation of PILS mediated auxin transport, together with reports of auxin function in unicellular algae, would suggest that auxin function preceded the advent of multicellularity. We find that PIN and PILS transporters form two eukaryotic subfamilies within a larger bacterial family. We argue that future functional characterisation of algal PIN and PILS transporters can shed light on a common origin of an auxin function followed by independent co-option in a multicellular context.

Auxin function in land plants and macroalgae

Auxin (see Glossary) was the first phytohormone to be discovered in plants [1] and plays a cardinal role in a plethora of developmental processes, including cell growth, differentiation and tissue patterning. In land plants, the compound acts by moving over both short and long distances and thereby establishes gradients maintaining cell and tissue polarity using a diverse range of auxin transporters (Box 1). When and how auxin evolved as a polarity-inducing factor, is still one of the most prominent enigmas of plant evo-devo [2–13].

Complex multicellular plant systems evolved multiple times in the eukaryotic tree of life (Key Figure 1B). In the green lineage (Viridiplantae), multicellular plant systems evolved from unicellular ancestors both in the chlorophytes as well as in the streptophytes [14]. Similarly, the brown lineage (Phaeophyceae) evolved complex multicellularity with strong convergent features [15-19]. While a role for auxin in development of some of these macroalgal clades (*e.g.* Rhodophyta [20–22], Chlorophyta [23–24]) still needs to be firmly established, there is evidence that auxin plays a role in cell polarisation and differentiation in the brown lineage [25–28]. Like in basal land plants (*Marchantia, Physcomitrella*) [29–31], exogenous application of auxins stimulates rhizoid production in the oogamous embryos of brown seaweeds (*Fucus, Dictyota)* [25,26,28] (Key Figure 1A). A similar stimulating effect of IAA on tip growth has been observed in the brown seaweed *Ectocarpus* [32]. The uncanny resemblance of embryogenesis in brown algae and land plants is also reflected by the auxin function in the apical-basal patterning in both groups [7,26,28,33].

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Auxin function as a homoplasious or a homologous character?

The striking similarity in auxin response between brown algae and bryophytes (Key Figure 1A) raises the question whether auxin as a polarity establishing phytohormone - together with its molecular machinery - may have a common origin (**homology**) or evolved convergently (**homoplasy**)*.* The auxin function in multicellular patterning may have evolved in parallel from an existing and more general growth regulating function already present in unicellular ancestors. It is conceivable that auxin may have acted as an ancient signalling molecule serving as a growth regulating pheromone [2], playing a role in chloroplast development in unicellular ancestors [34] or even in the zygotic developmental mechanism of the **LECA** [7]. Alternatively auxin may have an origin in interspecific [41] and intraspecific interactions [4]. An increasing number of reports from a diverse range of unicellular algae suggests auxin promotes growth rate and oil content, which a.o. has led to the application of auxin in industrial microalgal cultivation [10,35]. Cell division is promoted in unicellular microalgae such as *Pseudo-nitzschia, Chlorella* or *Micrasterias* following addition of **IAA** [36–38]. In the chlorophyte algae *Desmodesmus* and *Scenedesmus*, the addition of auxin induces a phenotypic response from larger colonial morphologies to unicells [39,40]. Next to a role in promoting growth, auxin has also been suggested to regulate signalling between cell types in the haptophyte *Emiliania* [41].

Alternatively, the omnipresence and physicochemical characteristics of IAA could make it destined to serve as a phytohormone among small multicellular aquatic organisms [2]. Auxin may have been convergently recruited for patterning in multicellular organisms, without a pre-existing function depending on cellular perception or intercellular transport [2]. Most organisms – from animals, fungi, bacteria to unicellular algae and plants – are able to produce auxins (reviewed in [42]) as a by-product of tryptophan biosynthesis [3] or protein hydrolysis [43]. IAA is a small organic molecule with a pKa of 4.75 and has the potential to rapidly diffuse in aquatic environments and across membranes. In acidic cell walls, IAA becomes protonated and readily diffuses through cell membranes to the cytosol where it loses its proton and may accumulate. Also, the similarity with tryptophan renders IAA some affinities for existing amino acid transporters [2].

The key to distinguish between the different alternative explanations (convergent evolution or parallel co-optation of an ancestral unicellular auxin function) might lie in the evolutionary conservation of genes involved in auxin transport and signalling pathways of the different clades [2,7]. In the case of a common origin, the molecular machinery for an ancient ancestral auxin function must have been independently co-opted and expanded for patterning in a multicellular context in land plants and brown seaweeds.

We argue that the conservation of auxin transporters (Box 1) rather than signalling pathways can give credit to a common origin of an auxin function followed by independent co-option in a multicellular context. This view is motivated by the recent discovery of PILS homologues outside the green lineage [28] and PINs outside the **streptophyte** lineage [4,45]. These two protein families are of special interest given the conservation of auxins as a substrate among all gene family members in *Arabidopsis*. While the auxin response system is the most popular line of enquiry, it has been demonstrated repeatedly that the canonical nuclear auxin response pathway is not conserved in charophytes [3,6,8,10–12,44] nor in brown algae [27,28]. Auxin function and PIN mediated auxin transport have been demonstrated in *Klebsormidium* (charophytes) [12,13]. Therefore charophytes have most likely developed their own signalling pathway different from the canonical response pathway of land plants [44], obscuring inferences on the origin of auxin function from conservation of the auxin signalling system. This leaves the auxin transport system as the best line of enquiry.

While previous reviews have concentrated mainly on the green lineage [4,46–51], in this opinion, we discuss the evolutionary origin of auxin transporter families using *in silico* analysis of a broad selection of eukaryotes (Figure S2, Table S1). We highlight an early eukaryotic origin of PILS transporters - together with the increasing evidence for an auxin function in growth and chloroplast development in unicellular algae - as putative evidence for an early origin of auxin function. We further argue that the proposed origin from a larger family of bacterial transporters with an attributed specificity for malate/malonate, warrants studies into the substrate specificity and role of these algal PILS transporters that could shed light on possible homology in auxin function in the brown and green lineage.

PINs and PILS: origin and conservation

PIN proteins have only been reported in streptophyte algae such as *Spirogyra, Penium, Chara* and *Klebsormidium* [48,49,52,75], but more recently chlorophyte PINs were identified [4,45]. Therefore, the origin of PINs likely predates the streptophyte-chlorophyte divergence (Key Figure 1, Figure 2). Bacterial transporters with the same characteristic 10 transmembrane helix topology (Box 1) exemplified by MleP*,* MdcF and MJ1031 (Table S2, II Additional sequences) have been identified as homologs of PIN proteins [6,53–56]*,* suggesting PINs may have originated in the green lineage by lateral gene transfer.

The more recently described PILS proteins [57], however, are known to be present in both streptophytes and chlorophytes [47,48]. Recently, putative homologs of the PILS proteins have been discovered in the brown algae *Ectocarpus* and *Dictyota* [28]. The presence of putative homologs of a transporter class, which has been uniquely involved in the transport of auxin, outside the green lineage opens up the question on the evolutionary origin of PILS proteins: (i) whether or not they may have been acquired from *e.g.* bacteria or land plants via **HGT** (Horizontal Gene Transfer) or (ii) whether they may represent evidence of a deep origin of auxin transport machinery (and by the same token auxin function).

We screened recursively (Figure S2) for PILS-like (Figure 2A) and PIN membrane proteins (Figure 2B) and inferred the phylogenetic relationships of the hits and a random subset of bacterial members of the EggNOG5.0 family COG0679 (Table S2) [58] (Figure 2C, Figure S2). Interestingly, almost all eukaryotic PILS are members of this large gene family, together with the above mentioned bacterial PIN homologs (Table S3). The phylogenetic tree including PIN, PILS and bacterial malate/malonate transporters shows that PILS and PIN form two monophyletic clades branching from the bacterial malate/malonate transporters on relatively short branches. Our analysis corroborates that of Feraru et al. [47], who showed the distinct nature of PILS and the PIN protein family, but also suggests a putative common origin from the same bacterial gene family. While PINs have likely been acquired in the green lineage given the predominance of streptophyte sequences (Figure 2B), the PILS family is present in almost all eukaryotic clades (but notably lost in Metazoa (animals)) and therefore likely to be present in the last common ancestor of unikonts and bikonts. In the yeast *Schizosaccharomyces pombe* the gene *Auxin Efflux Like* (AEL1) (SPAC5D6.04) has been described [59,60]. The mutant *ael1* shows a higher retention of [^{3}H]IAA in comparison to the wild type and therefore has a demonstrated auxin transporter activity. While the similarity with PIN proteins has been pointed out, this protein turns out to be a member of a fungal PILS clade (Figure 2A), suggesting a widespread auxin transporter activity.

In contrast to earlier studies [6,48,61], phylogenetic analyses discerned PIN hits in chlorophytes and also the diatom *Thalassiosira* and *Fistulifera* (Figure 2B). The bootstrap support values and short branch lengths suggest diatoms may have acquired the PIN protein via HGT rather than that PINs were acquired before the common ancestor of green algae and diatoms. Based on the length of the hydrophilic loop region, PIN proteins have been subdivided in **PM**-localised canonical PIN proteins (200-300 amino acids), noncanonical **ER**-localised PINs (~50 amino acids), and semicanonical PIN proteins with intermediate loop length and dual ER and PM localization [49,61,62]. While some reports have stated that the "long" canonical PINs evolved from "short" algal PINs like those of *Klebsormidium* [6,48], other reports disagree both on the similarity between algal PINs and the status of noncanonical PINs as "short" [49,61]. Deeper and wider sampling in the land plant clade of PIN proteins showed that the evolution of the PIN protein structure is rather conservative with occasional extreme events of reductive evolution towards non-canonical PIN protein structures [49,61]. In our dataset, the size of the hydrophilic loop region is <200 amino acids for 11 out of the 18 chlorophyte/diatom sequences, which is considered the lower-end cut-off for "long" canonical PINs (Figure 2B; [49]). Although the majority of sequences are categorized as semicanonical or noncanonical, the sequence divergence and especially long loops in *Chara* and strong divergence of some chlorophyceaen putative orthologues make conclusions difficult. This calls for experimental characterisation of intracellular localisation and functional role of these putative PIN proteins. It is conceivable that ER-localised PINs that mediate intracellular auxin transport are more relevant for unicellular algae such as *Chlamydomonas* and *Chlorella*.

Other transporter families

The AUX1/LAX family may have evolved before the evolution of land plants [63], because putative homologs have been identified in *Chlorella* [3]. In agreement with [6], we only find AUX1/LAX sequences in a limited collection of clades in the chlorophytes (Figure 3). Of particular interest are the AUX1/LAX homologs in some of the diatom genomes. At least for the occurrence in diatoms HGT may be the most parsimonious way to explain the lack of AUX1/LAX genes in other heterokont species, especially given that ~1200 genes in diatoms have been attributed to ancient HGT from the green lineage [64]. Therefore, we conclude that the AUX1/LAX family with auxin transport functionality most likely evolved in the green lineage.

The functional diversity within the widely conserved ABC transporter family [6,27] and its size – being one of the largest known protein superfamilies [65] - makes sequence-based function assignment nearly impossible [6]. Moreover, we have only started to identify the auxin transporters among the superfamily [66]. Therefore, the conservation of ABCB auxin transporters beyond land plants has remained a matter of pure speculation. The position of all ABCB transporters of *A. thaliana* with a demonstrated auxin transport functionality in a phylogenetic tree (Figure 3) in the streptophyte clade does suggest that evolution of auxin as a substrate occurred in the land plants.

Two other auxin transporters (NRT1.1 and WAT1) [62] have been described during the last decade. Both transporters emerged from larger streptophyte clades with diverse affinities and no algal or seaweed sequences show a topology that suggests a close phylogenetic relationship with these two auxin transporting members (Figure 3). Therefore we conclude there is no evidence that these distant homologs transport auxins.

Auxin and malate

The hypothesis that a deep origin of PILS genes is a testimony of an early auxin function relies on substrate specificity for auxin. Members of the PIN and PILS gene family in land plants are almost exclusively linked to auxin transport (Box 1, Figure I). Having evolved from a bacterial family with a demonstrated function as malate/malonate transporters (Figure 2C), one may ask when PINs and PILS acquired their auxin transport function. Or, alternatively, to what extent are transporters substrate specific and how evolvable this specificity is. The similarity of malate and auxin at the chemical level is reflected in the competition of both molecules for the same binding site in the voltage-gated anion channel GCAC1 of *Vicia fabia* [67]. Among the ABCB-type auxin transporters, malate and auxin are substrates that are at least evolutionary interchangeable. While AtABCB14 has been shown to be a malate uptake transporter in guard cells [68], the knockouts of the very same gene were reported to be affected in auxin transport in the stem [69]. The difficulty to reconcile two functions to the same transporter (see [69]), has led to the conclusion that altered auxin transport in AtABCB14 knockout is an indirect effect [66]. Nevertheless, *abcb14* mutants show a reduced malate-inhibitable auxin transport in the shoot [70] and its rice ortholog, OsABCB14, was recently shown to exert an auxin transport function [71].

Zazímalová et al. [70] suggested that there could be a mechanism of substrate competition between malate and auxin in AtABCB14. To date, there is no evidence that either AtABCB14 or OsABCB14 are specific for either malate or auxin respectively. Among the PILS transporters, in *Cucumis*, orthologs of PILS6 are involved in fruit acidification and knockouts were correlated with a decrease in malate and citrate accumulation in *Cucumis* fruit, but experiments trying to identify the direct substrate have been inconclusive [72].

Concluding Remarks and Future Perspectives

The origin of auxin's role in growth and development is a long-standing question of great interest to plant evo-devo [2] and aquaculture [10]. Elucidating the potential common molecular machinery has been hampered by the replacement of the poorly characterized ancestral charophyte auxin response [73] with the canonical nuclear auxin response pathway (NAP) in bryophytes [44, 74]. In this opinion we focused on the auxin transporters using a broad taxonomic resolution and found indication for a deep homology of auxin function in the conservation of the PIN-LIKES. Other auxin transporter families appear to be acquisitions of the green lineage (AUX1/LAX, PINs), with a surprising putative HGT event to diatoms. Other streptophyte transporters from expanded families of ABCB, SLC15A (NRT1.1) and vacuolar (WAT1) transporters evolved affinity for auxins in single or multiple clades along with many other transporter functionalities.

Future research (see Outstanding Questions) should aim to determine the role of putative algal auxin transporters (especially PILS), their subcellular localisation and especially their substrate specificity for auxin versus other organic acids, using forward genetics in selected algal model species, land plants [13], and heterologous models like yeasts or *Xenopus* [57,59,60]. Further elaboration of the auxin signalling pathways and transporters in algae and seaweeds will ultimately provide us with more detailed evidence for a putative ancient origin and a better understanding of the evolution of auxin function in algae and seaweeds.

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Supplemental information

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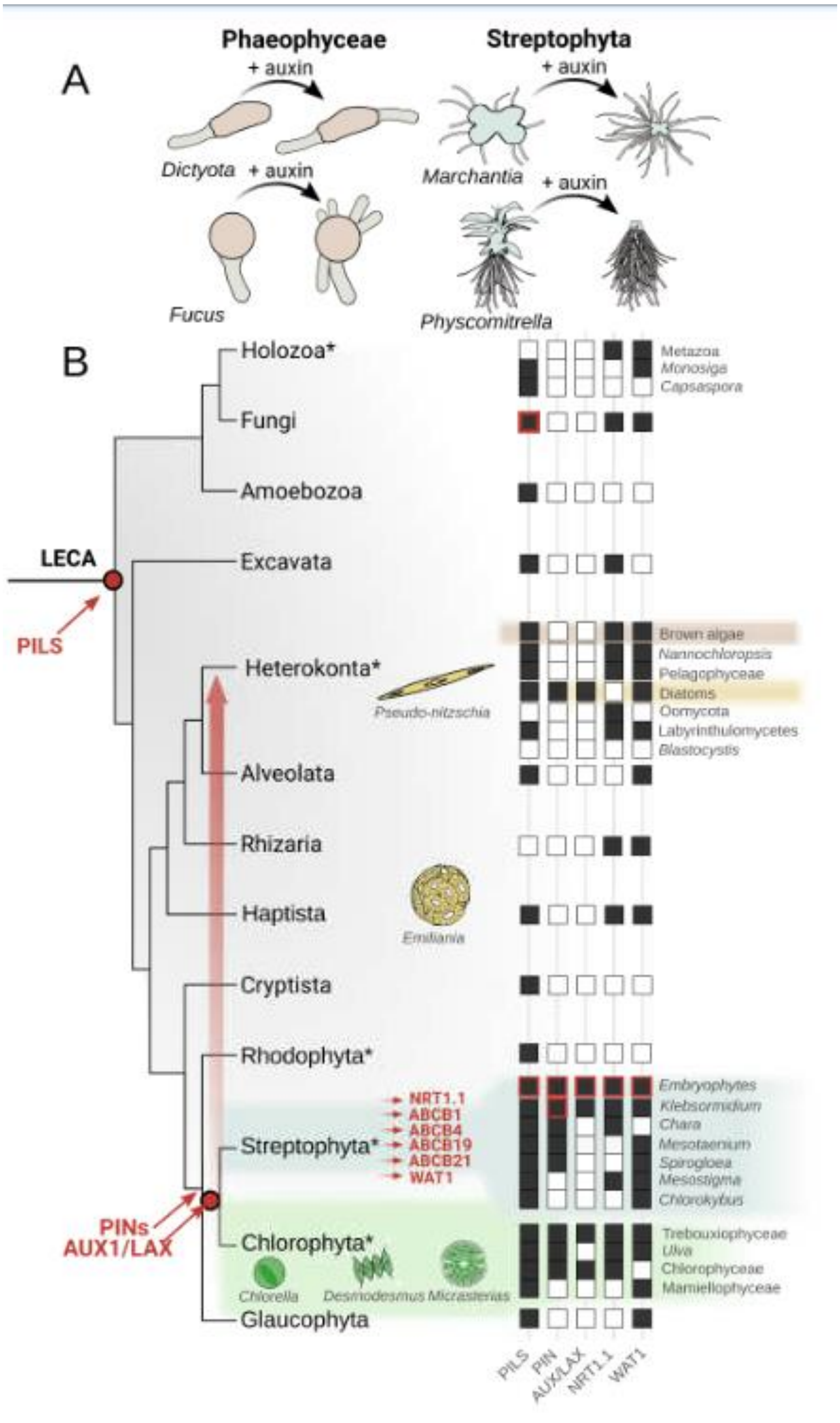

Figure 1. Convergent evolution in rhizoid promoting role of auxin in both the brown and green lineages and presence of auxin transporters. (**A**) Phenotype upon application of exogenous auxin in selected brown algal (*Fucus*, *Dictyota*) and bryophyte model systems (*Marchantia*, *Physcomitrella*). (**B**) Presence of auxin transporters (ABCB1,4,19,21; NRT1; WAT1) or transporter families (PINs; AUX1/LAX; PILS) mapped on a schematic eukaryotic tree of life. Presence of auxin [3, 43] is denoted by an asterisk in the clades. Unicellular examples with a phenotypic response upon exogenous auxin

addition are depicted: *Emiliania* [41], *Pseudo-nitzschia* [38], *Desmodesmus* [39,40], *Chlorella* [37], *Micrasterias* [36]. Based on phylogenetic analysis of putative homologs (See Online Supplemental Information Figure S2): auxin transporter NRT1.1, the ABCB auxin transporters and the WAT1 vacuolar transporter evolved from their respective transporter superfamilies within Streptophytes; auxin transporter families PINs and AUX1/LAX transporters arose in the green lineage; PILS transporters can be found in almost all major branches of the tree of life. Black squares depict the absence or presence of transporter families. Red strokes around the black squares denote these contain homologs with a demonstrated auxin specificity. See also Online Supplemental Information Figure S1 and Table S1. Small red arrows depict the putative origins of the various auxin transporters. The thick red arrow pointing from the streptophytes and chlorophytes to diatoms depicts the postulated HGT of PINs and AUX1/LAX members from green algae to diatoms.

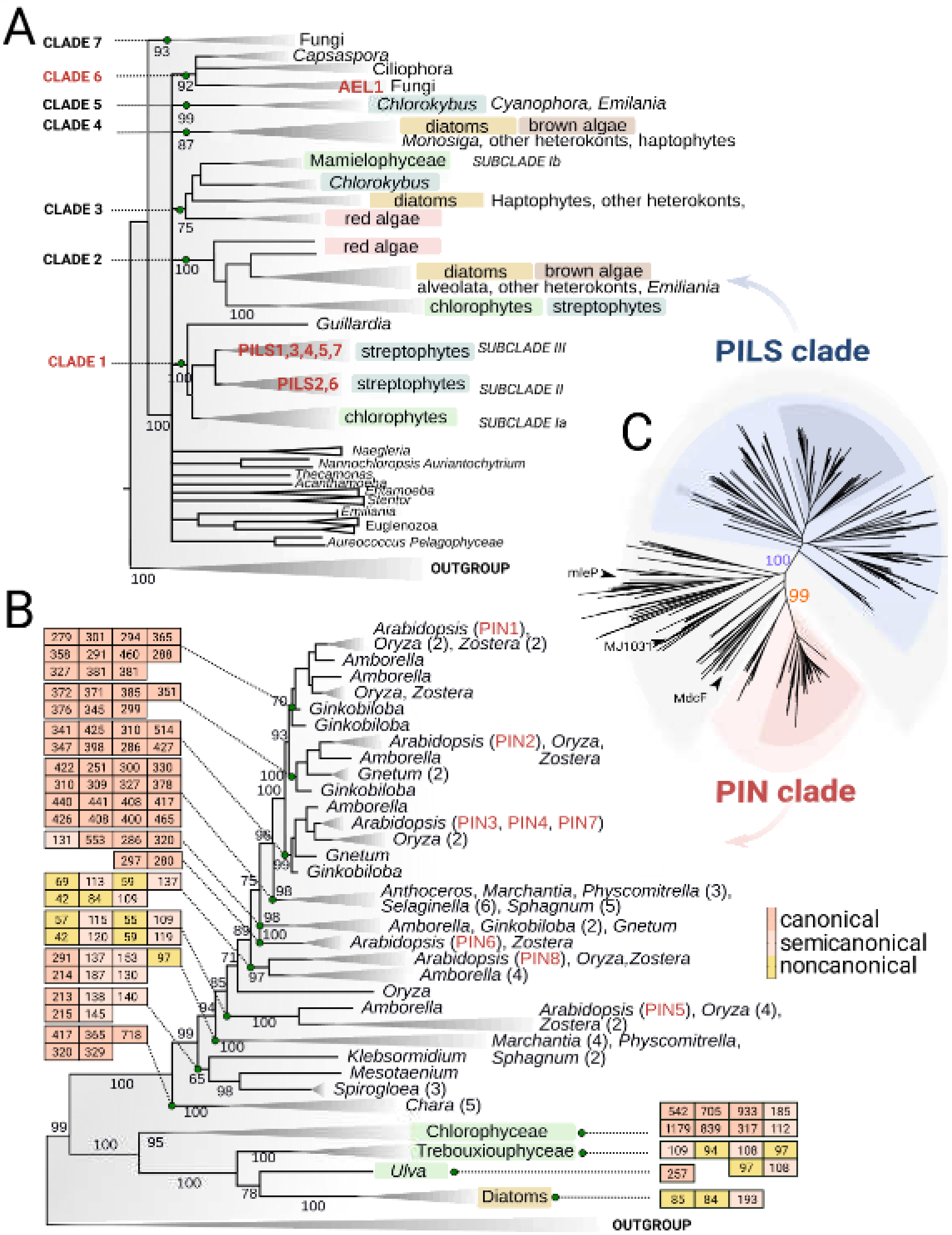

Figure 2. Evolution of PILS and PIN proteins. (**A**) ML-tree of PILS-clade. Red sequences denote family members with a demonstrated role in intracellular auxin accumulation: *Arabidopsis* (PILS1-7) [51, 57, 78, 79] and *Schizosaccharomyces* proteins (AEL1) [59,60]. 7 clades are delineated based on their bootstrap support (numbers below branches). The subclades Ia, Ib, II and III denote PILS clades previously described by Feraru et al. [47]. Colours depict relevant phylogenetic clades. Green, streptophytes; light green, chlorophytes s.s.; yellow-brown, diatoms; red, red algae; brown, brown algae.

Outgroup denotes the collapsed PINs and COG0679 genes. (**B**) ML-tree of PIN protein sequences. Length of the hydrophilic loop (as assessed using TMHMM2.0 software is depicted for each clade. Colored boxes denote the category of PIN genes according to the length of the loop. Loop lengths are given in each box per sequence. Number of boxes per clade and numbers behind species denote the number of sequences in collapsed end nodes. Red sequences denote the position of all *Arabidopsis* PIN auxin transporters in the tree. Outgroup denotes the collapsed PINs and COG0679 genes. (**C**) ML-tree of identified PILS and PIN hits with 100 randomly selected sequences of the COG0679 protein family and reported bacterial putative PIN homologs MdcF, MJ1031 and MleP [6,53–56]. Red, PIN-clade; blue, PILS-clade; grey clade, COG0679 family including the PILS. Currently described streptophyte PINs [61,75] and PILS [47] family members are denoted in respectively darker red or blue shading. (See also Online Supplemental Information Figure S2, Table S2, Table S3, Table S4).

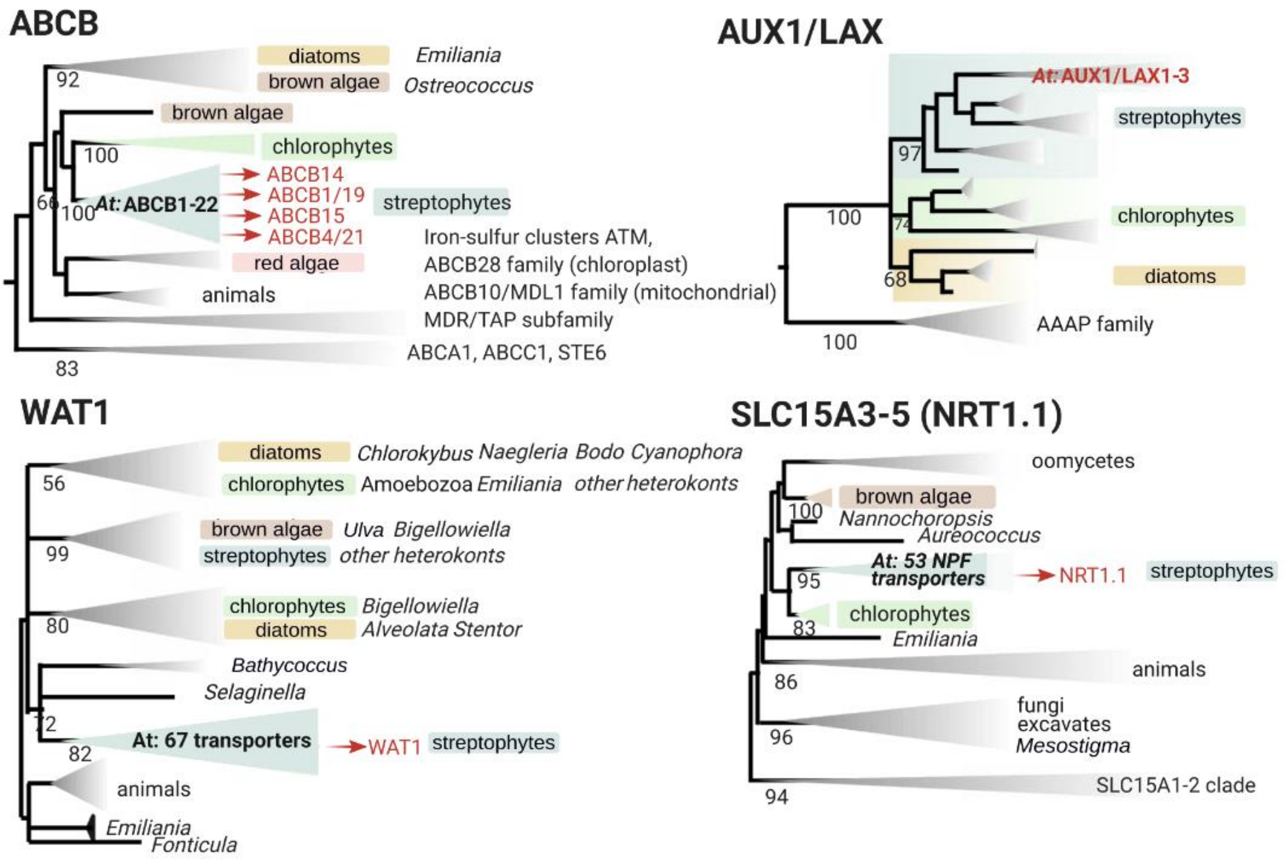

Figure 3. Evolution of AUX1/LAX, ABCB, WAT1 and NRT1.1 auxin transporters. While sequences homologous to land plant ABCB, WAT1, NRT1.1 auxin transporters (red) can also be found in several non-streptophyte clades, an auxin transport function cannot be deduced. Topology and close relationship of diatom, chlorophyte and streptophyte AUX1/LAX1-3 members can be most parsimoniously explained by postulating a HGT event. Collapsed ML trees, numbers denote bootstrap support for selected clades. Red proteins denote the transporters with demonstrated auxin transport activity. ABCB, WAT1 and NRT1.1 mediated auxin transport emerged from a larger family of streptophyte transporters. Colours depict relevant phylogenetic clades. Green, streptophytes; light green, chlorophytes; yellow-brown, diatoms; red, red algae; brown, brown algae. This figure was created using BioRender (https://biorender.com/). (See also Online Supplemental Information Figure S2, Table S4).

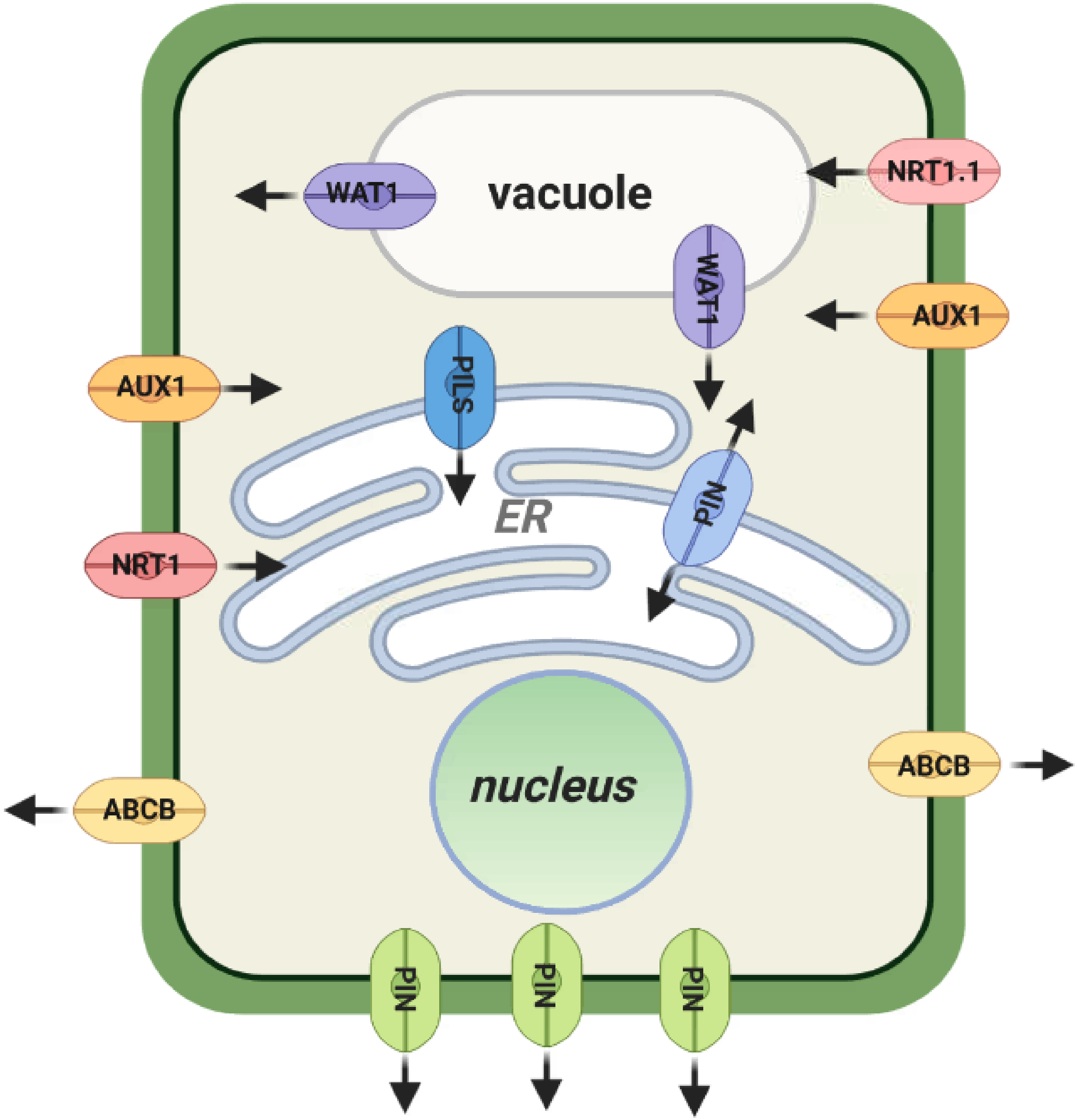

Figure I. Auxin transporters control auxin distribution at the plasma membrane, endoplasmic reticulum and vacuole. Schematic representation of a land plant cell with position of different auxin transporters in the plasma membrane and endomembrane system. Directional transport results from the polar distribution of PIN transporters at the plasma membrane. Arrows depict the directionality of transport. Blue PINs depict short PINs (PIN5, PIN6, PIN8), with PIN8 transporting auxin also into the cytoplasm [76]. Most PINs, PILS, ABCB transport auxin out of the cytoplasm, while other transporters auxin into the cytoplasm. Passive diffusion over the PM is not depicted. ER, endoplasmic reticulum. This figure was created using BioRender (https://biorender.com/).

BOX 1. Protein families linked to auxin transport

The chemiosmotic hypothesis implies the involvement of active transport of auxin molecules. In land plants, six protein families have been attributed an auxin-efflux activity (Figure I). Members of the PIN and PILS gene family in land plants are almost exclusively linked to auxin transport, while the four other transporter gene families have a much broader or evolvable substrate specificity.

PINs

Members of the PIN protein family were the first to be described as auxin transporters. They are classified in two to three classes: the “short”, the “long” PINs and additionally “semicanonical” PINs with an intermediate loop length. The canonical “long” PINs (in *A. thaliana*: PIN1-PIN4 and PIN7) are characterized by a long loop and tend to localise to the plasma membrane where they coordinate a multitude of developmental processes by canalizing polar auxin transport. The “short” noncanonical PINS (in *A. thaliana:* PIN5-6, 8) have a reduced loop and are characterized by an ER localization.

PILS transporters

PILS (PIN-LIKES) transporters have been identified based on their similarity to the PIN proteins relatively recently [57]. All 7 Arabidopsis gene family members have been demonstrated to contribute to intracellular auxin transport and are presumed to transport auxin into the ER and attenuate cellular response to auxins [51,57,77,78]. While PILS proteins appear structurally similar to PIN proteins, they are thought to have evolved independently and have been reported in the entire chlorophyte clade [51, 57] in contrast to the PINs which are thought to be an early streptophyte innovation [13].

AUX1/LAX transporters

AUX1/LAX genes are members of the large amino acid/auxin permease (AAAP) family of proton symport permeases. Together with the three LAX genes, AUX1 forms a subfamily of transporters which have subfunctionalized to transport auxin in different organs and tissues in Arabidopsis.

ABCB transporters

Uptake and efflux of auxin is also mediated by some members of the ATP-binding cassette superfamily B (ABCB). Of the 21 full-size ABCB transporter genes, so far there is solid evidence for the involvement in auxin transport of four (ABCB1, ABCB4, ABCB19 and ABCB21) and some evidence for 2 additional (ABCB14 and ABCB15) [66].

WAT1

The vacuole harbours auxin transporters as well. The tonoplast auxin transporter WALLS ARE THIN 1 (WAT1) has been implicated in auxin transport from the vacuole into the cytoplasm.

NRT1.1

Among SLC15 family members, the NITRATE TRANSPORTER 1.1 (NRT1.1) has been attributed a dual auxin/nitrate transport function. In absence of nitrate, NRT1.1 transports auxin.

Glossary

Auxin: any of a group of compounds with similar growth regulating action described in land plants such as indole-3-acetic acid (IAA), phenylacetic acid (PAA) or indole-3-butyric acid (IBA).

Canonical nuclear auxin response pathway: The well characterized auxin response pathway in which IAA mediated assembly of Transport Inhibitor Response 1 (TIR1)/Auxin F-Box (AFB) proteins with AUX/IAA transcriptional regulators activates gene expression.

Charophyte: a group of freshwater green algae, the paraphyletic restgroup of streptophytes without the land plants.

Chlorophyte: a taxon of green algae (Chlorophyta)

ER: endoplasmic reticulum, cellular organelle.

HGT: Horizontal gene transfer or lateral gene transfer is the evolutionary event in which genetic material is exchanged between multicellular organisms other than from parent to offspring.

Homology: a similar trait of different species as a result of descent from a common evolutionary ancestor

Homoplasy: a similar trait of different species that is absent in their common ancestor

IAA: indole-3-acetic acid, the most well studied auxin.

LECA: last eukaryotic common ancestor.

Malate: ionised form of malic acid ($\mathrm{C4H4O5}^{-2}$), a dicarboxylic acid with molecular formula $\mathrm{C4H6O5}$

Malonate: ionized form ($\mathrm{C3H2O4}^{-2}$) of malonic acid, a dicarboxylic acid with molecular formula

PM: plasma membrane, cellular organelle.

Streptophyte: a taxon of green algae consisting of charophytes and land plants.