Characterization of MADS-box genes in charophycean green algae and its implication for the evolution of MADS-box genes

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The MADS-box genes of land plants are extensively diverged to form a superfamily and are important in various aspects of development including the specification of floral organs as homeotic selector genes. The closest relatives of land plants are the freshwater green algae charophyceans. To study the origin and evolution of land plant MADS-box genes, we characterized these genes in three charophycean green algae: the stonewort *Chara globularis*, the coleochaete *Coleochaete scutata*, and the desmid *Closterium peracerosum–strigosum–littorale* complex. Phylogenetic analyses suggested that MADS-box genes diverged extensively in the land plant lineage after the separation of charophyceans from land plants. The stonewort *C. globularis* mRNA was specifically detected in the oogonium and antheridium together with the egg and spermatozoid during their differentiation. The expression of the *C. peracerosum–strigosum–littorale*-complex gene increased when vegetative cells began to differentiate into gametangial cells and decreased after fertilization. These expression patterns suggest that the precursors of land plant MADS-box genes originally functioned in haploid reproductive cell differentiation and that the haploid MADS-box genes were recruited into a diploid generation during the evolution of land plants.

charophytes | land plants | *Chara* | *Coleochaete* | *Closterium*

Diversity in form, a hallmark of extant species, is probably caused by modifications of ancestral gene networks regulating development and by the generation of novel developmental processes (1). The evolution of transcription factors, which have critical functions in development, through gene duplication and subsequent functional divergence has been hypothesized to be a major force in developmental evolution (reviewed in ref. 2). The adaptation of green plants to a terrestrial environment and their subsequent diversification are tightly linked to the evolution of the body plan of land plants (3, 4). Members of the MADS-box gene family regulate various aspects of development in flowering plants and therefore were probably involved in the evolution of the morphology of land plants.

MADS-box genes are characterized by the conserved MADS domain and are found in a wide range of eukaryotes including metazoans, fungi, slime mold, and green plants (5). These genes have been classified into several groups (6). MIKC^{C}- and MIKC*-type MADS-box genes contain intervening (I), keratin-like (K), and C-terminal (C) domains (7, 8). They are present in all major land plant taxa including seed plants, pteridophytes, and bryophytes but have not been found in other organisms such as green algae (5, 6, 9). The *Arabidopsis* genome (10) contains ≈38 MIKC^{C}-type and 5 MIKC*-type genes (6, 9). The MIKC*-type genes tend to form a monophyletic group, including several subgroups of genes with unknown functions (9), but the MIKC^{C}-type MADS-box genes have been classified into about a dozen subfamilies with diverse functions and expression patterns (reviewed in ref. 6). The floral homeotic genes are well characterized MIKC^{C}-type MADS-box genes. The ABC model of flower development (11, 12) postulates that the expression of one or more of the MIKC^{C}-type genes termed class A, B, and C genes, together with other transcription factors, controls the development of the floral organs sepals, petals, stamens, and carpels. MIKC^{C}-type MADS-box genes are also involved in floral transition, cell differentiation in fruits, establishment of root architecture, and other developmental processes (reviewed in ref. 13). Based on a molecular clock, orthologs of the A and C classes of floral homeotic genes are estimated to have diverged either ≈490 million years ago (14) or 570 million years ago (15), older than the oldest known fossil land plants 470 million years old (3).

Although the functions of many MADS-box genes in flowering plants have been studied extensively, related genes in nonflowering plants are largely unknown (13). In particular, no MADS-box genes have been reported from green algae, and it is still not known whether the MIKC^{C}- and MIKC*-type genes were already established in the most recent common ancestor of land plants and green algae. According to phylogenetic analyses based on both morphological and molecular data, the freshwater green algae charophyceans are the closest living relatives of land plants (reviewed in ref. 4). Charophycean green algae are paraphyletic and contain several monophyletic groups. Charales (stonewort) and *Coleochaete* (coleochaete) are highly derived charophyceans. Charales is likely most closely related to land plants, and *Coleochaete* is sister to the Charales + land plant assemblage. The unicellular desmid *Closterium* is related more distantly to land plants than stonewort and coleochaete (16). Here we report MADS-box genes from three charophycean green algae: the stonewort *Chara globularis*, the coleochaete *Coleochaete scutata*, and the desmid *Closterium peracerosum–strigosum–littorale* complex. These genes were characterized to help elucidate the origin and evolution of the functionally diverse MADS-box gene family.

Materials and Methods

Strains and Culture Conditions. *C. globularis* was collected from a pond at Chiba University (Chiba, Japan) and was aseptically

Abbreviations: I, intervening; K, keratin-like; C, C-terminal; *CgMADS1*, *Chara globularis* MADS-box gene 1; *Cgrpl6*, *Chara globularis* ortholog of the ribosomal protein L6 gene; *CpMADS1*, *Closterium peracerosum–strigosum–littorale* complex MADS-box gene 1; *CsMADS1*, *Coleochaete scutata* MADS-box gene 1.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AB035567–AB035569 and AB091476).

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cultivated in an aquarium. A strain of *C. scutata* (UTEX 2567) obtained from the Culture Collection of Algae at the University of Texas (Austin) was cultured in aerated nitrogen-supplemented medium (C medium; ref. 17). The heterothallic *C. peracerosum–strigosum–littorale* complex strains NIES-67 (mt^+) and NIES-68 (mt^-) were obtained from the National Institute for Environmental Studies (Ibaraki, Japan). Clonal cultures were grown in C medium as described (18). To induce *C. peracerosum–strigosum–littorale* complex sexual reproduction, vegetatively grown cells of each strain were harvested in the late logarithmic phase, washed three times with nitrogen-deficient medium [MI medium (17)], and suspended separately in MI medium under continuous light. After 24 h of incubation, cells of both mating types (5.4×10^5 each) were mixed in 75 ml of fresh MI medium in 300-ml Erlenmeyer flasks and incubated under continuous light.

Isolation of MADS-Box Genes. Total RNAs were extracted from various tissues in each species. MADS-box genes of the three charophyceans were isolated from fresh materials as described (19). 3′ RACE was performed by using two MADS-domain-specific nested primers, duMADS2-2 and AllMADS2 (19). The PCR conditions were one cycle at 94°C for 1 min, followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1.5 min, and a final step at 72°C for 5 min. The 5′ regions of the cloned genes were isolated by using the 5′ RACE system (Invitrogen). Genomic DNA fragments corresponding to the three cDNAs were PCR-amplified, sequenced, and compared with the cDNAs to determine the exon–intron junctions. Because of potential PCR errors, at least two independently amplified clones were sequenced in every experiment.

***C. globularis* MADS-Box Gene RT-PCR and *in Situ* Expression Analyses.** Total RNA from different tissues was treated with RNase-free DNase (Invitrogen) and used for cDNA synthesis (19). For RT-PCR analysis, the *C. globularis* MADS-box gene 1 (*CgMADS1*) cDNA was amplified by using the *CgMADS1*-specific primers CgMF1 (5′-ATGGGTCGAGCTAAGATAGAGAT-3′) and CgMR1 (5′-TCTCCATTTGCATCACCTCTCTC-3′). The *C. globularis* ortholog of the ribosomal protein L6 gene (*Cgrpl6*), which is constitutively expressed in all tissues, was used as a positive control. The *Cgrpl6* cDNA was amplified by using the *Cgrpl6*-specific primers Cgrp16F1 (5′-AAGTTGCCTAAGTTCTACCCCG-3′) and Cgrpl6R1 (5′-AAGTTCATGGGGCTTCATGCCG-3′). The PCR conditions consisted of one cycle at 94°C for 3 min, followed by 25 or 30 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1.5 min, and a final step at 72°C for 5 min. For Southern analyses of the RT-PCR products, a 382-bp *CgMADS1*-specific probe was PCR-amplified by using the *CgMADS1*-specific internal primers CgMF2 (5′-CAATGCCACGAGTCGCCAAGTTAC-3′) and CgMR2 (5′-CCGAAACCTTATTTTCCTCAAAGC-3′), with the *CgMADS1* cDNA as a template. The Cgrpl6F2 (5′-ACGATGTGCCCAAGCCACGTC-3′) and Cgrpl6R2 (5′-TTTCTCGATCACTGGAAGTAGC-3′) primers were used to produce a 419-bp *Cgrpl6*-specific probe. The amplified *CgMADS1* and *Cgrp16* fragments were isolated and labeled with [^{32}P]dCTP (Amersham Pharmacia Biosciences) by using the random primer DNA-labeling kit (version 2.0; Takara Bio, Otsu, Japan). Hybridization and washing were performed at 65°C (20). *In situ* hybridization was performed as described (19) by using a *CgMADS1*-specific RNA probe. A *CgMADS1*-specific DNA fragment for an RNA probe was PCR-amplified by using the CgMF3 (5′-ATCCTTGATCGGTATCATAGTTGT-3′) and CgMR3 (5′-GTACAAAGGCAGGAGTTCAATCC-3′) primers. All sections were 8 μm thick.

Quantitative RT-PCR of *C. peracerosum–strigosum–littorale* Complex MADS-Box Gene. Real-time PCR analysis was performed by using the ABI PRISM 7000 (Applied Biosystems) according to manufacturer instructions. The TaqMan probes [5′-FAM-TATCACTCCGATCTCCACGTCGCACA-TAMRA-3′ and 5′-FAM-ATCACGGGCCCTTGTTCCGCTG-TAMRA-3′ (FAM is 6-carboxyfluorescein, and TAMRA is tetramethylrhodamine)] and two sets of primers (5′-AGCCTGTTGGTCAGCCTTCA-3′/5′-TCCGACGCAAGCTAAGCAT-3′ and 5′-TGGAGCTGTCGAGGCTTCA-3′/5′-CAGGCCCTCGAATGATTCTG-3′) were designed based on sequences in the MADS and K domains of the *C. peracerosum–strigosum–littorale* complex MADS-box gene (*CpMADS1*), respectively. Total RNA was extracted from conditioned cells that had been cultured in the C medium from each mating type. Conditioned cells were collected for total RNA extraction at 2, 4, 8, 12, and 72 h after the mating reaction began. After cDNA synthesis using random hexamers, PCR was performed with a treatment of 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The signals were detected as fluorescent light generated by the dissociation of fluorescent chemicals from the TaqMan probes. In cycles in which all signals were amplified exponentially, the signals were converted into numeric values and divided by the values for the *C. littorale* 18S ribosomal RNA gene (GenBank accession no. AF115438) in each sample to normalize the signals.

Phylogenetic Analyses. MIKC^C- and MIKC*-type MADS-box genes were collected from DNA databases by using the program BLASTX (21) and aligned by using MAFFT 4.21 (22). All of the alignment gaps were eliminated, and unambiguously aligned regions containing 90 amino acids were selected for the phylogenetic analysis. Maximum likelihood distances were calculated by using the program PROTML (23) with the conditions of the JTT model (24), and a neighbor-joining tree was generated by using the program NJDIST (23). Bootstrap data sets were prepared with SEQBOOT in PHYLIP 3.61 (23). The neighbor-joining tree was calculated for each set and the obtained trees were subject to CONSENSE to calculate the bootstrap probability of each branch.

Results

Exon–Intron Structures and Phylogeny of Three Charophycean MADS-Box Genes. We isolated one MADS-box cDNA from each of the three charophyceans, *C. globularis* (*CgMADS1*), *C. scutata* (*CsMADS1*), and *C. peracerosum–strigosum–littorale* complex (*CpMADS1*), from the reproductive apical part of the thallus with the oogonia and antheridia, the flat thallus with zoospores, and the cells 2 h after mixing in nitrogen-deficient medium, respectively. No other MADS-box genes were isolated, despite attempts with total RNAs extracted from other tissues, various PCR conditions, and six other degenerate primers. Under high-stringency genomic Southern hybridization conditions at 65 and 60°C for hybridization and washing with the Church buffer (20), the only bands detected were those of *CgMADS1* or *CpMADS1* (data not shown). Insufficient amounts of genomic DNA for Southern hybridization were obtained from *C. scutata*. Genomic Southern hybridization experiments using a probe containing both MADS and K domains at 50°C were unsuccessful because of high background noises (data not shown).

The exon–intron structure of each MIKC^C- and MIKC*-type MADS-box gene group is well conserved (8). The homology of exons and introns of three charophycean MADS-box genes to other MIKC^C- and MIKC*-type MADS-box genes was inferred based on a multiple alignment of deduced amino acid sequences. The MADS, I, K, and C domains are present in all three charophycean MADS-box genes (Fig. 1). The putative homologous exons and introns are shown in Fig. 2. The exon–intron structures of the MADS, I, K, and C domains of the coleochaete *CsMADS1* gene are mostly identical to those of most land plant

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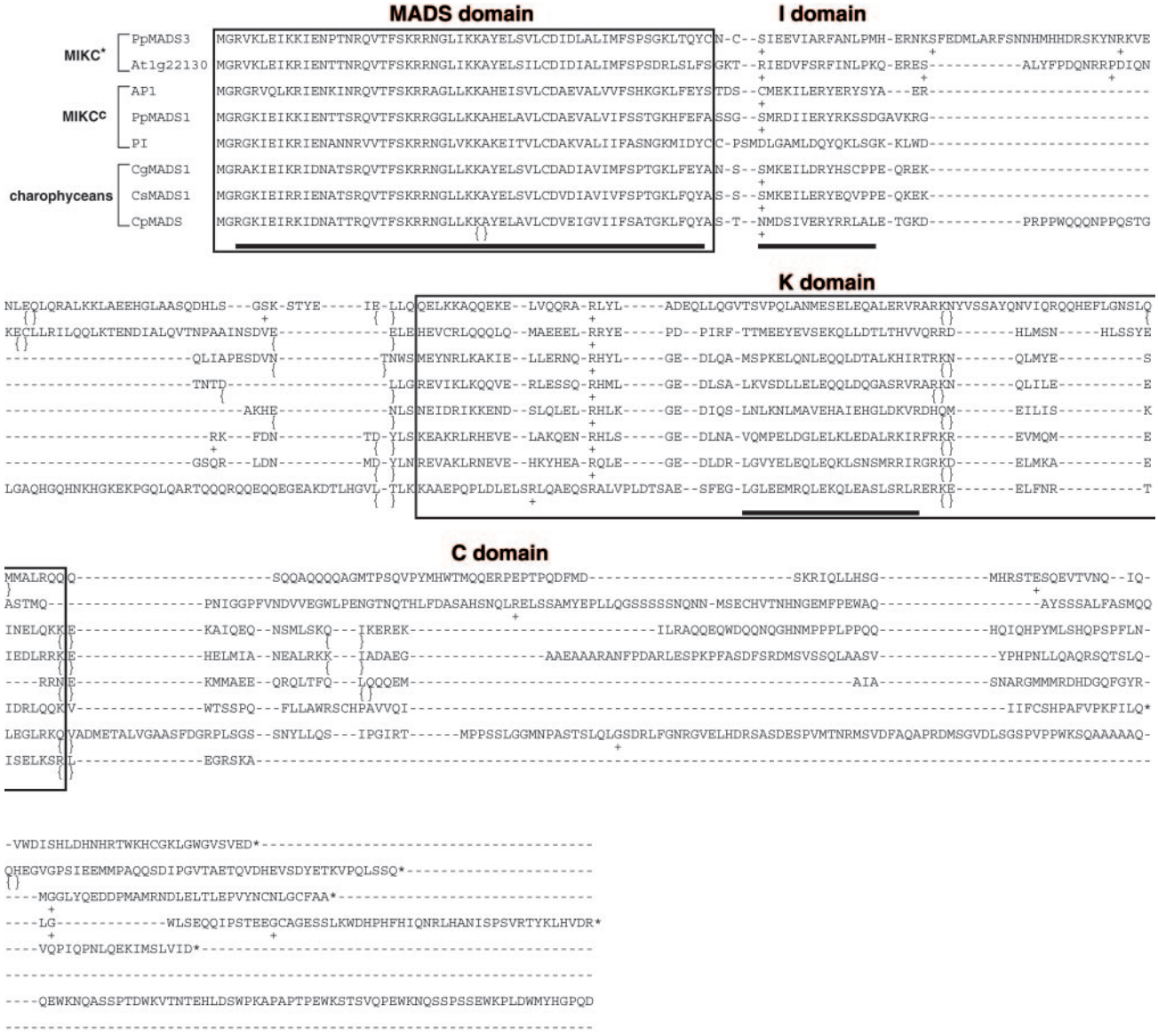

Fig. 1. Alignment of the deduced amino acid sequences of *CgMADS1* from stonewort, *CsMADS1* from coleochaete, *CpMADS1* from desmid, and the representative land plant MIKC^{C}- and MIKC*-type proteins *PpMADS1* and *PpMADS3* from the moss *Physcomitrella patens* and APETALA1 (AP1) and PISTILLATA (PI) from *Arabidopsis thaliana*. The underlined amino acids were used in the phylogenetic analysis shown in Fig. 3. The MADS, I, K, and C domains are marked. Brackets below the sequences indicate the positions of introns that are located between codons. Plus signs indicate the positions of introns that are located within codons.

MIKC^{C}-type genes, whereas the stonewort *CgMADS1* and desmid *CpMADS1* genes have an additional intron in the I and MADS domains, respectively. The first, second, third, and fourth through sixth exons of *CgMADS1*, the first, second, and third through fifth exons of *CsMADS1*, and the first and second, third, and fourth through sixth exons of *CpMADS1* correspond to the regions encoding the MADS, I, and K domains of other MIKC^{C}-type genes, respectively. The C domain of *CgMADS1* and *CpMADS1*, composed of a single exon, is shorter than those of *CsMADS1* and other typical MIKC^{C}-type genes.

We aligned the MADS, I, and K domains of the three charophycean genes and other representative MIKC^{C}- and MIKC*-type genes and generated neighbor-joining trees (Fig. 3). Phylogenetic relationships among the land plant MIKC^{C}- and MIKC*-type genes and the green algae MIKC^{C}-type genes were not solved with high bootstrap value.

Expression Patterns of Stonewort and Desmid MADS-Box Genes. To aid in determining the functions of charophycean MADS-box genes, the expression patterns of *CgMADS1* and *CpMADS1* were examined. Because of the difficulty of examining the complete life cycle of the coleochaete, *CsMADS1* was excluded from this analysis. The stonewort has multicellular gametophytes consisting of leaf- and stem-like organs, and the lower portion of the plant body is anchored to the ground with a root-like organ, the rhizoid. During the reproductive phase, egg cells and spermatozoid are formed in the oogonium and antheridium, respectively, both of which are multicellular reproductive organs. The fertilized egg, covered with oogonium tissue, falls from the gametophyte, and meiosis takes place after a period of dormancy, implying that only the zygote is diploid and that there is no sporophytic generation.

The expression of the stonewort gene *CgMADS1* in vegetative

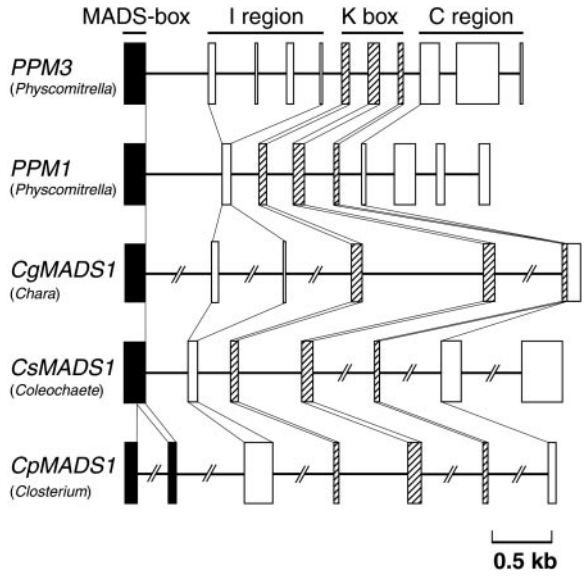

Fig. 2. The exon–intron structures of the charophycean MADS-box genes *CgMADS1*, *CsMADS1*, and *CpMADS1*. *PPM1* and *PPM3* from the moss *P. patens* represent land plant MIKC^{C}- and MIKC*-type MADS-box genes, respectively. Putatively homologous exon–intron borders are connected by vertical lines. Exons corresponding to the MADS, I, K, and C domains are indicated.

and reproductive organs was examined by RT-PCR (Fig. 4*A*) and *in situ* hybridization (Fig. 4 *B–D*). *CgMADS1* cDNAs were detected by RT-PCR in tissues containing oogonia and antheridia, whereas no transcripts were detected in gametophytic tissues other than these reproductive organs (Fig. 4*A*). To examine the expression in the only diploid cells of the life cycle, the zygotes were collected during the period of dormancy preceding meiosis. Again, no *CgMADS1* transcripts were detected. The expression patterns were examined in detail by *in situ* hybridization of the reproductive organs (Fig. 4 *B–D*). *CgMADS1* expression was detected in both oogonia and antheridia on the node of the stem-like branched filaments. *CgMADS1* signals were detected in oogonium and antheridium tissues during their development (Fig. 4*B*). Expression was detected in the egg cell and more weakly in the tube cells surrounding the egg cell (Fig. 4*C*). In the antheridium, *CgMADS1* mRNA was detected in the filaments that give rise to spermatozoid and very weakly in shield cells, which form the outermost layer of the antheridium (Fig. 4*D*).

C. peracerosum–strigosum–littorale complex is a unicellular, heterothallic green alga. When the vegetative cells of two different mating types (mt^- and mt^+) are cocultured in nitrogen-deficient medium, the cells divide and differentiate into two gametangial cells (25). Gametangial cells of opposite mating types form a pair and release their gametes, which conjugate to form a zygote. The temporal expression pattern of the desmid *CpMADS1* gene was quantified by using real-time PCR (Fig. 5). Two sets of primers that hybridize in the MADS or K domain of *CpMADS1* were used. *CpMADS1* expression gradually increased after cocultivation in nitrogen-deficient medium was begun, with the highest expression at 4 h after the beginning of cocultivation, at which point the vegetative cells were communicating with the opposite mating-type cells and beginning to differentiate into gametangial cells (25). *CpMADS1* expression decreased at ≈8 h after the onset of cocultivation, when most of the cells had begun to differentiate into gametangial cells but before fertilization (25). Expression analysis by *in situ* hybridization was not successful.

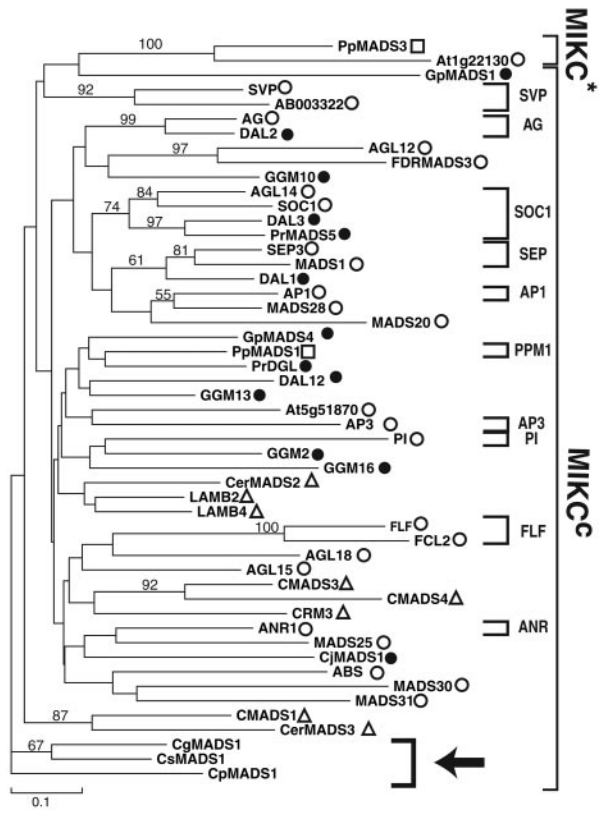

Fig. 3. A neighbor-joining tree showing the relationships among the three charophycean MADS-box genes and other MIKC^{C}- and MIKC*-type MADS-box genes from land plants using 90 amino acids as sequence data. This tree is unrooted. The taxa from which the genes were isolated are shown beside the protein names. Open circles, angiosperms; filled circles, gymnosperms; open triangles, pteridophytes; open squares, mosses. Charophycean genes are indicated by an arrow. Bootstrap values exceeding 50% are indicated on the branches. Subfamilies and types of MIKC^{C}-type MADS-box genes are labeled by brackets to the right. (Scale bar, 0.1 amino acid substitution per residue.)

Discussion

Evolution of MIKC^{C}- and MIKC*-Type Genes. Both MIKC^{C}- and MIKC*-type genes have been characterized from seed plants, pteridophytes, and mosses, indicating that the two types diverged before the divergence of mosses and other land plant lineages. The characteristics of the MIKC^{C}-type genes of the three charophycean green algae indicate that the common ancestor of the three charophyceans and land plants contained MIKC^{C}-type genes. If no other MADS-box genes including both MIKC^{C}- and MIKC*-type genes exist in the charophycean genomes, the MIKC^{C}-type genes are likely ancestral to the MIKC*-type genes. No MIKC^{C}- and MIKC*-type MADS-box genes could be identified in the entire nuclear genomes of the green algae *Chlamydomonas reinhardtii* (http://genome.jgi-psf.org/chlre2) and the red algae *Cyanidioschyzon merolae* (http://merolae.biol.s.u-tokyo.ac.jp), although each genome contains a single MADS-box gene lacking the I, K, and C domains (entry numbers C-1930010 and 4202, respectively). This finding suggests that the MIKC^{C}- and MIKC*-type MADS-box genes evolved in the charophycean–land plant lineage after its divergence from the *Chlamydomonas* lineage.

The MIKC^{C}-type genes have been classified into more than seven groups (13) that diverged before the divergence of angiosperms and gymnosperms. However, one monophyletic group of MIKC^{C}-type genes has been identified in mosses, one group from lycopods, and three monophyletic groups from

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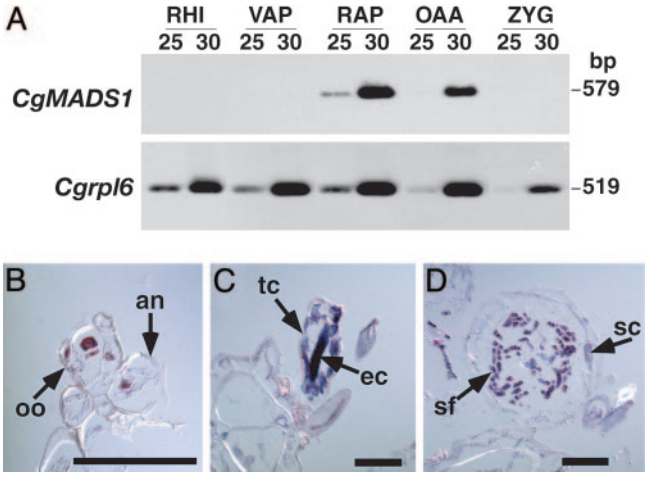

Fig. 4. Expression patterns of the stonewort *C. globularis CgMADS1* gene. (*A*) Southern blot analysis of *CgMADS1* RT-PCR products. Complementary DNA was synthesized from RNA isolated from rhizoids (RHI), the vegetative apical part of the thallus not including the oogonia and antheridia (VAP), the reproductive apical part of the thallus with the oogonia and antheridia (RAP), the oogonia and antheridia containing mature eggs and spermatozoids (OAA), and a zygote in the dormant period (ZYG). The stonewort L6 gene *Cgrpl6*, which encodes a ribosomal protein, was used as a positive control. Expression of *CgMADS1* mRNA in a young oogonium and antheridium (*B*), a mature oogonium (*C*), and a mature antheridium (*D*) was detected by *in situ* hybridization by using a *CgMADS1* antisense mRNA probe. oc, oogonium; an, antheridium; ec, egg cell; tc, tube cell; sf, spermatogenous filaments (which divide to form spermatozoid); sc, shield cell. (Scale bars, 100 μm.)

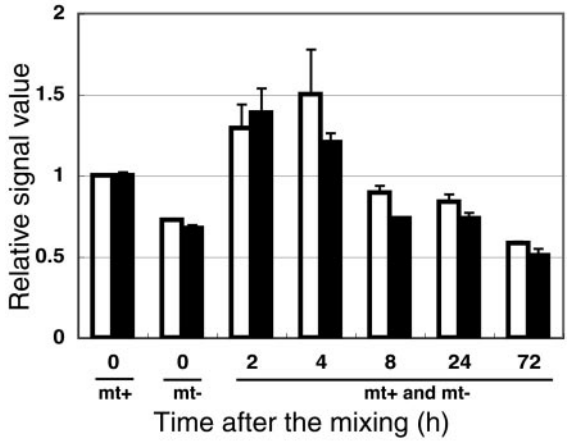

Fig. 5. Expression pattern of the *CpMADS1* gene. The relative signal values in real-time PCR are shown as means and standard errors. White and black bars represent signals for MADS and K domains, respectively. The signal values detected from mt^+ cells before the mating reaction were converted to values of 1.0.

ferns. These findings suggest that extensive diversification of the MIKC^C-type genes through gene duplication occurred after the divergence of the seed plant lineage from ferns, although we cannot eliminate the possibilities of extensive losses of genes in ferns and the moss lineages in parallel and of incomplete sampling of MADS-box genes in these species. Only one MIKC^C-type gene was found in each charophycean green alga, suggesting that the divergence of the MIKC^C-type genes in these organisms was not as extensive as in land plants and that the successive diversification of MIKC^C-type genes in the seed plant lineage was important for the evolution of more sophisticated developmental systems in higher land plants, which is in agreement with the fact that different MIKC^C-type genes are usually involved in different important processes in angiosperm development.

CgMADS1 and *CpMADS1* each contain a single exon downstream of the K box (Fig. 2), suggesting that the longer C region, including several exons, was likely acquired in land plants after their divergence from the lineage that led to extant charophyceans. Although the C domains are very diverse, they are quite conserved within each subfamily of MIKC^C-type genes, supporting the hypothesis that the acquisition and subsequent diversification of this domain was probably involved in determining the functions of the different subfamilies of MIKC^C-type genes (26–28). Thus, it is likely that the elongation and divergence of the C domains in the land plant MIKC^C-type genes after the divergence from charophyceans was important for the diversification of organs and body plans in land plants.

Putative Functions of MIKC^C-Type Genes in Charophytes. Expression of the desmid *CpMADS1* was induced just before gametangial cell differentiation from vegetative cells, and expression decreased when fertilization occurred. This expression pattern suggests that *CpMADS1* has a role in the differentiation of gametangial cells, which correspond to the egg and spermatozoid of charophytes and land plants. The stonewort *CgMADS1* was dominantly expressed in egg and spermatozoid, suggesting that it has a similar function as the desmid *CpMADS1*. It should be noticed that neither *CpMADS1* nor *CgMADS1* likely functions in the diploid generation because the expression level is lower than in the haploid generation. As transformation and RNA-interference techniques are not presently available in these green algae, additional functional analyses of these genes are goals for the future. The putative involvement of these genes in haploid reproductive cell differentiation is similar to that of the yeast MADS-box gene *MCM1*, which is required for the transcription of cell-type-specific genes (29). Similar to the expression of *CgMADS1* in both female and male reproductive cells, *MCM1* is expressed in both a- and α-type cells as well as diploid cells. Along with associated proteins, *MCM1* regulates cell-type-specific transcription of downstream genes. Additional studies on the charophycean MADS-box genes should provide insight into whether sex-specific haploid reproductive cell differentiation is controlled by similar gene cascades involving MADS-box genes in yeast and green plants; alternatively, it may be that two different gene cascades control similar differentiation processes in parallel, using members of the same gene family.

According to phylogenetic studies and paleobotanical evidence, the most recent common ancestor of land plants was probably a multicellular organism with a haplontic life cycle and oogamous reproduction (4). Thus, land plants inherited a multicellular haploid gametophyte from an ancestral charophycean-like green alga, whereas a multicellular sporophyte evolved after the land plant lineage diverged from the extant charophycean lineage (30). The gametophytic MADS-box genes reported in this study, therefore, may have more ancestral features than do sporophytic MADS-box genes that function in the development of multicellular sporophytic organs, including floral organs. The recruitment of gametophytic MADS-box genes for sporophytic development probably occurred during the early stages of the evolution of land plants. Recently, some MIKC^C-type genes from *Arabidopsis* and other angiosperms were shown to be expressed in pollen and the embryo sac, in which the male and female haploid generative cells differentiate, respectively (9, 31, 32). Comparisons of the functions of these genes with the charophycean genes will provide additional insight into the origin and evolution of plant body plans and of MADS-box genes.

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