Review

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The evolution of land plant cilia

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Summary

Eukaryotic cilia/flagella are ancient organelles with motility and sensory functions. Cilia display significant ultrastructural conservation where present across the eukaryotic phylogeny; however, diversity in ciliary biology exists and the ability to produce cilia has been lost independently on a number of occasions. Land plants provide an excellent system for the investigation of cilia evolution and loss across a broad phylogeny, because early divergent land plant lineages produce cilia, whereas most seed plants do not. This review highlights the differences in cilia form and function across land plants and discusses how recent advances in genomics are providing novel insights into the evolutionary trajectory of ciliary proteins. We propose a renewed effort to adopt ciliated land plants as models to investigate the mechanisms underpinning complex ciliary processes, such as number control, the coordination of basal body placement and the regulation of beat patterns.

I. Introduction

In eukaryotic biology, the terms cilia and flagella both refer to highly organized membrane-bound, microtubule-based organelles that project from cells. Often the terms are used interchangeably, but, in this review, we use the term cilia throughout. Cilia are of interest to cell biologists in terms of their essential roles in diverse cell types, the mechanisms that lead to their formation and the features that distinguish motile from sensory functions. Cilia were first studied in unicellular organisms, such as algae and trypanosomes, or in sperm cells (reviewed in Gibbons, 1981). These systems facilitated ultrastructural characterization, analyses of motility, forward mutagenesis screens for ciliary defects and the identification of genes encoding ciliary proteins. Subsequent studies in mammalian cell lines provided further insight into cilia assembly and function (reviewed in Satir & Christensen, 2007). In the last decade, however, the field has been revolutionized by the advent of proteomics and of bioinformatic approaches to genome interrogation. Not only are comparative analyses now possible between diverse species, but organisms that have previously been experimentally intractable can now be included in analyses that aim to identify novel ciliary components and to characterize the evolutionary trajectory of cilia across the eukaryotes.

In particular, the new technologies are enabling comparative analyses of ciliogenesis in the Viridiplantae (green algae and land plants). Although the green alga *Chlamydomonas reinhardtii* has long been a workhorse in the cilia field (reviewed in Silflow & Lefebvre, 2001), studies of ciliated land plants have previously been limited by the paucity of experimentally tractable systems. It should be noted, however, that the iconic 9 + 2 microtubule arrangement in cilia (see paragraph below) was first observed in plant sperm (Manton & Clarke, 1951). In this review, we provide an overview of cilia form, function and loss in the land plants, and we use insights gained from comparative genomic studies to speculate on how cilia evolved within the Viridiplantae. To underpin this discussion, we first outline our current understanding of cilia diversity in other lineages – in terms of structure, assembly and function.

1. Basic ciliary structure

A canonical cilium consists of a microtubule axoneme that extends from a basal body (Fig. 1) (reviewed in Satir & Christensen, 2007). The basal body comprises two structurally distinct regions that differ in microtubule composition; the proximal end contains nine symmetrically arranged triplets of A, B and C microtubules, whereas the more distal transition zone comprises nine doublet microtubules. This difference arises because only the A and B microtubules extend into the transition zone. The canonical axoneme is a ring of nine outer microtubule doublets that are connected via nexin links, surrounding a central pair of singlet microtubules (known as the '9 + 2' arrangement). Specialized inner- and outer-arm dynein motors are attached to the A microtubule of each doublet, and positioned in such a manner that the motor head domains are in close proximity to the B microtubule of the neighbouring doublet (Gibbons & Gibbons, 1973). Radial spokes attach the central pair microtubules to the surrounding doublets. In most species, bidirectional intraflagellar transport (IFT), which is based on the action of the specialized microtubule motors cytoplasmic dynein-2 and kinesin-2, carries cargo towards the distal tip of the axoneme and back (Kozminski *et al.*, 1993; reviewed in Rosenbaum & Witman, 2002).

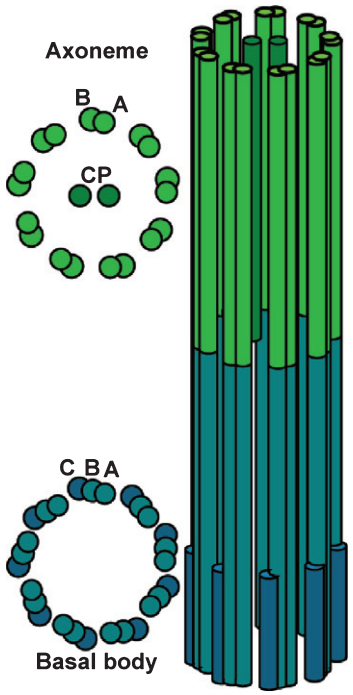

Fig. 1 Microtubule structure of a eukaryotic cilium. Diagrammatic illustration of the canonical microtubule arrangement in the basal body (blue) and axoneme (green) of a eukaryotic cilium. Basal bodies are composed of two structurally distinct regions: a proximal region of nine triplet microtubules (A, B, C) and a transition zone of nine doublet microtubules (A, B). Axonemes are composed of nine doublet microtubules (A, B) and a microtubule central pair (CP).

2. Diversity in cilia assembly

All cilia are formed by microtubule extension from basal bodies, but the process of ciliary assembly is variable across the eukaryotes. In many species, the basal body is formed from a triplet centriole that is embedded in the centrosome. In the slime mould *Physarum flavicomum*, when the amoeboid phase encounters water, the centriole pair moves from its juxtanuclear position, docks at the cell membrane and extends to form one long and one short cilium from the mature centriole and immature pro-centriole, respectively (Aldrich, 1968). When water is removed, the cilia are resorbed. This feature is also seen in other protists, such as amoeboflagellates that can switch between amoeboid and flagellate forms (Balamuth *et al.*, 1983). In these cases, ciliogenesis is thus induced by environmental cues.

In contrast with the inductive formation seen in *Physarum*, cilia are always present in trypanosomes. In these organisms, basal bodies are never found in a centrosomal (centriolar) context, but exist as a pair made up of a mature basal body subtending the axoneme and an associated (immature) pro-basal body. During the cell cycle, the pro-basal body matures to form the new cilium and new pro-basal bodies form next to each of the original basal bodies. In this way, each daughter cell has a basal body with a cilium and a pro-basal body (reviewed in Ginger *et al.*, 2008).

In animal cells, two distinct processes of basal body duplication and ciliogenesis operate depending on the cell type involved and developmental status. In most dividing cells, centrioles are produced in S phase by a process of templated duplication similar to that seen for the basal bodies of trypanosomes (Fig. 2a) (Cavalier-Smith, 1974; Dirksen, 1991; Quarmby & Parker, 2005). This duplication process involves the separation of existing centrioles and the formation of daughter centrioles orthogonally alongside the mature centrioles (reviewed in Cunha-Ferreira *et al.*, 2009). Ciliogenesis is thus linked to the cell cycle – occurring (if at all) by the formation of a transient primary cilium in G1 or a more permanent cilium when the cell exits the cell cycle in G0. Importantly, this assembly process has an inherent number control system because, for each cilium in the mother cell, duplication and subsequent mitosis result in a centriole pair in each daughter cell. Notably, the widespread phylogenetic

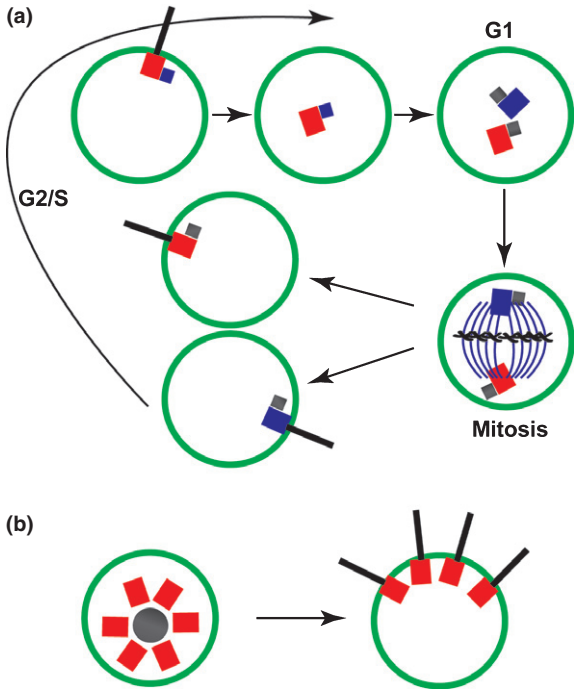

Fig. 2 Basal body duplication pathways in mammalian cells. (a) Template duplication pathway in dividing cells. In G2/S, cilia are disassembled and centriole (red)/pro-centriole (blue) pairs are released from the membrane. During G1, the pro-centriole matures into a centriole and duplication then occurs to form two new pro-centrioles (grey). After mitosis, each daughter cell inherits a centriole/pro-centriole pair that docks on the membrane to form a basal body and to elongate an axoneme. (b) *De novo* duplication pathway in nondividing (G0) cells. The deuterosome (grey) produces multiple centrioles (red) that dock at the membrane to form basal bodies and to elongate an axoneme.

distribution of the templated pathway suggests that this mechanism was present in the last eukaryotic common ancestor (LECA) (Cavalier-Smith, 1974).

An alternative pathway of basal body duplication is found in multiciliated animal cells, such as airway epithelial cells (Fig. 2b). This pathway allows for the rapid *de novo* formation of multiple centrioles around an electron-dense structure, termed the deuterosome (reviewed in Beisson & Wright, 2003; Bettencourt-Dias & Glover, 2007; Nigg & Stearns, 2011). Once formed, centrioles migrate to the cell membrane where they dock and act as basal bodies for axonemal elongation. An interesting feature of metazoa is that the *de novo* formation of centrioles is possible in most differentiated cell types, unless the process is actively suppressed by pre-existing centrioles (reviewed in Nigg & Stearns, 2011). The inhibitory effect of existing centrioles on *de novo* formation is further suggested by the observation that *de novo* assembly can occur in dividing cells if the centriole is removed (Khodjakov *et al.*, 2002).

Although the templated and *de novo* pathways of duplication predominate in the metazoa, variant pathways have been observed in the sperm cells of the termite *Mastotermes darwiniensis* (Riparbelli *et al.*, 2009) and in unfertilized eggs of some parthenogenetic insects (Riparbelli & Callaini, 2003). In these cases, centrioles are formed *de novo* without the need for deuterosomes or a template centriole. Notably, many of the regulatory components involved in the duplication process are conserved across species that use the templated, *de novo* and variant pathways (Azimzadeh & Marshall, 2010) and, as such, mechanistic differences may arise through the fine-tuning of a common set of key proteins.

Following centriole duplication, basal body maturation and axonemal elongation occur to form a cilium (Sorokin, 1962). Within animal epithelial cells, docking of the basal body is associated with the actin–myosin network and is mediated by the planar cell polarity (PCP) pathway. A key PCP protein, Dishevelled, has been shown to localize to the apical surface of multiciliated epithelial cells (Park *et al.*, 2006) and to regulate both the docking and planar polarization of basal bodies in *Xenopus laevis* epidermal cells (Park *et al.*, 2008). In most cases, mature basal bodies dock to the membrane and elongation then occurs through the delivery of ciliary components to the growing tip by a process of IFT (Kozminski *et al.*, 1993; Parker & Katsanis, 2011). This transport is bidirectional: kinesin-2 mediates anterograde movement, whereas cytoplasmic dynein-2 performs retrograde movement (Cole *et al.*, 1998; Pazour *et al.*, 1999). In some cases, however, such as in *Plasmodium yoelii* (Sinden *et al.*, 1976) and in *Drosophila melanogaster* sperm cells (Phillips, 1970), elongation occurs in the cytoplasm in an IFT-independent manner, and membrane docking occurs after axonemal elongation. In *Plasmodium*, cilia always form in the cytoplasm and IFT genes have been lost from the genome (Briggs *et al.*, 2004). By contrast, the *Drosophila* genome possesses the IFT gene cohort, and all cell types apart from sperm cells utilize the IFT elongation mechanism (Han *et al.*, 2003; Sarpal *et al.*, 2003).

3. Diversity of cilia form and function

In animal cells, cilia have distinct nonmotile and motile forms. In general, motile cilia possess axonemal dynein arms that generate sliding force and hence the ciliary beat, and also usually contain a central microtubule pair (reviewed in Satir & Christensen, 2007). In contrast, nonmotile cilia lack both the central apparatus and dynein motors associated with beating and, instead, carry out a sensory role (reviewed in Singla & Reiter, 2006). Because examples of motile cilia are found in extant species of all the major eukaryotic lineages, and overall cilia morphology is highly conserved both within and between lineages, it is widely believed that the LECA also possessed a motile cilium (Cavalier-Smith, 1978; Luck, 1984; reviewed in Satir & Christensen, 2007). Based on comparative analyses of ciliary structure and function across a range of eukaryotes, it is further hypothesized that cilia of the LECA could perform both motility and sensory functions (Cavalier-Smith, 1978; Hodges *et al.*, 2010; Pereira-Leal *et al.*, 2010; Carvalho-Santos *et al.*, 2011; Wickstead & Gull, 2011). Notably, the restricted distribution of immotile cilia within specific phylogenetic clades, such as in the evolutionary distant metazoa and centric diatoms (Jensen *et al.*, 2003), implies that nonmotile cilia evolved from motile ancestors on independent occasions. Despite this apparent parallel evolution, nonmotile cilia display a remarkable conservation of structure, possessing a '9 + v' (variable) microtubule arrangement (reviewed in Gluenz *et al.*, 2010).

In addition to the structural differences that are clearly associated with motile vs nonmotile ciliary function, other structural variations are apparent, even within functionally similar cilia. Such variation can be seen in the nematode *Caenorhabditis elegans*, where immotile sensory cilia lack a canonical triplet basal body structure (Perkins *et al.*, 1986). Other examples include the addition of nine outer dense fibres surrounded by a fibrous sheath in mammalian sperm tails (Fawcett, 1975; Eddy *et al.*, 2003), the para-axonemal rods of *Giardia* (Holberton, 1973) and the elaborate paraflagellar rod structure found in species such as the kinetoplastid protozoa (Bastin *et al.*, 1996).

4. Diversity of ciliomes

Consistent with the observed variations in ciliary form and function, there is considerable variation in the protein composition of cilia (the 'ciliome') in different species. Over the years, the characterization of genes encoding ciliary proteins has been facilitated by a number of experimental approaches, with mutant screens and protein purification being two of the most established (Lewin, 1952, 1953; Brokaw *et al.*, 1982; Brokaw & Luck, 1983). Examples of genes identified in this way include *uniflagellate-1* (Brokaw *et al.*, 1982) and *fla10* (Walther *et al.*, 1994) in *Chlamydomonas* (mutational studies reviewed in Dutcher, 1989), and the ciliary adenosine triphosphatase isolated from *Tetrahymena* (Gibbons, 1963). For many years, cross-species comparisons of genes such as these were limited by the extent to which gene sequences were conserved, because experiments often relied on DNA hybridization assays.

In another form of 'mutant' analysis, the study of ciliopathies (human diseases caused by defects in cilia) has identified ciliary proteins that can have tissue- and even cell type-specific roles (Fliegauf *et al.*, 2007). For example, mutations in genes encoding polycystin 1/2 proteins (PKD1/2) perturb ciliary function in kidneys, leading to polycystic kidney disease, yet cilia in the trachea and in sperm are normal (Yoder *et al.*, 2002). Similarly, Kartagener's syndrome is the result of reduced ciliary beat in the respiratory tract and sperm cells as a result of defects in inner/outer dynein arms (reviewed in Chodhari *et al.*, 2004). Such examples imply either that differences in cilia phenotypes result from variation in the role of ciliary proteins between cell types, or that some cell types may be more resilient to mutations in ciliary components than others.

Although mutant and biochemical analyses of individual proteins have been hugely important to our understanding of the cilium, the recent application of proteomic methods to the study of cilia has allowed catalogs of ciliary proteins to be compared between species and at great evolutionary distance (Keller *et al.*, 2005, 2006; Pazour *et al.*, 2005; Broadhead *et al.*, 2006; Kilburn *et al.*, 2007). Direct comparison between these ciliomes can identify proteins shared between sets, but can also be used to test whether ciliary proteins from one organism are encoded in the genome of another. Both of these types of comparison have revealed that, although the ciliary form is extensively conserved, there is a surprising lack of homology between the components of different cilia. For example, of 331 proteins identified as part of the ciliary matrix of the protist *Trypanosoma brucei* (Broadhead *et al.*, 2006), only 49 orthologues were identified in a similar preparation from the alga *Chlamydomonas reinhardtii* (Pazour *et al.*, 2005).

From the pool of ciliary proteins that are present in more than one species, bioinformatic analyses have facilitated the identification of genes that encode a core cohort that is shared across all extant eukaryotes (e.g. Avidor-Reiss *et al.*, 2004; Broadhead *et al.*, 2006; Merchant *et al.*, 2007). The phylogenetic distribution of genes encoding these core proteins implies that many were present in the LECA (Hodges *et al.*, 2010; Pereira-Leal *et al.*, 2010). These core ciliary proteins include some (such as tubulins) that have important cellular functions outside of their ciliary role, and others that have become highly specialized for their ciliary role (e.g. inner- and outer-arm dyneins, radial spoke proteins) (Piperno *et al.*, 1977; Gibbons, 1995; Kamiya, 1995; reviewed in Satir, 1998 and King, 2000).

Although the core protein cohort reveals conservation in cilia structure and function, and can be used to infer some of the likely properties of the LECA, differences between ciliomes are a reflection of evolutionary divergence. Ciliome divergence can be assessed in two complementary ways. In the first, genomes from a wide range of species can be scanned for the presence or absence of genes encoding proteins known to have a ciliary role in at least one of those species. For example, it has been shown that genes encoding the basal body proteins VFL1, SAS-4 and SAS-6, and also each of the axonemal dynein classes, are not ubiquitous to all ciliated species (Silflow *et al.*, 2001; Pfannenschmid *et al.*, 2003; Delattre *et al.*, 2006; Nakazawa *et al.*, 2007; Wickstead & Gull, 2007; Dammermann *et al.*, 2008; Hodges *et al.*, 2010). Furthermore, the absence of the triplet basal body structure in cilia of *Caenorhabditis elegans* has been shown to be associated with the loss of a cohort of genes encoding typical ciliary proteins (Perkins *et al.*, 1986; Hodges *et al.*, 2010; Pereira-Leal *et al.*, 2010). In the second approach, novel ciliary components can be identified through trait map comparisons of genomes of ciliated vs nonciliated organisms (Hodges *et al.*, 2010, 2011). In this way, 213 orthologues have been identified that each possess a phylogenetic distribution suggestive of conserved ciliary function. Many of these genes have not previously been associated with ciliogenesis.

5. Ciliary loss

Loss of cilia has occurred on multiple independent occasions during the evolution of eukaryotes, perhaps most notably in fungi and land plants (reviewed in Renzaglia & Garbary, 2001; Liu *et al.*, 2006). In the fungi, loss most probably occurred just once prior to the divergence of the ascomycetes, basidiomycetes and zygomycetes (Liu *et al.*, 2006). Given that cilia evolution is marked by both conservation and diversification, and that whole-genome analysis can identify associated changes in genome composition, it is now possible to associate changes in genome composition with the loss of the ability to form cilia.

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II. Plant cilia: a model for evolution and development

1. The ancestral state

The green algae and land plants together form the Viridiplantae, a group that is subdivided into the chlorophyte algae and the streptophytes (which include the charophyte algae and the land plants) (Fig. 3). The similarity of ciliary form in charophyte algae and the ciliated land plants contributed to the conclusion that land plants evolved from green algal ancestors (Graham, 1993).

Key ciliary traits that are likely to have been present in the most recent common ancestor of Viridiplantae can be inferred from the analysis of morphological structures present in evolutionary distant extant algal species, such as *Chlamydomonas* (Lang, 1963; Silflow & Lefebvre, 2001; O'Toole *et al.*, 2003; Geimer & Melkonian, 2004; Mitchell & Nakatsugawa, 2004), *Mesostigma* (Manton, 1965; Melkonian, 1989) and *Trentophilia* (Graham & McBride, 1975). Such comparisons suggest that the cenancestor of these organisms was most likely biciliated, with cilia possessing basal bodies of nine triplet microtubules that duplicated through the canonical, cell cycle-regulated template pathway. The basal body is likely to have been assembled through the formation of a cartwheel structure at the very proximal base, and axonemes most probably possessed both inner- and outer-arm dyneins plus ninefold doublet microtubule symmetry and a central pair. Alongside these canonical eukaryotic ciliary features, the cenancestor is also likely to have formed a star-shaped filamentous structure in the transition zone. This so-called stellate array, which is found in many species of green algae and ciliated land plants, is unique to the Viridiplantae. A perspective on how cilia evolved from this baseline cenancestor state has been gained from ultrastructural studies of a range of algal and land plant sperm cells (Table 1 and references therein).

Some species of chlorophyte algae can form cilia throughout their life cycle, but, in the streptophytes, cilia are only formed during spermatogenesis (Graham, 1993). Within the land plants, cilia are produced in the sperm cells of all nonseed plants (bryophytes, lycophytes and monilophytes), but the vast majority of seed plants are nonciliated. The most parsimonious explanation for this phylogenetic distribution is that ciliary loss occurred twice; once in gymnosperms following the split of conifers and Gnetales from cycads and *Ginkgo*, and once near the base of the angiosperms (Fig. 3) (Bremer *et al.*, 1987; Finet *et al.*, 2010). A third independent loss is seen in the sister lineage of land plants. Although the morphologically advanced Charales has traditionally been considered to be the charophyte sister group to land plants (reviewed in Lewis & McCourt, 2004), two recent genome-based studies have instead suggested that the Zygnematales is more closely related to land plants (Wodniok *et al.*, 2011; Timme *et al.*, 2012). Unlike all other charophyte orders, members of the Zygnematales do not form cilia (Fig. 3). All three losses are presumably reflected in changes in genome composition, and similar gene loss may have occurred in both the Zygnematales and seed plants.

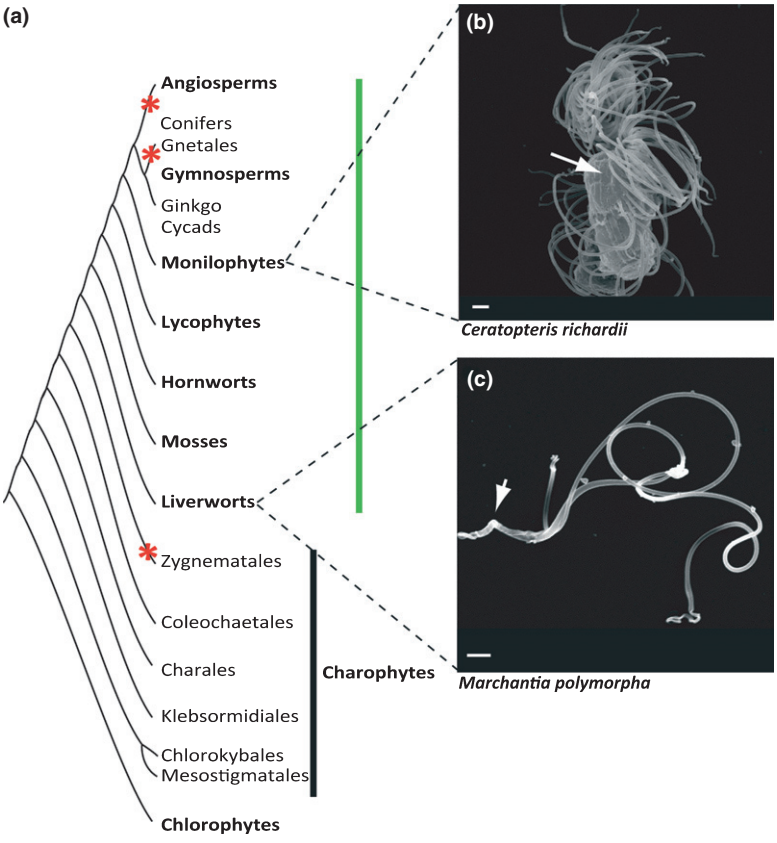

Fig. 3 Land plant phylogeny and the loss of cilia. (a) Cladogram showing the relationship between Chlorophyte, Charophyte (vertical black bar) and land plant (vertical green bar) groups. In both the Charophytes and land plants, where produced, cilia are restricted to sperm cells. The most parsimonious explanation for the distribution of cilia in land plants is two loss events (red stars): once at the base of the Angiosperms and once in the Gymnosperms, where Ginkgo and the cycads diverge from the nonciliated conifers and Gnetales. An independent loss occurred in the land plant sister lineage of Zygnematales. (b) Scanning electron micrograph of a sperm cell from the fern *Ceratopteris richardii*. (c) Scanning electron micrograph of a sperm cell from the liverwort *Marchantia polymorpha*. Arrows show sperm cell body. Bars, 1 μm.

Table 1 Key features of cilia structure in green algae and ciliated land plants

	Species	Formed *de novo*	Basal body number	Approx. basal body length (nm)	Proximal end	Structures around basal body	Stellate pattern	Cilia number	ODA	Tip	References
Chlorophytes	*Chlamydomonas*	No	2	300	O	E	D	2	Yes	9 + 2	Lang (1963), Geimer & Melkonian (2004), Silflow *et al.* (2001) and O'Toole *et al.* (2003)
	Mesostigma	No	2	600	O	E	D	2	Yes	9 + 2	Manton *et al.* (1965) and Melkonian (1989)
	Trentepohlia	No	2	200	O	E	D	2	Yes	–	Graham & McBride (1975)
Charophytes	*Coleochaete*	Yes	2	–	O	E	D	2	No	9 + 2	Graham (1978) and Graham & McBride (1975)
	Chara	Yes	2	250	O	E	T	2	No	9 + 2	Turner (1968) and Jin & Hasenstein (2009)
Bryophytes	*Marchantia*	Yes	2	250	D with PE	A	D	2	No	–	Carothers & Kreitner (1968) and Carothers *et al.* (1975)
	Aulacomnium	Yes	2	240	D with PE	A	D	2	No	–	Bernhard & Renzaglia (1995)
	Polytrichum	Yes	2	200	D with PE	A	D	2	No	–	Paolillo *et al.* (1968) and Miller *et al.* (1983)
	Sphagnum	Yes	2	240	D with PE	A	D	2	No	–	Manton (1957)
	Phaeoceros	Yes	2	170 – STZ, NS	D with PE	A	No	2	No	–	Carothers *et al.* (1977) and Renzaglia & Duckett (1988)
	Notothylas	Yes	2	190 – STZ, NS	D with PE	A	No	2	No	–	Carothers *et al.* (1977) and Renzaglia & Duckett (1987, 1988)
Lycophytes	*Lycopodium*	Yes	2	400	D	E	T	2	No	Tapers	Carothers *et al.* (1975), Hyams & Campbell (1985) and Maden *et al.* (1997)
	Selaginella	Yes	2	500	D	E	T	2	No	Tapers	Carothers *et al.* (1975)
	Isoetes	Yes	11	500	D	E	T	11	No	Tapers	Carothers *et al.* (1975)
Monilophytes	*Equisetum*	Yes	80–120	300	D	E	T	80–120	No	–	Duckett (1973) and Duckett & Bell (1977)
	Psilotum	Yes	36–40	350	D	E	T	36–40	No	–	Renzaglia *et al.* (2001)
	Ceratopteris	Yes	36–100	350–450	D	E+	C	36–100	No	Disorganized	Bilderback *et al.* (1974), Duckett *et al.* (1979), Hoffman *et al.* (1994) and Renzaglia *et al.* (2004)
	Marsilea	Yes	50–100	–	D	E+	T	50–100	No	Tapers	Bilderback *et al.* (1974), Hepler & Myles (1977) and Hyams & Campbell (1985)
	Lygodium	Yes	30–40	–	D	E+	T	30–40	No	Tapers	Bilderback *et al.* (1974)
Gymnosperms	*Ginkgo*	Yes	> 1000	850	D	E+	T	> 1000	No	Tapers	Li *et al.* (1989) and Vaughn & Renzaglia (2006)
	Zamia	Yes	> 10 000	–	D	E+	T	> 10 000	No	–	Norstog (1974)

All cilia examined possess a radially symmetrical nine triplet basal body and a 9 + 2 axonemal structure. The proximal end of the basal body is either organized (O) or lacking symmetry (D), and can be formed through proximal extension (PE). Associated with the proximal base, there are fibres (F), patches of amorphous electron-dense material (A) or an extensive electron-dense structure (E). The stellate pattern is restricted to the doublets of the transition zone (D), split between the doublet and triplet regions (T) or spans the doublet and triplet regions (C). ODA, outer dynein arms. Uncertainty or lack of information is represented by '–'.

2. Increase in cilia number

Sperm cells in the different land plant groups vary in the number of cilia per cell (reviewed in Renzaglia & Garbary, 2001). The number increases from two in bryophytes, such as liverworts (Fig. 3c), mosses and hornworts, to 40–80 in monilophytes, such as ferns (Fig. 3b), and on to thousands in the ciliated gymnosperms. In other gymnosperms and in angiosperms, sperm cells have no cilia. From the biciliated ancestral state, multiciliated sperm evolved at least three times: twice in the lycophytes and once in the common ancestor of monilophytes and seed plants (reviewed in Renzaglia & Garbary, 2001). The increase in cilia number correlates with an increase in both sperm cell body size and nuclear DNA content (in part caused by polyploidy) (Renzaglia & Duckett, 1991; Renzaglia *et al.*, 1995; Renzaglia & Garbary, 2001). Although there is no obvious evolutionary driver for this dramatic increase, it may have provided a way of enabling sperm cell motility in the reproductive context of seed plants. In contrast with all other land plants, motile gametes of seed plants are produced in male gametophytes that are 'trapped' in the developing ovule, and are subsequently released into a viscous matrix. Ciliary beating is thus required for movement of a short distance through a resistant medium. All other motile land plant sperm cells are released into the environment and may have to travel long distances. The evaluation of evolutionary trends across swimming plant sperm must clearly take into consideration these two fundamentally different environments encountered by sperm cells. In addition, it should be noted that cilia may play a role in fertilization, not just movement, a fact that could also account for variation in ciliary number. Interestingly, the giant sperm cells of *Ginkgo* and cycads show parallels with the evolution of ciliary structure in dipteran lineages, where some fly species produce giant, relatively immotile, sperm (Phillips, 1970; Bjork & Pitnick, 2006; Lopez-Fernandez *et al.*, 2007).

3. *De novo* basal body assembly

Centrioles are standard components of the cellular apparatus in most chlorophyte algae, and centriole duplication occurs in dividing cells through the canonical template pathway (Cavalier-Smith, 1974). Within ciliated streptophytes, however, basal bodies are produced *de novo* immediately preceding sperm cell formation. Basal body assembly occurs from bicentrioles in biciliated sperm or blepharoplasts (electron-dense microtubule-organizing centres) in multiciliated sperm, in a manner which is at least superficially similar to that seen in animal cells undergoing the transition to multiciliated forms (Graham, 1982; Graham & Wedemayer, 1984; Vaughn & Harper, 1998). The bicentriole is composed of two centrioles that are positioned end-to-end and are connected by a fibrous central core (Carothers *et al.*, 1977). In most cases, a pair of bicentrioles forms *de novo* within the sperm mother cell and one of the pair is distributed to each of the daughter sperm cells after mitosis (Robbins & Carothers, 1978). In hornworts, this process is modified slightly in that the bicentriole pair is formed in the cell generation preceding the sperm mother cell, but the end result in the sperm cells is the same (reviewed in Renzaglia & Garbary, 2001). These double centriolar units function in the biciliated sperm cells of both bryophytes and lycophytes, but bicentriole length is greater in lycophytes (Robbins & Carothers, 1978; Maden *et al.*, 1997).

In monilophytes, instead of bicentrioles, a pair of spherical blepharoplasts forms *de novo* in either the sperm mother cell or the preceding cell generation. An independently derived branched blepharoplast is produced in the multiciliated sperm of the lycophyte *Phylloglossum* (reviewed in Renzaglia & Garbary, 2001). Fern blepharoplasts contain a cylindrical structure embedded within an electron-dense matrix and, after the final cell division (i.e. in the daughter sperm cells), microtubule polymerization around the periphery of this matrix results in the formation of multiple centrioles (Hepler & Myles, 1977; Vaughn & Harper, 1998). By contrast, blepharoplasts of *Gingko* and cycads are large spherical organelles (> 8 μm in diameter) that arise in pairs in the sperm mother cell. Centrioles are tightly packed around the periphery of an electron-dense core, with the number of centrioles (and hence blepharoplast diameter) corresponding to the number of cilia present on the sperm cell (Norstog & Nicholls, 1997; Vaughn & Harper, 1998).

Like the deuterosomes of animals, the bicentriole and blepharoplast are evolutionary innovations that allow the *de novo* formation of centrioles. It is clear that the deuterosome of animals had an independent origin to that of bicentrioles and blepharoplasts, but the relationship between bicentrioles and blepharoplasts themselves is not obvious. The bicentriole arose within the land plants, and it is hypothesized that the blepharoplast is derived from that structure (reviewed in Renzaglia & Garbary, 2001). However, structural and functional differences between monilophyte and gymnosperm blepharoplasts indicate further modifications after the divergence of the two lineages. Immunolocalization studies have elucidated components of the different organizing centres and have shown, for example, that the monilophyte blepharoplast lacks tubulin (Pennell *et al.*, 1986; Hoffman & Vaughn, 1995a,b). However, a more comprehensive genomic analysis is needed to enable more general comparisons to be made.

4. Basal body positioning

Once centrioles are formed from either the bicentriole or the blepharoplast, they dock in the sperm cell membrane to form basal bodies, and axonemal extension then occurs. The Dishevelled–PCP pathway that mediates basal body docking in animals is absent in plants. Instead, docking is associated with a multilayered structure (MLS) that comprises a 'spline' subtended by a lamellar strip (Paolillo, 1974; Kreitner, 1977; Miller *et al.*, 1983). The lamellar strip is a highly specialized microtubule-organizing centre from which the spline is formed. As sperm cells mature, the lamellar strip extends leading to an increase in length (bryophytes) and/or number (tracheophytes) of spline microtubules. The mature spline thus comprises a band of regularly spaced microtubules that number around 10–25 in most bryophytes, typically 150–200 in monilophytes and

thousands in gymnosperms, Basal bodies become associated with the extending lamellar strip (Carothers *et al.*, 1977), such that the extent of elongation correlates with basal body number (Norstog, 1974; Li *et al.*, 1989; Renzaglia & Maden, 2000).

The MLS is present in all ciliated sperm of land plants and charophyte algae, but the angle of orientation of the lamellar strip relative to the spline varies (Pickett-Heaps, 1968; Paolillo, 1974; Maden *et al.*, 1997; Renzaglia & Maden, 2000). The angle ranges from 90° in charophytes (Graham, 1993), to 45° in bryophytes, to 28–45° in monilophytes (Renzaglia *et al.*, 2001, 2002) and 16° in gymnosperms (Norstog, 1974). This observed variation reflects the placement of few or many cilia around the sperm cell, with acute angles associated with tighter packing.

In addition to the MLS, basal bodies are often associated with other structures in the sperm cell membrane. In chlorophyte algae, such as *Chlamydomonas*, the proximal base of the basal body is typically associated with rootlets and fibres (Sleigh, 1979). These structures, which amongst other roles provide stability (Yang *et al.*, 2005), are also present in cilia of the charophyte algae *Chara* and *Coleochete* (Turner, 1968; Graham & McBride, 1979). In some algae, however, the basal apparatus is instead associated with a region of amorphous electron-dense material (Carothers & Kreitner, 1968). Although the protein composition and function of such amorphous regions are unknown, studies in the chlorophyte alga *Chlorogonium elongatum* appear to exclude a role in waveform changes or beat patterns (Hoops & Witman, 1985). Similar amorphous regions are associated with cilia in land plant species and, interestingly, the number and area of amorphous regions increases as the number of cilia per sperm cell increases. At the extreme, the cilia-packed membranes of gymnosperm sperm cells (thousands per cell) are associated with dense staining of the whole sperm cell body (Norstog, 1974; Vaughn & Renzaglia, 2006). Given the correlation between cilia number per cell and the size of the amorphous region, it is reasonable to suggest that the uncharacterized amorphous material is somehow required for fixing, positioning and/or coordination of basal bodies within the membrane.

Once docked, in most ciliated eukaryotes, the proximal end of the basal body is defined by triplet microtubules (Cavalier-Smith, 1974). In ciliated land plants, however, the proximal end of the basal body is typified by a loss in microtubule symmetry, such that transverse sections show doublet and even singlet microtubules (Fig. 4) (Manton, 1965; Carothers & Kreitner, 1968; Bilderback *et al.*, 1974; Norstog, 1974; Graham, 1978; Carothers & Duckett, 1980; Renzaglia *et al.*, 1999; Vaughn & Renzaglia, 2006). Although the evolutionary significance of this loss of symmetry is unclear, in bryophytes, it results from the growth of microtubules, a clear example of bidirectional microtubule assembly (Duckett & Carothers, 1982).

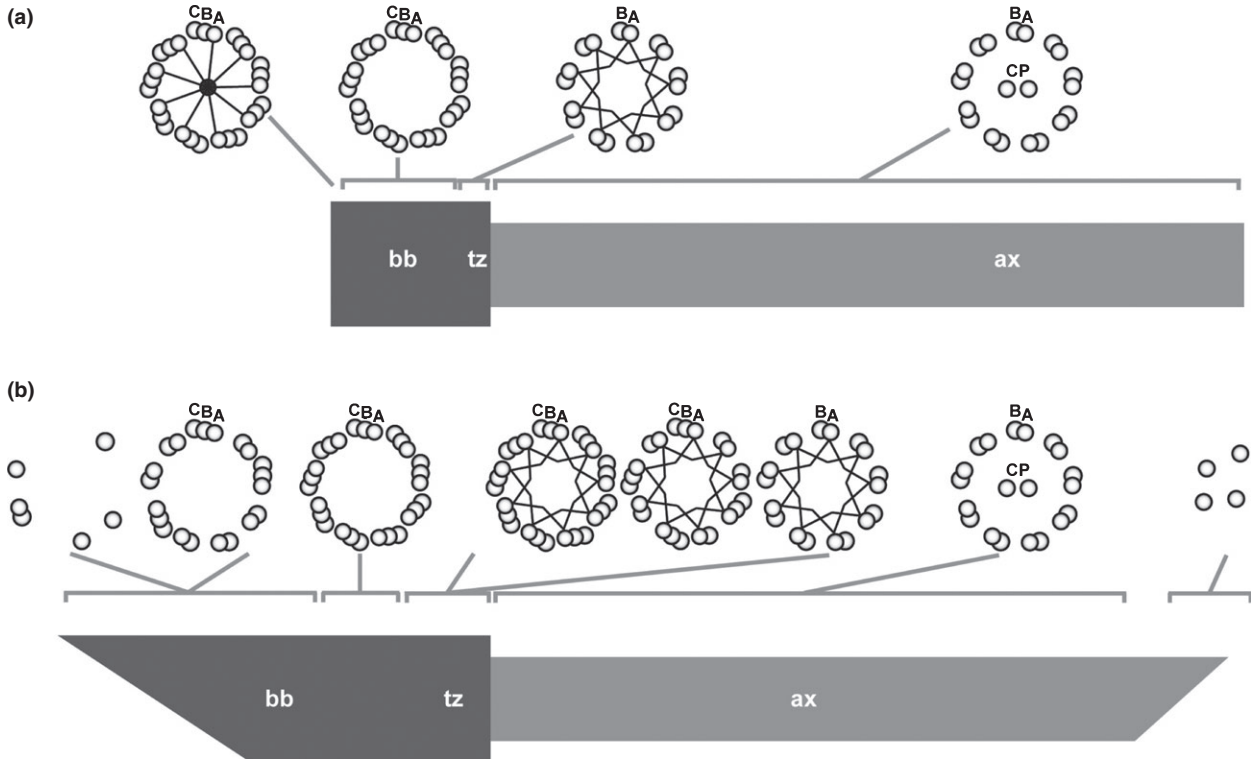

Fig. 4 Schematic representation of cilia structure in green algae and land plants (a) Cilia structure in the green alga Chlamydomonas. The basal body has a proximal triplet region with a cartwheel, and a distal doublet transition zone with a stellate array. The axoneme has a canonical 9 + 2 arrangement throughout. (b) Land plant cilium. Microtubules are disorganized and extended at the proximal end of the basal body, and an elongated stellate array spans from the triplet microtubule region to the doublet region of the transition zone. Microtubule arrangements are also disorganized at the tip of the axoneme. A, B and C microtubules, the central pair (CP), basal body (bb), transition zone (tz) and axoneme (ax) are shown in each case.

5. Elongation of the basal body

Basal body length varies between different species within eukaryotes, although length is generally constrained between 200 and 400 nm (Satir & Christensen, 2007). An examination of basal body length within ciliated land plants, however, reveals an increase in length across the phylogeny, again associated with increases in overall cilia number on each sperm cell (Renzaglia & Garbary, 2001). Mature basal bodies of the early divergent bryophytes (liverworts, mosses and hornworts) have an overall length of between 200 and 250 nm (excluding proximal extensions) (Carothers & Kreitner, 1968; Paolillo *et al.*, 1968; Paolillo, 1981; Renzaglia & Duckett, 1991; Bernhard & Renzaglia, 1995), similar to the lengths seen in charophyte algae (Manton, 1965; Melkonian, 1989; Geimer & Melkonian, 2004). Within lycophyte and monilophyte sperm cells, basal body length is extended to between 350 and 550 nm (Bilderback *et al.*, 1974; Carothers *et al.*, 1975; Duckett *et al.*, 1979; Hoffman *et al.*, 1994; Maden *et al.*, 1997; Renzaglia *et al.*, 2001, 2002, 2004) and, in the gymnosperms, even longer basal bodies, of between 800 and 900 nm in length, are formed (Norstog, 1974; Vaughn & Renzaglia, 2006). Although it is clear that much of this increase in length results from elongation of the transition zone, further analysis is required to fully understand changes in the triplet microtubule region of the basal body.

Within the transition zone of the basal body, the stellate array of filaments connects the A microtubule of the doublets to form a nine-pointed star structure in transverse section (Lang, 1963). This highly symmetrical array could be formed in a number of ways. For example, simple connections between every other A microtubule would produce a stellate pattern in transverse slices. Such connections could be mediated by a relatively small number of proteins. Alternatively, interactions could occur throughout the planar arrangement of the stellate array, potentially involving numerous proteins. Only a high-resolution electron tomographic study of this region will distinguish between these two scenarios. The exact composition of the stellate array is not known, but ultrastructural immunolocalization studies in *Chlamydomonas* (Geimer & Melkonian, 2005), *Chara* (Vouilloud *et al.*, 2005; Jin & Hasenstein, 2009) and *Ginkgo* (Vaughn & Renzaglia, 2006) have shown that centrin is localized to the same area. The occurrence of centrin is widespread in sperm cells and has been associated with fibres attached to basal bodies of charophycean algae, and the amorphous zone of monilophytes (Vaughn *et al.*, 1993). In green algae, centrin has many roles, including a role in the stellate pattern, where it is involved in microtubule severing during flagellar excision (Sanders & Salisbury, 1994). It is not clear, however, whether centrin is the principal component of the array, or what other components are also present. It has been hypothesized that the stellate array plays a role in the coordination of the cilia beat (Lang, 1963), but further study is required to determine the exact role played by this intriguing structure.

In the early divergent land plants, such as the liverwort *Marchantia* (Carothers & Kreitner, 1968) and the mosses *Aulacomnium* (Bernhard & Renzaglia, 1995) and *Polytrichum* (Paolillo *et al.*, 1968), the stellate array spans a longer domain than seen in charophyte algae, but is still only present in the doublet microtubule region of the transition zone. In contrast, the even longer stellate array of ciliated vascular plants, typified by the lycophytes *Lycopodium*, *Selaginella* and *Isoetes* (Carothers *et al.*, 1975), the monilophytes *Ceratopteris*, *Marsilea* and *Lygodium* (Bilderback *et al.*, 1974; Duckett *et al.*, 1979; Hoffman *et al.*, 1994; Renzaglia *et al.*, 2002, 2004) and the gymnosperms *Ginkgo* and *Zamia* (Norstog, 1974; Vaughn & Renzaglia, 2006), spans both the doublet and triplet regions of the transition zone. The increase in stellate domain length is again correlated with increased numbers of cilia present on the sperm cells. Stellate pattern length is a result of the stage in which stellate growth occurs, and the distance to the plasma membrane. Basal bodies in bryophytes are repositioned by proximal microtubule growth and are adjacent to the plasma membrane when the stellate pattern and axoneme develop. As such the stellate pattern develops only in the doublet microtubule region of the transition zone. By contrast, the stellate pattern of monilophytes elongates during basal body positioning, such that it is positioned from the triplet to the doublet microtubules of the transition zone. In seed plants, the larger cell size and distance from the MLS to plasma membrane result in extensive stellate pattern growth throughout the transition zone. Interestingly, the stellate array is absent from the cilia of all examined hornworts – an example of independent loss of this structure within the land plants (Carothers & Duckett, 1980). Comparative studies between ciliary protein preparations from mosses, liverworts and hornworts may thus elucidate potential candidates for stellate array components.

6. Axonemal tips

The distal tips of cilia in *Chlamydomonas* and many other eukaryotic species exhibit a discrete end point that is coincident with the termination of the central pair microtubules and is associated with a cap structure positioned at the axoneme tip (Dentler, 1980a). As a result, the axonemal 9 + 2 organization of microtubules is maintained from the appearance of the central pair (i.e. just distal to the transition zone) through to the tip of the cilium (Dentler, 1980a,b). These axonemal capping structures have been hypothesized to stabilize microtubule disassembly and growth (Rosenbaum & Child, 1967), a suggestion supported by the lack of caps in mammalian sperm cells (Woolley & Nickels, 1985). Given that these sperm cells only have to be motile for a short time, there would presumably be limited need for a mechanism that stabilizes the axoneme tip.

Axonemal caps are also absent from land plant cilia, and transverse sections of the tip region show that the doublet microtubules terminate at different positions (Fig. 4). For example, in the fern *Ceratopteris*, the central pair microtubules stop proximally to the outer doublets (Duckett *et al.*, 1979). The outer doublets terminate in a seemingly random order, but the B microtubule of the doublet ends proximally to the A microtubule. The result is a breakdown of 9 + 2 symmetry at the end of the axoneme (Renzaglia & Garbary, 2001) – an example of evolutionary convergence of a feature seen also in mammalian sperm cells (Woolley & Nickels, 1985).

7. Rotation of the central pair

Rotation of the central pair microtubules in relation to the nine outer doublets of the axoneme during the beating of cilia was first reported in the protist *Paramecium*, and has since been shown in a number of algal species, including *Chlamydomonas* (Omoto & Kung, 1980; Omoto & Witman, 1980, 1981; Omoto *et al.*, 1999; Mitchell, 2003; Mitchell & Nakatsugawa, 2004). Central pair rotation has not been reported previously in ciliated land plants, but serial sections of cilia in fixed sperm cells of the fern *Ceratopteris* show that the central pair is twisted in relation to the outer doublet microtubules (M. E. Hodges *et al.*, unpublished). Although it is possible that the central pair is fixed, but twisted in this case, the most probable interpretation is that the central pair of these axonemes rotates in a manner similar to that seen in algae. Given that central pair rotation has been proposed to help facilitate complex ciliary waveform changes, such as changes in beat direction (Mitchell, 2005), this would imply that land plant sperm cells are able to undergo changes in beat type or direction.

8. Ciliome changes during land plant evolution

As discussed above, cilia apparently increased in number during the first 300 million years of land plant evolution. Specific cellular structures associated with cilia (including bicentrioles, blepharoplasts, basal bodies and the stellate array) became progressively larger, concomitant with increases in cilia number per sperm cell. Between 130 and 150 million years ago, the ability to produce any form of ciliary structure was lost independently on at least two occasions. As more plant genomes are sequenced, comparative analyses are starting to illuminate the genomic footprint of this evolutionary trajectory.

Bioinformatic analysis has shown that key ciliary protein components were lost within the land plants, even before the ability to produce cilia was lost (Bettencourt-Dias & Glover, 2007; Hodges *et al.*, 2010). For example, over a third of ciliary proteins that are highly conserved in ciliated eukaryotic species, but not in nonciliated species, are absent from ciliated land plant genomes (Hodges *et al.*, 2010, 2011). Examples of proteins with a known ciliary role that are absent from land plants include: Bardet–Biedl syndrome (BBS) proteins (BBS 1/2/5/7/8/9), which play important roles in trafficking cargo to the basal body and axoneme (Fan *et al.*, 2004; Mykytyn *et al.*, 2004; Blacque & Leroux, 2006); kinesin-II associated protein, which functions in anterograde IFT and sensory transduction (Pan & Snell, 2002); Rab-related GTPases (such as RABL2A and RBJL1), which play a role in the regulation of vesicle transport from the Golgi apparatus to the cilium base (Li & Hu, 2011); proteins of the outer-arm dynein complex and tektin, which are built into motile axonemes (Yano & Mikinoumura, 1981; Kamiya & Okamoto, 1985; Pirner & Linck, 1994); and certain radial spoke proteins (e.g. PF1), which are involved in ciliary movement (Hyams & Campbell, 1985; Diener *et al.*, 1993; Wickstead & Gull, 2007). The absence of these proteins reveals a number of biological features of land plant cilia. For example, compared with the inferred ancestral eukaryotic cilium, land plant cilia most probably have a reduced sensory role because of the absence of BBS proteins. Motility is also likely to be altered because of a lack of outer-arm dyneins, tektin and certain radial spoke proteins. Potentially different Golgi to cilia trafficking could result from a reduction in Rab-related GTPases, and the loss of proteins, such as Ca^{2+} ion channels, may influence the coordination of ciliary beat. In combination, the loss of these proteins suggests a regulatory shift in ciliogenesis during the transition from water to land.

9. Ciliary proteins conserved in nonciliated plants

The loss of cilia in the majority of land plants was presumed to be accompanied by a similar loss of genes encoding ciliary proteins. However, although the majority of ciliary function proteins are lost, bioinformatic analyses of the genomes of nonciliated seed plants have shown that genes encoding certain ciliary function proteins are retained (Hodges *et al.*, 2011). Retention of these genes suggests that the encoded ciliary proteins perform additional roles in a nonciliary context. The expression patterns of the retained genes in the model angiosperm *Arabidopsis thaliana* reveal high levels of mRNA accumulation in mature pollen (Hodges *et al.*, 2011). Given that sperm are formed in pollen grains by a division of the generative cell, this expression profile suggests that prior to the loss of cilia, the 'ciliary' proteins may have carried out dual roles within sperm cells. Interestingly, angiosperm generative and sperm cells share a distinctive arrangement of microtubule bundles that is not seen in somatic cells (reviewed in Southworth & Cresti, 1997). These bundles must therefore form *de novo* in the generative cell progenitor of the sperm cells. The similarity with *de novo* formation of the blepharoplast in sperm mother cells of ciliated seed plants is striking, and it may be that proteins required for blepharoplast formation are also needed for the assembly of cytoskeletal bundles in nonciliated plants.

In support of the suggestion that 'ciliary' proteins have a more general cytoskeletal function in nonciliated seed plants, at least some of the retained genes are predicted to encode proteins with cytoskeletal/microtubule roles. They include a putative katanin – a class of microtubule-severing proteins (McNally & Vale, 1993); *TONNEAU2/FASS1* which, when mutated, causes cytoskeletal abnormalities in *Arabidopsis thaliana* (Camilleri *et al.*, 2002); and a homologue of FUSED, which was identified as a component of the hedgehog signalling pathway in *Drosophila* and localizes to the cilium in many species (Oh *et al.*, 2005). Perhaps counter-intuitively, therefore, the nonciliated angiosperms, such as *Arabidopsis thaliana*, present an opportunity for ciliary research. Specifically, they provide a system for the study of how ciliary genes are utilized for other cellular functions, particularly following cilia loss.

III. Future perspectives: *Marchantia* and *Ceratopteris* as models for cilia research

Chlamydomonas is an excellent model system for the study of cilia form and function in Viridiplantae (reviewed in Silflow & Lefebvre, 2001). However, of the hundreds of cilia mutants that

have been isolated in this system, only two perturb basal body formation (Dutcher & Trabuco, 1998; Harper, 1999). This low number reflects the fact that centrioles are a pivotal component of the vegetative cell cycle in *Chlamydomonas* and therefore centriole/basal body mutants are normally lethal. Given that centrioles are formed *de novo* in the sperm cells of ciliated land plants, these organisms provide a system to study ciliogenesis in a context that is isolated from the cell cycle, and thus to identify novel components of cilia form and function. In addition, because ultrastructural studies have shown that numerous changes to cilia have occurred within the land plant lineage (summarized in Fig. 4), this group can also be used to increase our knowledge of evolutionary changes. By revisiting these organisms in a modern molecular context, questions about *de novo* basal body formation and migration/docking of multiple basal bodies to the cell membrane could be investigated through the characterization of mutants with no or fewer cilia per cell. Genes that affect migration and placement might also be identified through mutant screens that select for altered swimming phenotypes. Even in species with large genomes, the ease with which transcriptome datasets can be generated would permit phenotypes to be associated with underlying changes in gene function.

Species with sperm representing both the ancestral biciliated and derived multiciliated condition need to be included in any comparative analysis, because significant changes in ciliogenesis occurred during the transition. The liverwort *Marchantia polymorpha* produces biciliated sperm (Fig. 3c) in large numbers on morphologically recognizable male plants. The species can be transformed (Ishizaki *et al.*, 2008), populations of insertional mutants are easy to generate (Y. Yasumura, University of Oxford, Oxford; pers. comm.) and the mutated genes can be identified using the inserted tag. Importantly, the 290-Mb genome is currently being sequenced (K. Ishizaki, Kyoto University, Kyoto; pers. comm.). *Marchantia* is thus an obvious model organism for cilia studies, and is a significantly better choice than the model moss *Physcomitrella patens* because the separate male and female plants make genetic crosses and sperm isolation much easier.

For studies of multiciliated sperm, the fern *Ceratopteris richardii* is a particularly attractive choice because it has a relatively short life cycle compared with other ferns (3–4 months), can be propagated vegetatively through plantlets that are formed on the leaf margins and produces many easily isolated sperm (Fig. 3b) (Hickok *et al.*, 1995). There is even a closely related rapid cycling species that can complete a spore to spore cycle in 60 d (http://www.c-fern.org). Forward genetics is straightforward, mutant populations already exist (Hickok *et al.*, 1995) and mutations that disrupt cilia formation on sperm cells have been isolated (Renzaglia *et al.*, 2004). Furthermore, perturbation of gene function through RNAi silencing has already been reported (Stout *et al.*, 2003) and stable transformation is now possible (L. Huang, A. Plackett & J. Langdale, unpublished). Although the genome is extremely large (39 chromosomes and a haploid size of 11 Gb), *de novo* assembly of transcriptome data is feasible, as seen with the closely related fern *Pteridium aquilinum* (genome size, 9.8 Gb) (Der *et al.*, 2011), and comparisons of mutant and wild-type sperm would be possible. With the decreasing cost of sequencing, the existence of a genetic map (Nakazato *et al.*, 2006) and increasing sophistication of genomic assembly, even the large genome may not hinder the potential of *Ceratopteris* for ciliary research for much longer.

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