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Identification and characterization of a rice MCM2 homologue required for DNA replycation

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The pre-replication complex (pre-RC), including the core hexameric MCM2-7 complex, ensures that the eukaryotic genome is replicated only once per cell division cycle. In this study, we identified a rice minichromosome maintenance (MCM) homologue (*OsMCM2*) that functionally complemented fission yeast MCM2 (CDC19) mutants. We found *OsMCM2* transcript expression in roots, leaves, and seeds, although expression levels differed slightly among the organs. Likewise, the OsMCM2 protein was ubiquitously expressed, but it was downregulated when nutritients were limiting, indicating that MCM2 expression (and therefore cell cycle progression) requires adequate nutrition. Yeast two-hybrid and GST pull-down assays demonstrated that OsMCM2 interacted with the COP9 signalosome 5 (CSN5). Taken as a whole, our results indicated that OsMCM2 functions as a subunit of the rice MCM complex and interacts with CSN5 during developmental regulation. [BMB reports 2008; 41(8): 581-586]

INTRODUCTION

Eukaryotic cells precisely coordinate their chromosomal replication events to generate only one chromosome set per cell division cycle (1, 2), and the minichromosome maintenance (MCM) proteins play a major regulatory role in this process. DNA duplication begins at replication origins that serve as scaffolding for the origin-recognition complex (ORC). The ORC oscillates between two states, the pre-replication and post-replication complexes (pre-RC and post-RC, respectively), depending on whether the cell is in the G1 or the S phase of the cell cycle. During G1, ORC and MCM10 bind to the replication origin and recruit cdc6 and cdt10, which in turn induce the MCM complex to load onto the replicating DNA. After the MCM complex is recruited, it in turn recruits the cdc7-dbf4 kinase, which replaces cdc6. Once the cell enters S phase, cdc7-dbf4 phosphorylates the MCM complex to mediate a conformational change that unwinds the DNA at the replication origin (3-6). MCM then recruits cdc45, which dissociates MCM10, enabling DNA polymerase α and primase to initiate DNA synthesis. Once DNA replication begins, the MCM proteins exit the nucleoplasm into the cytoplasm, and they are not able to reassociate with DNA until the cell completes a full round of mitosis (7). This process is conserved in plants; the maize MCM6 homologue (ZmMCM6) re-enters the nucleus during G1 phase in preparation for the assembly of the pre-RC (7).

MCM proteins were initially identified in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* and dubbed the MCM and CDC proteins, respectively. Later, homologues were identified in all types of eukaryotes (1) including plants (8-10). Within the cell, six of the MCM proteins (MCM2-MCM7) interact with each other in equimolar quantities to form a relatively stable globular structure. This complex has licensing activity, as was shown in previous *in vitro* replication assays using *Xenopus* egg extracts (11 and references therein), and it is subdivided into three parts: the MCM4/6/7 core complex, MCM3/5, and MCM2 (3, 12, 13). Since the latter two associate weakly with the core complex, some have proposed that they might regulate the DNA helicase activity of the MCM4/6/7 complex (14). MCM2 interacts with many nuclear proteins, such as H3 (15, 16), histone aetyltransferase (17), and RNA polymerase II (18). MCM2 also interacts with cdc7/dbf4 and is required for their phosphorylation, a critical step preceding DNA replication (19). These previous results demonstrate a crucial role for MCM2 in DNA replication.

A plethora of studies in many organisms report structure-function relationships of the MCM proteins, but only a limited number were conducted in plants. One relatively well-characterized plant MCM protein, *PROLIFERA* (*PRL*), encodes an *Arabidopsis* MCM7 homologue (9, 10, 20). *PRL* functions in early embryogenesis (9) and cytokinesis during seed development (20), implicating MCM7 in rapid cell division. Consistent with the function of AtMCM7, a maize MCM7 homologue (*ZmPRL*) is preferentially expressed during early embryogenesis (21). Another member of this MCM gene family (*MCM3*) is also predominantly expressed in proliferating cells, similarly to *MCM7*, in both maize (8, 22) and Arabidopsis (23). A homologue of a third class of MCM proteins, MCM6, has also been identified in a plant, namely maize (*ZmMCM6*) (7). *ZmMCM6*,

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which is highly expressed in the actively-dividing developing kernel, exhibited nucleo-cytoplasmic transporting activity.

Herein, we identified a rice gene as a homologue of MCM2 (*OsMCM2*) by demonstrating that it complemented the *cdc19* mutant in fission yeast and that it interacted with the COP9 signalosome 5. Our result is one of only a few reports characterizing a plant MCM protein, and thus will help further our understanding of the cell division process in plants.

RESULTS AND DISCUSSION

Structural and functional features of *OsMCM2*

We conducted a screen for genes preferentially expressed early in developing rice seeds, using a library constructed from five-day-old rice embryos (24). This screen yielded a putative 2,525-bp MCM2 homologue (*OsMCM2*), with an open-reading frame encoding a 674-amino acid protein with a calculated molecular mass of 75 kDa (Fig. 1A). Genomic DNA gel blot analysis, using 264-bp or 1,813-bp probes from the 5'-upstream region (including the 5' UTR), indicated that it was derived from a single copy gene (data not shown). A search of the rice genome sequence revealed that this gene was annotated as Os06g11500 (TIGR locus numbers TC307083 and TC287555), comprising 21 exons (Fig. 1A). The putative OsMCM2 protein contained an MCM motif, a DNA-dependent ATPase motif, and two zinc-finger motifs (Fig. 1A), all of which are characteristic of MCM family proteins (1).

To gain further insight regarding the identity of the putative OsMCM2, we generated a phylogenic tree by comparing the OsMCM2 polypeptide with different types of core MCM polypeptides (MCM2-MCM7) from various species (Fig. 1B). OsMCM2 exhibited the highest homology to the MCM2 protein family, but it diverged from the rest of the family at a rather early point.

The remarkable homology between OsMCM2 and the budding and fission yeast MCM2 (known as CDC19 in *S. pombe*) prompted us to examine whether OsMCM2 could complement a yeast CDC19-deficient mutant, in order to verify the function of OsMCM2. In these experiments, we introduced *OsMCM2* into a *ts* mutant lacking CDC19 activity (25), using an *OsMCM2* construct under the control of the thiamine-repressible *nmt* promoter (Fig. 2, upper panel). With this experimental design, withholding uracil and thiamine induced OsMCM2 expression. Although a *cdc19* mutant cell transformed with the empty SLF172 vector was not viable at restrictive temperature (37°C), cells expressing pOsMCM2 (pSLF172 with *OsMCM2*) grew normally at 37°C (Fig. 2). These results demonstrated that

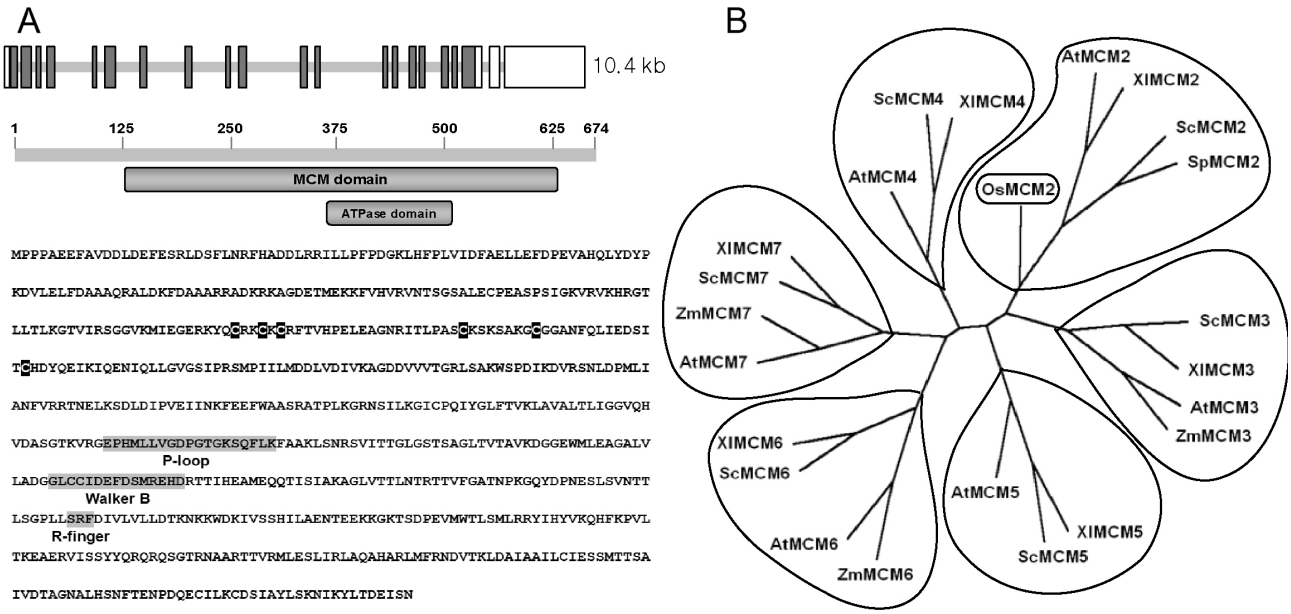

Fig. 1. Gene and protein structure of OsMCM2, and phylogeny of MCM proteins from various species. (A) Gene structure of *Os06g11500*, which encodes OsMCM2 (upper panel); a diagram of the putative domain structure of the OsMCM2 protein (middle panel); and motif locations within the OsMCM2 polypeptide (bottom panel). Amino acid residues in black indicate the Cys residues of the zinc-finger motifs. The P-loop and Walker B motifs constitute the ATPase domain required for NTP binding, whereas the R (Arg)-finger is the catalytic domain. (B) An un-rooted phylogeny drawn using ClustalW program, based on representative amino acid sequences of the MCM2- MCM7 core complexes from various species: *Arabidopsis* [AtMCM2-NM_103572, AtMCM3-NM_123997, AtMCM4-NP_179236, AtMCM5- NP_178812, AtMCM6-NP_680393, AtMCM7 (PRL)-L39954], rice [OsMCM2-NM_001063693], budding yeast [ScMCM2-P29469, ScMCM3- P24279, ScMCM4-S56050, ScMCM5-P29496, ScMCM6-S64219, ScMCM7-S34027], fission yeast [SpCDC19-SPU08048], frog [XlMCM2- U44047, XlMCM3-U26057, XlMCM4-T47223, XlMCM5-PC4225, XlMCM7-T47221, XlMCM6-AF031139], and maize [ZmMCM3 (ZmROA)- AAD48086, ZmMCM6-AAW55593, ZmMCM7 (ZmPRL)-CAC44902].

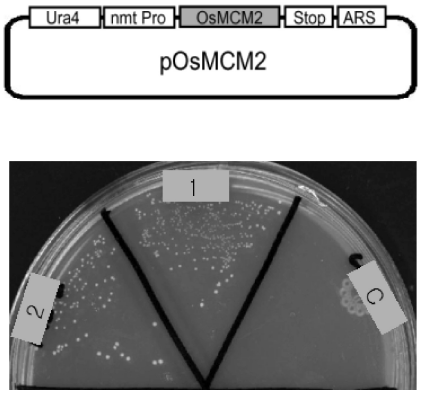

Fig. 2. *OsMCM2* cDNA functionally complemented *cdc19* fission yeast mutants. *OsMCM2* was expressed under a full-length thiamine-repressible promoter in pSLF172 (upper panel), and resulting transformants were grown at 37°C on EMM plates without uracil and thiamine (bottom panel). C; pSLF172 vector, 1 and 2; pOsMCM2 (*OsMCM2* in pSLF172). *OsMCM2* rescued the growth of *ts* mutant fission yeast defective in *cdc19* gene expression, suggesting that *OsMCM2* is homologous to *MCM2* in budding yeast.

OsMCM2 encodes a functional homologue of the CDC19 fission yeast protein, consistent with the structural relationship between OsMCM2 and MCM2 and CDC19 in budding and fission yeast.

OsMCM2 transcripts and proteins were ubiquitously expressed in rice plants

To more thoroughly understand the roles of the *OsMCM2* gene in *planta*, we next examined the *OsMCM2* expression pattern in rice plants (leaves, seeds, stems, and roots) using RT-PCR analysis. All tissues examined in this study contained *OsMCM2* transcripts (Fig. 3A), suggesting this gene is ubiquitously expressed in rice. Given the important role of MCM2 during cell cycle progression in dividing cells, this result is not surprising. Previous reports revealed that maize *MCM7*, a subunit of MCM2-7, was also expressed throughout plant development (21), similarly to *OsMCM2*. Despite its ubiquitous expression, the actual expression levels varied between the organs examined; specifically, *OsMCM2* transcripts were expressed in a lower level in the mature seeds, stems, and roots than in leaves (young and expanding) and milky-stage seeds. It therefore appeared that young or expanding tissues expressed higher levels of *OsMCM2* transcripts than older tissues.

We next sought to examine OsMCM protein expression in developing rice plants, and to address whether expression levels depended on nutritional status. For this purpose, we generated a polyclonal OsMCM2 antibody and utilized it in immunoblot analyses of soluble proteins from rice seeds [12-15 days after fertilization (DAF)], roots, and leaves (Fig. 3B, control tissue). In agreement with the RT-PCR results, all organs examined in the study expressed OsMCM2 proteins, with the lowest expression levels in mature leaves.

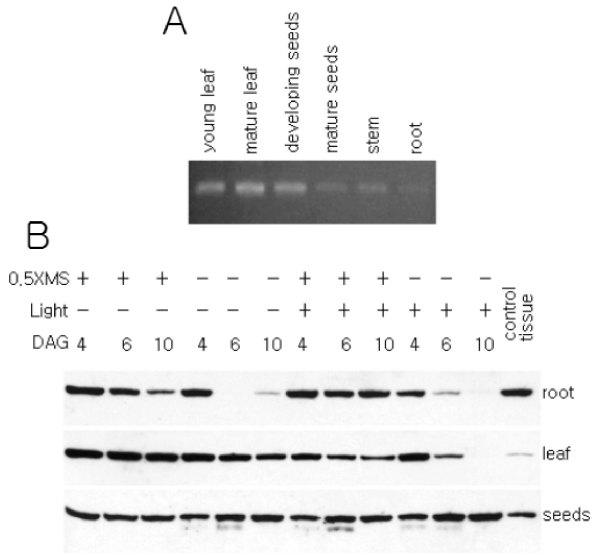

Fig. 3. OsMCM2 was ubiquitously expressed in rice, but expressed at higher levels in developing tissues. (A) Using RT-PCR, expression of *OsMCM2* mRNA was examined in leaves (young seedling leaves and mature expanding leaves), 10- to 15-day-old developing seeds, fully matured seeds, stems, and mature roots. (B) Seeds were germinated under the indicated conditions, and the roots, coleoptiles (including leaves), and seeds were separated. Immunoblot analysis was conducted on 20 μg of total soluble protein per lane. Control tissue was from mature plants. DAG; days after germination.

Because nutrient and carbon sources reportedly regulate the expression of many cell cycle-related genes (26-29), we investigated whether limiting nutrients altered the OsMCM2 protein expression pattern in germinating seeds. Withholding nutrients dramatically reduced OsMCM2 expression in roots, but it did not significantly affect levels in germinating seeds, which most likely utilized stored nutrients for cell growth. This result is consistent with previous studies, in which nutrient limitation arrested many plant cells at G1 phase (26).

OsMCM2 interacted with CSN5

To identify OsMCM2 interacting partners, we carried out a yeast two-hybrid screen, using OsMCM2 as the bait and a rice embryo cDNA library as the prey. Interestingly, the screen suggested that OsMCM2 interacted with JAB1 (Jun activating binding protein), also known as COP9 signalosome 5 (CSN5), corresponding to TIGR annotation Os10g41390 (GenBank accession AK099088). Subsequent domain mapping experiments demonstrated that the full-length CSN5 polypeptide is required for the MCM2 interaction (Fig. 4A). Because JAB1/CSN5 interacted with Gal4 DNA binding protein in a previous study (30), we verified the interaction between CSN5 and OsMCM2 using a GST pull-down assay (Fig. 4B).

CSN5 participates in a variety of signaling pathways, such as light-responsive signaling (30) and cell cycle control (30-

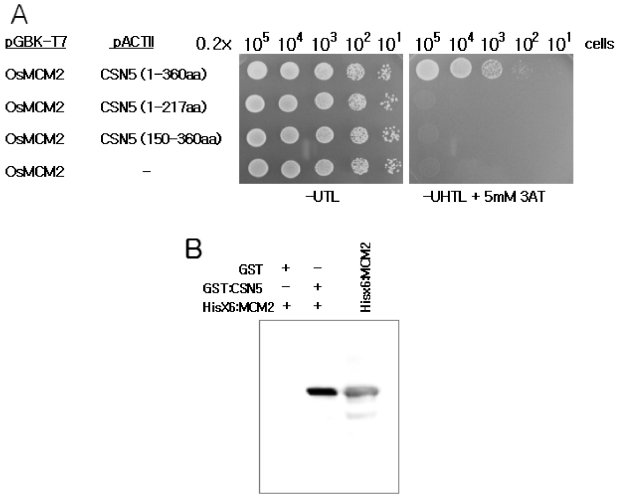

Fig. 4. OsMCM2 interacted with CSN5. (A) In a yeast two-hybrid assay, OsMCM2 interacted with only full-length CSN5. U, uracil; H, histidine; T, tryptophan; L, leucine; 3AT, 3-amino-1,2,4-triazole. (B) This interaction was verified with a GST pull-down assay using immobilized GST-CSN5 fusion proteins and OsMCM2 proteins. Note that the GST-CSN5 fusion interacted with His-tagged OsMCM2 proteins, but that control GST proteins did not. Hisx6: MCM2, His-tagged OsMCM2 positive control protein expressed in *E. coli*.

33). Rice CSN5, the same protein identified in our screen, can also interact with PCNA (proliferating cell nuclear antigen) (31), implicating CSN5 in plant cell division activity as well. It is therefore possible that (a) OsMCM2 may be degraded after firing the pre-RC, and/or that (b) CSN5 indirectly phosphorylates OsMCM2.

In conclusion, *OsMCM2* is the rice homologue of yeast *MCM2*. OsMCM2 functionally complemented fission yeast MCM2 mutants, strongly suggesting that it might play a role in G1 to S phase transition during the cell cycle. Further, we showed that OsMCM2 interacts with CSN5. These results add significantly to our understanding of cell cycle progression in plants.

MATERIALS AND METHODS

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated according to a protocol described by Pawlowski *et al*. (34). First-strand cDNA was synthesized with 3 μg of total RNA using the ImProm-II™ Reverse Transcription System (Promega; Madison, WI, USA) followed by PCR amplification with Taq DNA polymerase, according to the manufacturer's recommendations. PCR amplification of the *OsMCM2* gene was performed as follows: 25 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C, using the primer pair 5'-CCTG AAATGTGATTCCATAGCTTACTTATCCAAGA and 5'-AGGGAG TAGTTGC TATTTCAGACCTTCAAGT.

Production of polyclonal OsMCM2 antibody

Recombinant *N*-terminal Hisx6-tagged OsMCM2 was expressed in *E. coli* as follows: The DNA insert was PCR amplified with F5-Bam (5'-TTGGATCCACCATGCCGCCGCCGGC-GGAGGAGT) and F3-Not (5'-AAGCG GCCGCTTATCAGT-TCGAGATTTCATCAGT) primers using pfu DNA polymerase and an *OsMCM2* cDNA template. The PCR product was digested with *Bam*HI/*Not*I, cloned into pET30a(+), and transformed into *E. coli* BL21 (DE3). Transformed bacteria were cultured to an OD_{600} of ~0.5, and protein expression was induced by exposing them to 0.5 mM IPTG for 3 hours. The Hisx6-tagged antigen was purified using a nickel-NTI affinity column (Novagen; Madison, WI, USA) according to the manufacturer's instructions. The partially-purified protein was separated from contaminating proteins with SDS-PAGE, after which the 100-kDa band corresponding to OsMCM2 was cut out of the gel with a razor blade, incubated in water for an hour, and ground to small pieces in PBS solution overnight. This purified protein was used to immunize rabbits, and antiserum was collected from the rabbits after the second boost.

Immunoblotting for OsMCM2

To fractionate soluble proteins, total protein extracts were prepared according to Martinez-Garcia *et al*. (35) and resolved in 8% SDS-PAGE. Immunoblots were performed as previously described (36), using the anti-OsMCM2 described above at a 1 : 3,000 dilution and horseradish peroxidase-conjugated anti-rabbit IgG (Sigma; St. Louis, MO, USA) at a 1 : 5,000 dilution.

Complementation test in *CDC19* mutant yeast (*Shizosaccharomyces pombe*)

To evaluate whether *OsMCM2* complemented the budding yeast mutant *CDC19-P1*, the *OsMCM2* cDNA insert was first amplified as described above, digested with *Bam*HI/*Not*I, and cloned into the *Bgl*II/*Not*I restriction sites of pSLF172 (ATCC 87609) (37) to produce pSLF172-OsMCM2 (pOsMCM2). This construct, in which OsMCM2 expression was driven by the full-length *nmt* promoter, was transformed into *CDC19* mutant fission yeast (FY360h+ *cdc19-P1 leu1-32 ade-M210 ura4-D16 ts*) according to the previously-described TRAFO protocol (38). Transformed yeast was plated on Edinburgh minimal medium (EMM) supplemented with adenine and leucine (39) and incubated at the restrictive temperature (37°C) for about 60 hours.

Yeast two-hybrid assay

The *OsMCM2* coding sequence (CDS) was amplified using PCR with MCM2-NcoI (5'-TTCCATGGAGATGCCGCCGCCG-GCGGAGGAGT) and MCM2-BglII (5'-CCAGATC TGGCAAG-AACATCCATATACGTGGCACTACAG) primers, digested with *Nco*I/*Bgl*II, and cloned into the *Nco*I/*Bgl*II site of the DNA-binding domain vector pGBKT7 (Clontech; San Jose, CA, USA). To screen for MCM2-interacting proteins, this bait plasmid was transformed into AH109 yeast containing a pACTII library con-

structed from RNAs from five-day-old rice embryos (24).

MCM2-CSN5 interaction analysis was performed in AH109 cells harboring pGBKT7-OsMCM2 and one of the following constructs: pACTII-CSN5F (full-length), pACTII-CSN5N (*N*-terminal region), or pACTII-CSN5C (*C*-terminal region). Primers used to amplify the CSN5 DNA fragments are as follows: CSN5BamHI (5'-ACCGGATCCGGATGGAGCCCACCTCGTCG), CSN3XhoI (5'-ACCCTCGAGTCATGCTTCAACCATAGGCTC), CSN5BamHI-1 (5'-AC CGGATCCAACTGGTTATCAGGCATT-GA), and CSN5 XhoI-1 (5'-ACCCTCGAGTCTA TCTTGTTG-AGTGGTAT). Inserts were amplified using CSN5BamHI/CSN3XhoI for CSN5F (1-360 aa), CSN5BamHI/CSN3XhoI-1 for CSN5N (1-217 aa), and CSN5BamHI-1/CSN3XhoI for CSN5C (150-360 aa). The resulting DNA fragments were digested and cloned into the *Bam*HI/*Xho*I site of pACTII.

GST pull-down assay

To express GST:CSN5F fusion proteins, a *Bam*HI/*Xho*I DNA fragment in pACTII-CSN5 was cloned into pGEX-5X-1 (GE Healthcare; Buckinghamshire, UK), and 1 mL GST:CSN5F lysate in lysis buffer (20 mM Tris-Cl, pH8.0, 10 mM EDTA, 30 mM NaCl, 2 mM PMSF) was incubated with 10 µL of glutathionine Sepharose beads at 4°C for about 12 hours. Beads were then washed with lysis buffer containing 150 mM NaCl, incubated with equal amounts of 6xHis:MCM2 proteins at 4°C for about 6 hours, and washed three times. Proteins were eluted from the beads with 10 µL of elution buffer containing 10 mM glutathionine (GE Healthcare). Proteins were fractionated on 10% SDS-PAGE and hybridized as previously described (36), except that primary and secondary antibodies were used at dilutions of 1 : 7,500 and 1 : 5,000, respectively.

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