The *Parachlorella* Genome and Transcriptome Endorse Active RWP-RK, Meiosis and Flagellar Genes in Trebouxiophycean Algae

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Summary   The genus *Chlorella* is a well-known member of the green algal class Trebouxiophyceae, which is characterized by an immotile and asexual life cycle. Here, we performed an analysis of the whole genome and transcriptome of *Parachlorella kessleri* NIES-2152 with emphasis on the evolution of meiosis and the flagellar proteins. The *Parachlorella* transcriptomic data showed that the MID-related RWP-RK genes and meiosis-specific and flagellar proteins were expressed; at the transcriptional level, the DNA repair protein *RAD50* was upregulated in the stationary phase, with four-fold more reads per kilobase of transcript per million mapped reads (RPKM) compared with the early stage of culture. In contrast, radial spoke protein genes were down-regulated in the stationary phase. These results suggest that genes for meiotic and flagellar proteins are culture stage-dependent and retain their functions. We presume that the algae lost some of the genes for meiosis and the flagella during asexual evolution, but other genes still possess biological functions other than those related to the flagellum and meiosis.

The flagellum is an important structure that enables swimming, and which must have originated before the last common ancestor of all extant eukaryotes (Cavalier-Smith 2006). Meiosis is likely to have arisen prior to the origin of the nuclear envelope during the early evolution of eukaryotes, and the development of mitochondria, nuclei, and flagella was virtually simultaneous (Cavalier-Smith 2010). Thus, meiosis first evolved following evolution of the eukaryotic flagellum. After diversification, completely immotile and asexual groups evolved in some eukaryotic lineages, such as Trebouxiophyceae of the green plants (Friedl 1995, Lewis and McCourt 2004). The class Trebouxiophyceae is a major subset of the green algal division Chlorophyta, along with the classes Chlorophyceae and Ulvophyceae (Lemieux *et al.* 2014). The trebouxiophycean genus *Chlorella* and its sister genus *Parachlorella* are well known among algae due to their high growth rates and excellent biomass productivities (Guschina and Harwood 2006, Hu *et al.* 2008, Lang *et al.* 2011, Přibyl *et al.* 2012, 2013). *Parachlorella kessleri*, one of the three currently described *Parachlorella* species (Krienitz *et al.* 2004, Bock *et al.* 2011), is an spherical unicellular alga with cell wall (Fig. 1), with the highest biomass and lipid and starch productivity within the *Chlorella* and *Parachlorella* species (Mizuno *et al.* 2013, Takeshita *et al.* 2014). The *P. kessleri* genome and transcriptome were published recently with particular emphasis on sulfur deprivation to enhance lipid production (Ota *et al.* 2016a).

In *Chlamydomonas reinhardtii* and its close relatives (Volvocales), haploid vegetative cells differentiate into
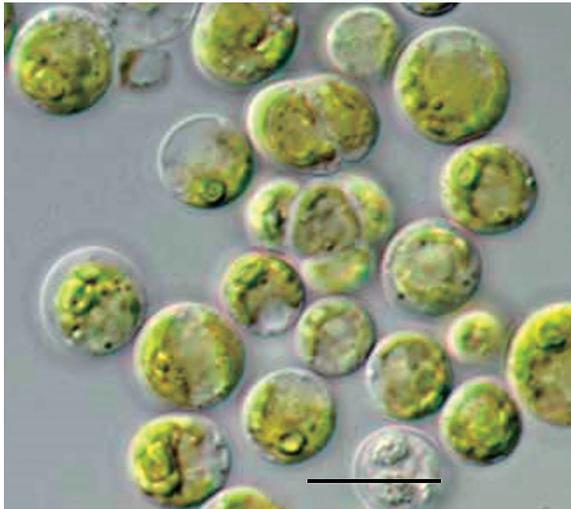
Fig. 1. Morphology of *Parachlorella kessleri* cells. Scale bar = 10 μm.

gametes of two mating types (mt^+/mt^-) in response to nitrogen starvation. Ferris and Goodenough (1997) reported the characterization of *mid*, the minus-dominance gene of *Chlamydomonas*. The MID sequence was found to be similar to that of the plant RWP-RK domain-containing proteins that are involved in nitrogen-responsive processes, implying that MID activity may be nitrogen sensitive (Lin and Goodenough 2007). Since the discovery of *MID* in *Chlamydomonas*, *MID* orthologues have been found in *Pleodorina* (Nozaki *et al.* 2006), *Gonium pectorale* (Hamaji *et al.* 2008), and *Volvox* (Ferris *et al.* 2010), indicating that *MID* is a sex-determining factor in the Volvocales lineage. Based on phylogenetic and domain analyses, RWP-RK proteins are classified into two subfamilies: the nodule inception (NIN)-like proteins (NLPs) and the RWP-RK domain-containing proteins (RKDs), and the RKDs are divided into three subgroups: RKD (A), RKD (B), and RKD (C) (Schauser *et al.* 1999, Chardin *et al.* 2014). The evolutionary phylogenetic position and functions of the trebouxiophycean RWP-RK proteins remain unclear.

It is widely accepted that meiosis arose very early during eukaryotic evolution and that the core gene set for meiosis was likely present in the last common ancestor of eukaryotes (Bernstein and Bernstein 2010, Speijer *et al.* 2015). Villeneuve and Hillers (2001) defined the core meiotic recombination machinery whose functions are widely conserved among eukaryotes. Later, a core set of putative meiotic genes was found in the diplomonad *Giardia intestinalis*, which is not known to have a sexual cycle (Ramesh *et al.* 2005). Schurko and Logsdon (2008) described a meiosis detection toolkit that helps to detect sexual reproduction in diverse taxa. The latest meiosis detection toolkit includes homologs of 29 components of the meiotic recombination machinery (Malik *et al.* 2008).

Trebouxiophycean algae are mostly asexual. In a literature review (Fučíková *et al.* 2015), direct observation of syngamy, the formation of a zygote, or stages presumed to be the result of syngamy was reported for a limited number of species belonging to four lineages: Chlorellales, the Elliptochloris clade, the Prasiola clade, and Trebouxiales (Fučíková *et al.* 2015). However, despite the asexual phenotype, the review indicated that homologs of meiosis-specific genes remain encoded in trebouxiophycean genomes (Fučíková *et al.* 2015), including that of *Chlorella variabilis* (Blanc *et al.* 2010). Whether trebouxiophycean algae are truly asexual, and whether the meiotic genes are coded in their genomes, remain open questions.

The "9+2" microtubule axoneme is the typical structure of a flagellum (Ginger *et al.* 2008). In motile axonemes, the formation of transient bridges between dynein arms on outer-doublet A tubules with the B tubules of the adjacent nine outer-doublet microtubules causes sliding of the microtubules (Downing and Sui 2007, Ginger *et al.* 2008). During assembly and maintenance of flagella, intraflagellar transport (IFT) plays a mediating role by delivering axonemal precursors to the distal tip of the growing organelle (Cole 2003). In *Chlamydomonas*, 360 flagellar proteins were identified with high confidence based on proteomic analyses using mass spectrometry (Pazour *et al.* 2005). Blanc *et al.* (2010) identified many putative orthologues of *Chlamydomonas* flagellar proteins (103 of 360 flagellar proteins) in the genome of *Ch. variabilis*, despite *Chlorella* being phenotypically non-motile and lacking a flagellar stage. This discovery led to the following hypotheses, which should be verified empirically: (1) the conserved flagellar proteins might have acquired other biological roles when the flagellar apparatus was lost; and (2) given that *Chlorella* is probably capable of sexual reproduction, the genus likely retained the ability to form possibly motile, rudimentary flagella.

To better understand the evolution of the trebouxiophycean algae, we examined common orthologues from among the published genomes and compared Trebouxiophyceae and Chlorophyceae. We also used a phylogenetically informed approach to investigate evolutionary aspects of the RWP-RK, meiosis, and flagellar genes. Our data provide clues aiding the understanding of whether the trebouxiophycean flagellar and meiotic proteins retain their functions.

Materials and methods

Growth conditions and DNA and RNA extraction

Parachlorella kessleri NIES-2152 was obtained from the National Institute for Environmental Studies (NIES) in Tsukuba, Japan. *P. kessleri* cells were grown at 20°C under a 70-μmol photons m^{-2} s^{-1} 12-h light (L) : 12-h dark (D) cycle in TAP medium (Ota *et al.* 2016b). For RNA extraction, the cells were grown in TAP or dSTAP (Ota

et al. 2016b) medium in a 500-mL flask (Iwaki, Tokyo, Japan) at 20°C under a 100-μmol photons m^{-2} s^{-1} 12-h L : 12-h D cycle. DNA and RNA were extracted as described previously (Ota *et al.* 2016a).

Construction of protein families and KEGG analysis of orthologues

Annotated genes of *P. kessleri* were compared to those of representative sequenced chlorophyte algae [*Chlorella variabilis* NC64A (Blanc *et al.* 2010), *Coccomyxa subellipsoidea* C-169 (Blanc *et al.* 2012), *Chlamydomonas reinhardtii* (Merchant *et al.* 2007), *Ostreococcus tauri* (*Palenik et al.* 2007), and *Micromonas* sp. CCMP1545 (Worden *et al.* 2009)] based on shared sequence similarity (E-value <1×10^{-15}) using OrthoMCL (Li *et al.* 2003). Groups that contained proteins from more than one organism were defined as homologous. The proteins in these groups were defined as paralogues, and proteins in groups that contained only proteins from single organisms were defined as specific. The proteins were then grouped using OrthoMCL, and the groups and numbers of proteins in each group were counted using a custom Python script.

For the functional category analysis based on the KEGG database, sequences of proteins (except those of *P. kessleri*) were retrieved from the Phytozome v.11.0 database, and orthologues of Trebouxiophyceae (*P. kessleri* and *Co. subellipsoidea*) and Chlorophyta (*C. reinhardtii* and *Micromonas* sp. CCMP1545) genes were identified using a best reciprocal BLASTP approach (E-value <1×10^{-10}). These common orthologues were classified into functional categories using the KEGG database (Table S1), and the number of orthologues in each category was tallied. The presence or absence of putative orthologues against the *Chlamydomonas* flagellar proteins (Pazour *et al.* 2005), RKDs, and meiosis-related proteins defined by a meiosis detection toolkit (Schurko and Logsdon 2008) was determined according to BLASTP with a cut-off E-value of 1×10^{-10}.

Taxon and orthologue sampling and phylogenetic analysis

Amino acid sequences were collected from NCBI (http://www.ncbi.nlm.nih.gov) and the Joint Genome Institute (http://genome.jgi.doe.gov) and aligned with MUSCLE (Edgar 2004). Informative positions were selected using Gblocks v.0.91b (Castresana 2000) with the default setting. Maximum likelihood phylogenetic analyses were performed in IQ-TREE v.1.6.10 (Nguyen *et al.* 2015) with 1000 bootstrap replications according to the optimal substitution models.

Accession numbers

For *P. kesseleri*, the DDBJ accession numbers were: PkRWP1 (LC214364), PkRWP2 (LC214365), PkRWP3 (LC214366), PkRWP4 (LC214367), PkRWP5

(LC214368), and PkNit2 (LC214750). The entire *P. kessleri* NIES-2152 genome is available in the DDBJ/EMBL/GenBank under the accession numbers BBXU01000001–BBXU01003651. The other accession numbers are shown in Table S2.

Results and discussion

General genome structure

The total nuclear genome size of *Parachlorella* was estimated to be 62.5 megabase pairs (Mbp), and 13057 genes were identified (Ota *et al.* 2016a). To analyze the general genome composition, we compared the *Parachlorella* genome with other published genomes of the Trebouxiophyceae and Chlorophyta (*Ch. variabilis*, *Co. subellipsoidea*, *Micromonas pusilla*, and *C. reinhardtii*) (Fig. 2). Genes that had multiple copy numbers were combined into gene families using OrthoMCL (E-value <1×10^{-15}). Among the trebouxiophycean algae, 4618 unique and 2179 shared gene families were found in the *Parachlorella* genome compared to the *Chlorella* and *Coccomyxa* genomes (Fig. 2). In pairwise comparisons among the Trebouxiophyceae, 2338 gene families were shared with *Chlorella*, and 899 gene families were shared with *Coccomyxa* (Fig. 2A). In the pairwise comparison between the chlorophytes *Chlamydomonas* and *Micromonas*, 2566 gene families were shared between them (Fig. 2B).

Functional annotation analysis

After the functional annotation analysis using the KEGG database, we examined genome-wide evolution among green algae, and we evaluated the number of orthologues gained or lost in the lineages. Orthologues in some functional categories were scarce in the Trebouxiophyceae, suggesting the loss of orthologues in the lineage (Fig. 2C, Table S1). Focusing on the categories that had larger differences in the orthologue counts between the Trebouxiophyceae and Chlorophyceae, the largest difference was in the category of "aminoacyl-tRNA biosynthesis" (seven differences), indicating that genes involved in this biosynthesis have been lost in Trebouxiophyceae. In terms of meiosis- and flagellum-related categories, there were six fewer meiosis-related, and three fewer flagellum-related orthologues in the Trebouxiophyceae than in the Chlorophyta (Fig. 2D). There were no orthologues in the category "IFT complex B" (intraflagellar transport) in the Trebouxiophyceae.

Distribution of orthologues of RWP-RK, meiosis, and flagellar proteins

A comparative genomic analysis of sequenced green algae was performed focusing on genes related to RKDs (Chardin *et al.* 2014), meiosis, and flagellar proteins. RKDs include MID, which is crucial for sex determination in Volvocales (Ferris and Goodenough 1997,
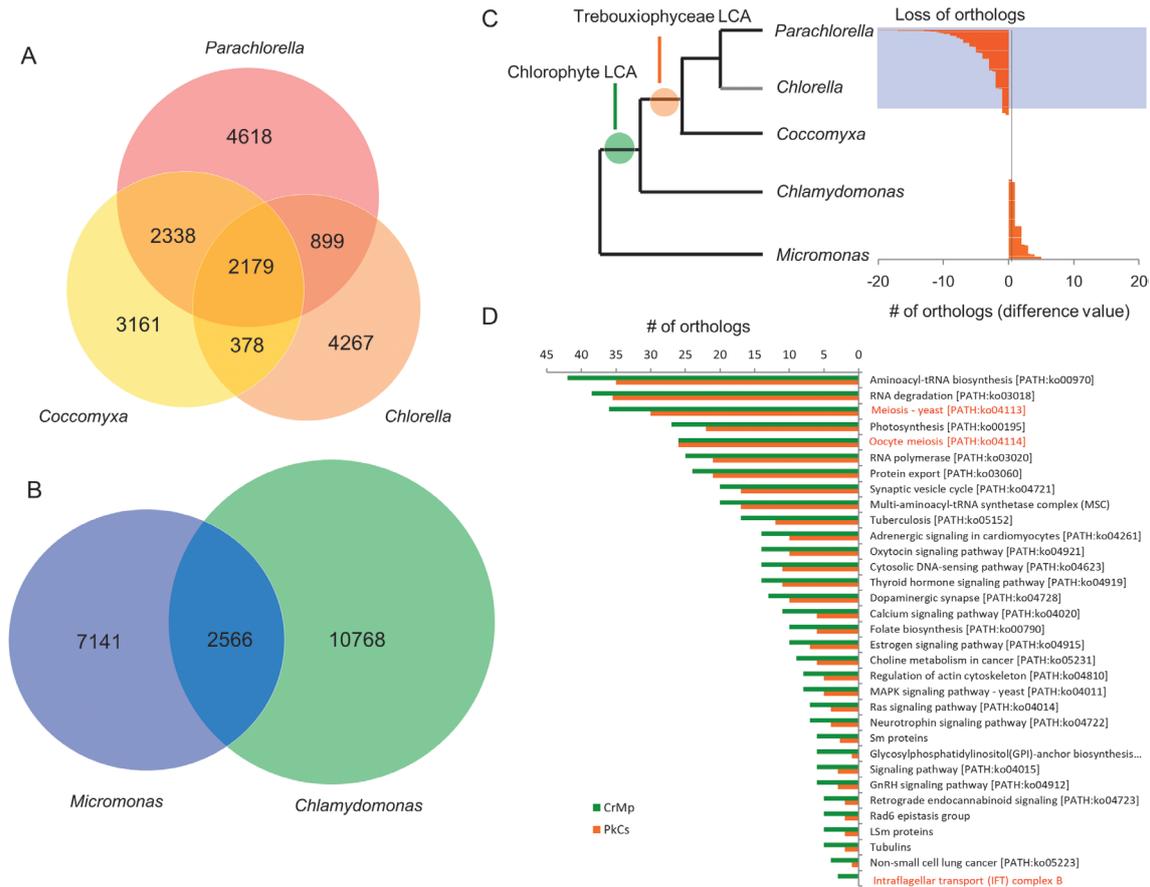
Fig. 2. Comparison of the *Parachlorella* genome with other green algae. (A, B) Shared and unique genes between trebouxiophycean and chlorophycean algae. The number of gene families is indicated in each section of the Venn diagram. (C) Phylogenetic and KEGG category analysis of the orthologues among the Trebouxiophyceae and Chlorophyceae. The trebouxiophycean (*Parachlorella* and *Coccomyxa*) and chlorophycean (*Chlamydomonas* and *Micromonas*) orthologues were identified using reciprocal best-hit BLASTP searches. The bar graph indicates differences in orthologue counts (Table S1) between Trebouxiophyceae and Chlorophyceae. Orthologues were counted only if the genes were assigned to KEGG orthology (KO) numbers, and showed a functional annotation of category 3. The negative values indicate fewer orthologues from Trebouxiophyceae compared to Chlorophyceae. Reference phylogenetic tree is shown in Fig. S1. (D) The number of orthologues if the difference was >2, corresponding to the blue-shaded box in C. Green and orange bars indicate Chlorophyceae and Trebouxiophyceae orthologue numbers, respectively. Meiosis and flagellar categories are shown in red. CrMp: orthologues shared between *Chlamydomonas* and *Micromonas*; PkCs: orthologues shared between *Parachlorella* and *Coccomyxa*.

Nozaki *et al.* 2006, Hamaji *et al.* 2008, 2013). Based on phylogenetic and domain analyses, Chardin *et al.* classified the RWP-RK proteins into two subfamilies (Chardin *et al.* 2014): NLPs and other RKDs: RKD (A), RKD (B), and RKD (C) (MID). The present molecular phylogeny shows two homologs belonging to the NIT2 subclade in the NLP group, and five *Parachlorella*-unique homologs (Fig. 3A). There were no MID homologs in the *Parachlorella* or *Coccomyxa* and *Micromonas* genomes (Figs. 3A, 4A). This result enabled us to infer that RKDs are not used as a sex-determining factor in the trebouxiophycean and prasinophyte algae.

Putative meiotic genes were found using a meiosis detection toolkit (Schurko and Logsdon 2008). A BLASTP search identified 26 gene families out of 29 meiosis-related genes in the *Parachlorella* genome (E-value < 1×10^{-10}) (Table S1, Fig. 4B). Of the 26 genes, six genes with functions restricted to meiosis were found in the

Parachlorella genome. Recent studies revealed that meiotic genes are present in the genomes of trebouxiophycean species, including *Ch. variabilis*, which presumably has an asexual life cycle (Blanc *et al.* 2010, Fučíková *et al.* 2015). A similar evolutionary history appears to be shared by flagellar proteins in the Trebouxiophyceae. Based on the BLAST search, and similar to the findings from the *Chlorella* genome, many putative orthologues of *Chlamydomonas* flagellar proteins (116 of 337 flagellar proteins) were identified (Fig. 3C). The presence and absence of genes encoding flagellar proteins were similar between *Parachlorella* and *Chlorella*. However, many radial spoke (7–9 of 12 homologs) and IFT (6–8 of 12 homologs) proteins were absent in the genomes of *Ostreococcus*, *Coccomyxa*, *Chlorella*, and *Parachlorella*. Thus, the core flagellar proteins were clearly lost in the lineages that lost the flagellum (the trebouxiophyceans and *Ostreococcus*). The only conserved flagellar
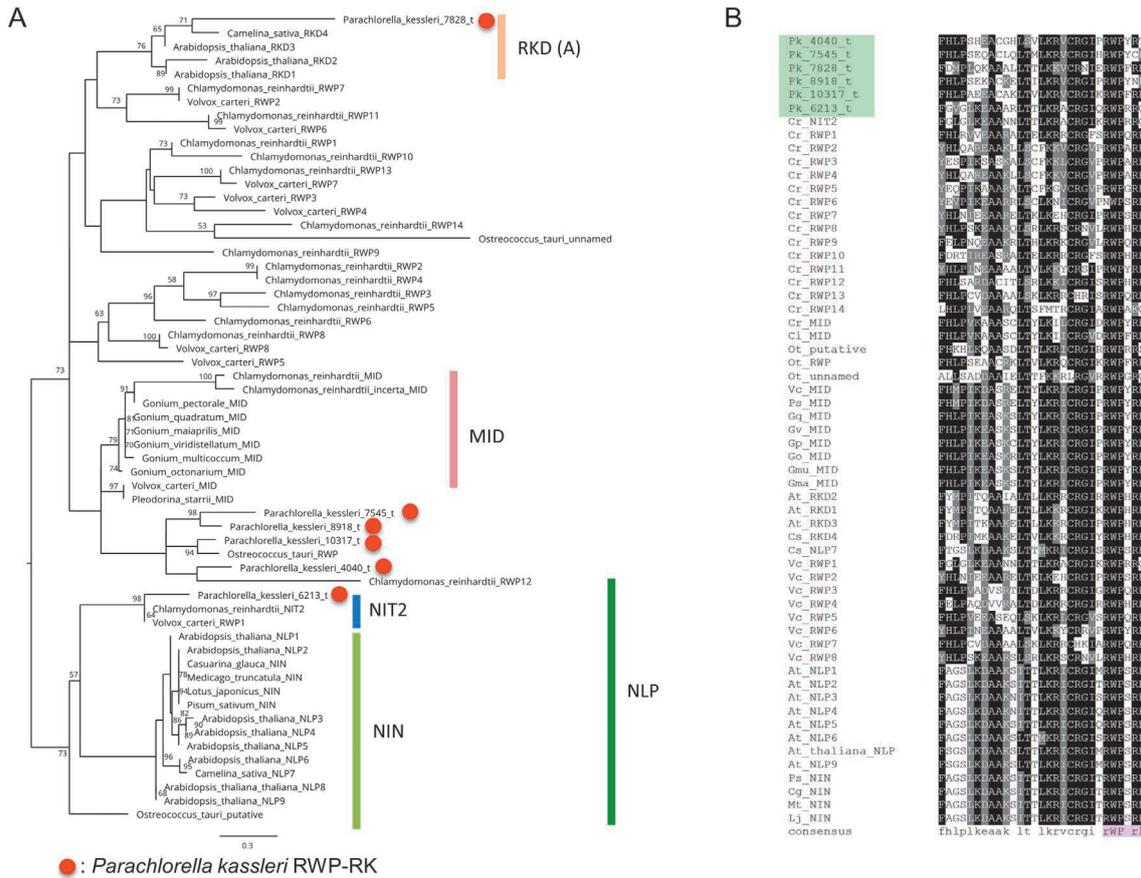
Fig. 3. Phylogeny of the RWP-RK transcription factors. (A) Phylogenetic tree inferred from the amino acid dataset using the WAG+G4 model. The bootstrap values (>50) are shown near the branches (1000 replicates). Abbreviations and accession numbers used here are given in Table S2. Scale bar denotes the number of amino acid substitutions per site. NLP: NIN-like proteins; NIT: nitrilase; NIN: nodule inception. (B) Amino acid sequence alignment of the RWP-RK domain-containing proteins. This alignment corresponds to the amino acid dataset from the phylogenetic analysis in A. *Parachlorella* RWP-RKs are highlighted in green. Red highlighting indicates the RWP-RK consensus motif. Species abbreviations are defined in Table S2.
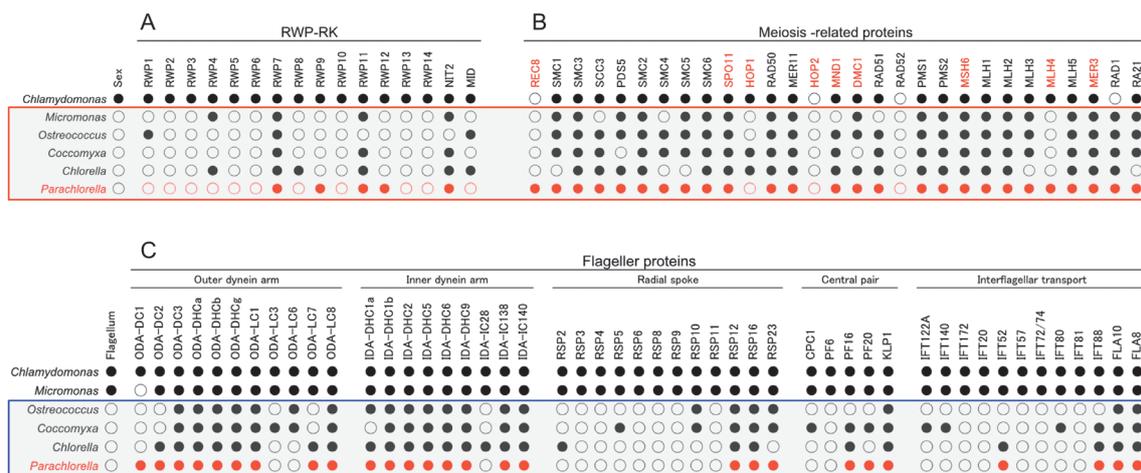

Fig. 4. Presence or absence of putative orthologues related to RWP-RK, meiosis, and flagella. (A) Genes for RWP-RK domain-containing proteins. (B) Meiosis-related genes. (C) Flagellar protein-related genes. Solid circles indicate the presence and open circles indicate absence. The presence or absence of *P. kessleri* genes is indicated in red. Meiosis-specific genes (core-meiosis genes) are shown in red. The presence or absence of putative orthologues against *Chlamydomonas* flagellar proteins, RWP-RK-domain-containing proteins, and meiosis-related proteins was determined based on BLASTP searches against *Chlamydomonas*. Species framed by red and blue boxes reflect the phenotypic absence of sexual reproduction and flagella, respectively.
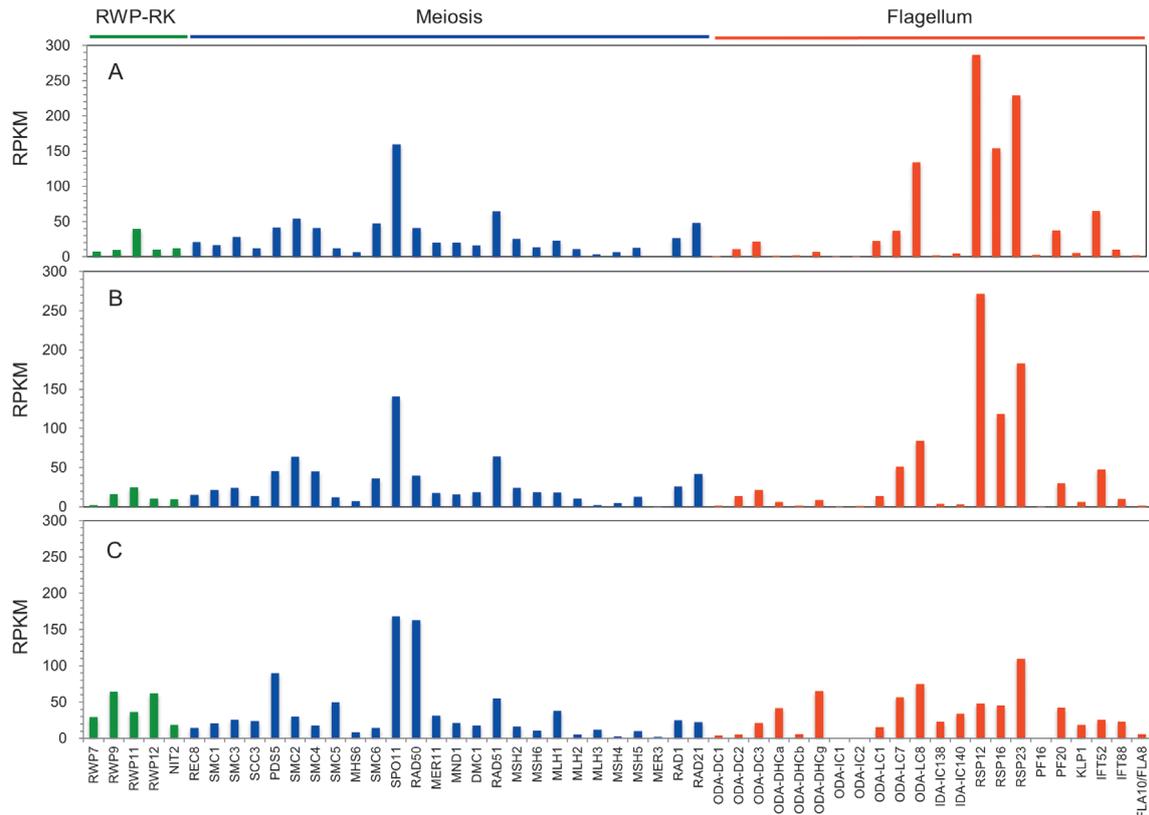
Fig. 5. Expression profiles of genes related to RWP-RK, meiosis, and flagellar proteins in *P. kessleri*. Five RWP-RK transcription factors (green), eight core meiosis genes (blue), and 14 flagellar genes (red) are indicated. Flagellar genes were selected by a BLAST search against *Chlamydomonas*. RPKM values are shown at the early-log phase (2-d-old, A), late-log phase (4-d-old, B), and stationary phase (8-d-old, C) cultures.

proteins were orthologues of the motor protein dynein, which may be crucial for cargo movement on microtubules, a required function that is independent of the presence of flagella.

Transcriptomic profiles

To examine transcriptomic regulation of genes for RWP-RK, meiosis, and flagellar proteins, we analyzed the *Parachlorella* transcriptome data. Despite the asexual life cycle of *Parachlorella*, the present reanalysis of transcriptome data (Ota *et al.* 2016a) confirmed that those RWP-RK, meiotic, and flagellar genes were expressed (Fig. 5). The transcriptional levels of the four *Parachlorella* homologs related to RKD (B) and *NIT2* were upregulated in the stationary phase (Fig. 4C), suggesting that these RKDs are regulated in response to nitrogen limitation. The transcriptional level of the DNA repair protein RAD50 was also upregulated in the stationary phase, with four-fold more RPKM compared with the early stage of culture. In the flagellar genes, genes of radial spoke proteins (RSPs) were down-regulated in the stationary phase. For example, the transcript level of RSP12 had 0.17-fold RPKM compared with the early stage of culture. The authors of a recent review argued that asexual organisms could be as persistent as organisms with conventional sexual cycles through the use of other mechanisms, *e.g.*, genomic rearrangement,

to promote adaptation (Seidl and Thomma 2014). The finding that *RAD50*, the DNA protein repair gene, was upregulated in *Chlorella* supports the idea that genome rearrangements foster an evolution in the absence of a meiotic life cycle.

Phylogeny of the flagellar and meiosis genes

We used a phylogenetically informed approach to investigate the evolutionary relationship between meiosis and flagellar proteins using the two representative genes of meiosis and flagellar proteins, *SPO11* and *ODA-DHCg* (Fig. 6). SPO11 is the meiosis-specific topoisomerase-like DNA transesterase (Seidl and Thomma 2014), and ODA-DHCg is the outer dynein arm heavy chain protein. They act as centers of sexual reproduction and flagellar structure, respectively; in particular, SPO11 is known to be a meiosis-specific gene (Fig. 3). In the molecular phylogenetic analysis of SPO11, sexually reproducing *Chlamydomonas* and *Volvox* were grouped into a single clade of trebouxiophyceans with 100% bootstrap (Fig. 6A). *Micromonas* was in a sister relationship with the trebouxiophycean clade and sexual green alga but had a relatively long branch (Fig. 5A). On the other hand, flagellated organisms formed a single clade in the molecular phylogenetic analysis of ODA-DHCg (Fig. 6B). The phylogenetic analysis suggested that the two genes are very close evolutionarily, as the group with fla-
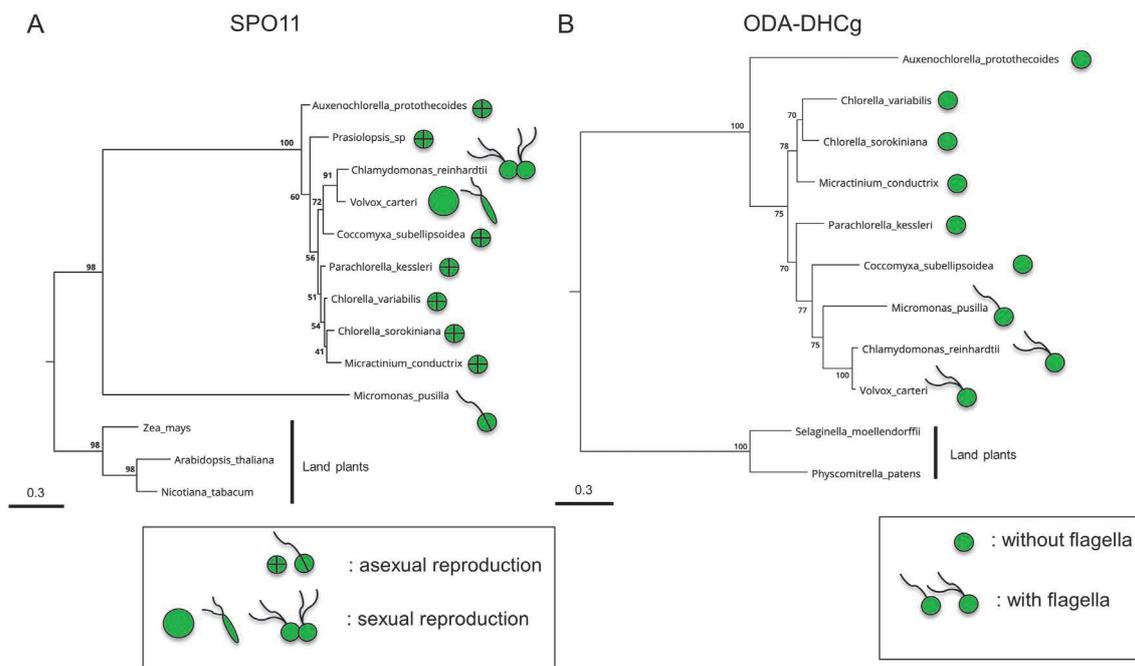
Fig. 6. Phylogeny of the meiosis and flagellar proteins. (A) Phylogenetic tree inferred from the SPO11 amino acid dataset under the LG+G4 model. The bootstrap values (>50) are shown near the branches (1000 replicates). (B) Phylogenetic tree inferred from the amino acid dataset of ODA-DHCg under the LG+G4 model. The bootstrap values (>50) are shown near the branches (1000 replicates). Scale bars denote the number of amino acid substitutions per site.

gella and sexual reproduction was nested in the trebouxiophyceans. In addition, considering that flagellar genes encode structural proteins, their grouping together with the flagellar phenotype suggests that flagellar evolution is conserved.

In summary, our phylogenetic study showed that *Parachlorella* RKDs are separate from *MID* genes and may not be used as a sex-determining factor (Fig. 3). The trebouxiophycean algae lost some meiosis and flagella genes during asexual evolution. In contrast, the expression of genes related to flagellar and meiotic proteins was regulated by culture stage. Evolutionarily, it was related closely to the group with flagella and sexual reproduction. These findings suggest that these genes are not pseudogenes, but have unknown biological functions. Future studies are expected to clarify the specific functions of these genes in asexual *Chlorella*.

Acknowledgements

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