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Specific expression of *ZmPRL*, the maize homolog of MCM7, during early embryogenesis

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Abstract

The maize cDNA sequence (ZmPRL) homologous to the Arabidopsis thaliana PROLIFERA or to the different MCM7 genes (yeast cdc47, as an example) has been identified by differential display due to its increased mRNA accumulation in the apical region of the maize immature embryo. Probably two sequences coding for ZmPRL exist in the maize genome and when comparing the different MCM protein sequences available, the protein clusters with other MCM7 proteins already described. Analysis by in situ hybridization shows that the pattern of ZmPRL mRNA accumulation is in agreement with the pattern observed for genes related to cell cycle in plants and that the expression is mostly found in the apical meristem. It is proposed that this is a consequence of the mechanisms that control the formation of the apical-basal pattern in plant embryo acting upon the regulation of the cell cycle. © 2002 Published by Elsevier Science Ireland Ltd.

Keywords: Maize; Embryogenesis; Cell cycle; Mcm proteins

1. Introduction

The control of cell cycle is a highly conserved process between different organisms. The cycle may be divided in two phases, one of synthesis of DNA and another one of mitosis. They are separated by control gaps, G1 and G2, which are checkpoints that ensure that the process of duplicating the genome and division of the cell is taking place in a correct way. One important control is that each fragment of DNA making up the genome is replicated only once in each cycle [1]. It was proposed [2] that there should exist a replication licencing factor that during G1, right before S phase, binds to DNA and allows it to replicate. Once replication has started, the licencing factor is displaced from chromatin, either inactivated, degraded or re-exported out of the nucleus, leaving DNA in a non-permissive fashion for replication [3,4].

Searching for mutants in yeast defective in DNA synthesis, or mutants that showed cell cycle arrest at

the G1/S transition, the MCM (minichromosome maintenance) family of proteins was identified [5]. MCM mutants are unable to sustain the autonomous replication of minichromosomes bearing certain types of autonomously replicating sequences [6]. MCM proteins are required for the initiation of DNA replication at the multiple origins of replication that the genome contains, by binding to the origin recognition complex (ORC) during G1/S phase and, once replication has started, they are displaced from chromatin [7-9]. It has recently been shown that MCM proteins are not only required for the initiation of DNA replication, but they are also necessary for DNA elongation. In fact, it has been demonstrated that the complex has helicase activity in vitro and MCM members share a DNA dependent ATPase motif resembling the one found in some DNA helicases [10-12]. This family of proteins has been studied in different eukaryotes such as human [6], mouse [13], Drosophila [14,15], Xenopus [16], yeast [4,7], and plants [17-20], and it has been shown that they share a high degree of conservation among the different species analyzed, indicating their important role in the control of the replication of DNA.

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It has also been reported that CDK-mediated phosphorylation of MCM2-7 complex plays an important role in the control of DNA replication. These proteins can only bind chromatin in an underphosphorylated state, and this only happens during G1 [1,16]. On the other hand it has also been postulated that the retinoblastoma protein (Rb), a highly conserved tumor growth suppression factor having a central role in the regulation of G1/S phase, is also involved in the control of initiation of replication. During G1, Rb in mammals is phosphorylated by CDK4 and CDK6 cyclinD dependent kinases, releasing transcription factors such as E2F, that are involved in the transcription of many genes [21-23]. It has recently been shown by a two hybrid analysis that Rb interacts with a member of the MCM family, MCM7 [24]. During G1, when Rb becomes phosphorylated, it will release MCM7 allowing it to form the hexameric MCM2-7 complex required for the initiation of DNA replication. During S phase, when Rb is again hipophosphorylated, it will recruit MCM7, dissociating it from chromatin. Therefore, Rb is negatively regulating gene transcription by E2F binding and DNA replication by MCM7 binding [24].

The control of cell cycle in plants is not so well characterized as it is in other organisms. However, as more information is available, it is becoming evident that there are many parallels between plant cell cycle and animal or yeast cell cycle. This relation reinforces the idea that cell cycle is such an important process for the development and survival of the organism that it should be highly conserved among eukaryotes. In the majority of organisms, the most important control of the cell cycle occurs late in G1, when a committed decision to divide is made by the cells. This regulation is of particular interest because of their significance in the context of plant development and cell differentiation, specially during embryogenesis. In fact, the rate and direction of cell division and elongation are the essential mechanisms that plants use during embryogenesis in order to define the basic elements of the structure of the adult plant.

Little is known about the molecular mechanisms controlling G1/S transition in plants, although several D-type cyclins have been isolated, as well as different CDKs [25]. In maize and other plant species, the homolog of Rb has been reported [22] and recently, the existence of E2F in plants has also been described [26]. During G1, there is an increase in the levels of D-type cyclins. It has been proposed that cyclin D can associate with Cdc2a to phosphorylate the Rb protein, inactivating it and releasing E2F transcription factors. Several genes could then be transcribed and the cell cycle allowed to progress through the S phase [27].

Here, we report the cloning and characterization of MCM7 from maize embryo. It has been cloned by differential display and it encodes a protein, which is

highly similar to the PROLIFERA protein of Arabidopsis and other MCM7 proteins from various organisms. In yeast it corresponds to the cdc47 gene. PROLIFERA in Arabidopsis was identified using gene trap tagging and was shown to be required for megagametophyte and embryo development, having also a more general role in cell proliferation throughout the plant [17,18]. Up to now, only another member of the MCM family of proteins, MCM3, has been reported in maize and it is also required for proliferation in the meristematic tissues of the plant [19]. In our group, we are interested in the different mechanisms controlling embryogenesis of maize and defining the pattern of the embryo. Genes with a function in the control of cell division are essential for these purposes. Following the cloning of the maize analog of MCM7 by differential display, its expression during maize early embryogenesis was analyzed.

2. Materials and methods

2.1. Biological material

The plant material that has been used in this work is derived from seeds of *Zea mays* cv W64A pure inbred line grown in the greenhouse of the Departament de Genètica Molecular (IBMB, CSIC) in Barcelona, Spain. Seeds were germinated for 5 days under the following cyclic conditions, 16-h light at 26 °C and 08-h dark at 24 °C.

2.2. Differential display

RNA was obtained from 20 days after pollination (DAP) embryos that had been dissected in three regions, the apical, the node and the basal regions. Differential display procedures were carried out as described by Liang and Pardee [28]. Reverse transcription was performed on 0.2 µg of total RNA and using a T₁₂MN oligonucleotide as anchor-primer. Two microliter of this cDNA was used as template for the polymerase chain reaction (PCR), in which an arbitrary decamer oligonucleotide was used in combination with the T₁₂MN oligonucleotide. Products were labeled with ³⁵S-dATP and separated on a denaturing polyacrylamide gel. This gel was exposed to an X-ray film and bands of interest were cut out, reamplified with the same combination of oligonucleotides and cloned into a pBluescript vector.

2.3. Molecular cloning, cDNA library screening, RACE and sequencing

General recombinant techniques were performed as described by Sambrook et al. [29]. A cDNA library from 12 DAP maize embryos was screened with the cDNA fragment obtained with the differential display. The remaining 5' end of the cDNA was cloned using the 5'-RACE technique (Life Technologies, Inc.) following the manufacturers' protocol. DNA sequencing was carried out using the automatic fluorescent sequencing (ALF, Amersham Pharmacia Biotech).

2.4. Sequence alignment and phylogeny

ZmPrl was aligned to different MCM7 amino acid sequences using the CLUSTALW multiple-alignment program (version 1.5; [30]). PROTDIST in Felsenstein's [31] PHYLIP package was used to generate a distance matrix based on the Jukes–Cantor algorithm [32]. This was used to generate neighbor-joining trees [33]. Bootstrap analyses were performed using the Seqboot and Consense programs from Felsenstein [31] PHYLIP package

2.5. RNA blot and Southern blot analysis

Tissues were collected from different stages of embryogenesis and from different parts of the germinating plant. Material from the apical, the central and the basal regions of 20 DAP embryos was also collected. All the material was frozen in liquid nitrogen and kept at -80 °C until use. Total RNA was extracted and 10 µg of it were denatured and fractionated on a denaturing gel as described by Logemann et al. [34]. The gel was transferred to a nylon membrane (Nytran; Schleicher and Schuell) using $20 \times$ SSC as a transfer buffer. Maize genomic DNA was isolated as described by Chen and Dellaporta [35] and transferred under alkaline conditions into a nylon membrane (Nytran; Schleicher and Schuell) following the manufacturer's protocol. Northern and Southern blots were fixed, hybridized and washed as described by Church and Gilbert [36]. Probes were labeled using the random priming method following the protocol of the manufacturer (Random Primed DNA Labeling Kit, Boehringer, Mannheim).

2.6. In situ hybridization

Tissues from different developmental stages were collected and fixed in an ethanol:formaldehyde:glacial acetic acid (80:3:5:5) fixative solution for 1 h at room temperature and during 1 week at 4 °C. Once the fixative solution was removed, the material could be stored in 70% ethanol at 4 °C. Paraffin inclusion, tissue sectioning and tissue pretreatment was performed according to Langdale [37]. Riboprobes, hybridization, washes, blocking, antibody incubation and detection was carried out according to the suppliers' protocols (RNA color kit for non-radioactive in situ-hybridization, Amersham). Digoxigenin labeled hybrids were viewed using bright field microscopy and photographed using Kodak Ektachrome 160 films.

3. Results

3.1. Sequence of the cDNA coding for ZmPRL

In order to isolate and characterize genes involved in maize early embryogenesis, and/or genes with a specific expression in the different regions of the immature embryo, a differential display approach was used. A number of bands were detected as being preferentially expressed in the apical region of the embryo. One of the bands cloned and sequenced showed high homology to the PROLIFERA (PRL) gene from Arabidopsis [17]. For this reason, it has been named ZmPRL. The protein it encodes, Zm-PRL, belongs to the highly conserved MCM family of proteins that have been described in many organisms and are important for the control of the cell cycle [38]. ZmPRL, as well as AtPRL, shares homology to the MCM7 proteins of different organisms.

After the screening of a maize embryo cDNA library and the use of a RACE (rapid amplification of cDNA ends) approach, the full length cDNA coding for the ZmPRL was obtained. The deduced amino acid sequence from this cDNA is presented in Fig. 1 and has the AJ313531 Accession Number in the EMBL Database. The alignment with MCM7 from other organisms is presented in Fig. 1 where the amino acid sequences for the other MCMs homologs that has been cloned in maize, MCM3 (called ROA in the original article and in the figure), is also included.

The identity of ZmPRL with the other MCM7 ranges from 77% in *Arabidopsis* to 43% in *Caenorhabditis elegans*. However, the identity with the maize MCM3 homolog is 32%. This result indicates that the degree of conservation is higher between the same MCM member in different organisms than between different MCMs from the same organism. This observation is more clearly presented in Fig. 2, with the phylogenetic comparison of different MCM sequences. As stated above, MCMs are not grouped according to the species, but according to the member of the family they represent in agreement with the specificity of the function that the proteins perform in each organism.

The central region of the sequence, from T^{141} to Y^{640} represents the MCM domain. The central region, from G^{370} to D^{523} , contains a DNA-dependent ATPase motif, which includes four motifs A–D, thick line above the sequence in Fig. 1, that are similar to a modified version of the Walker-type NTP-binding domain [39]. This domain, highly conserved in the MCM family and in a vast range of ATPases, has a potential intrinsic helicase activity [40]. Labeled with asterisks are the four cysteines of the zinc finger type motif present in the different MCM7 sequences, and also in the MCM2,

MCM4 and MCM6 proteins [15]. This motif is of the type $CX_2CX_{18}CX_2C$, and it probably has an structural role or a role in protein–nucleic acid or protein–

protein interactions. The ZmPRL sequence also contains multiple sites of phosphorylation (not shown in the figure).

ZmPr1	MKNPDFALDEALAEDFLSNTTGPHGE	PEYLNILQDVANRKIRATQ	45
AtPr1	MKDHODDGDKGLAGGLADGRADANGR	SIMMEIDQEVSNRKIRADQ	45
SpMcm7	MALPTIDLKIPE EECQHKITDELSH KQEQVQDO	GOONODISMSDAGDEPFLKS WMDILOKISNRESNVIN	72
ScMcm7	MSAALPSIQLPV-D NNLFNEITDFLVT KQDTLSSDAT	RNENEDENLDAENIEQHLLEKGP MMAM QK NRELNS I	79
HaMem7	MALKONALEKEKVISKELQENYQDDELEK	KOP WOR VE WEROUN Y	49
V1Mcm7	MPRIMONERERCO TEL OF SYNDDEF SK-	ENE NOVO AND HEEOVIL C	48
DmMcm7	MARRONAODRESISTELSESCKCDDDK	KEFVNGSO VK HBEOVLIT	49
CeMcm7	METTTYNTHAAFETEINSFEDENYVDNEDESG-	KAFPWRDOUFEL REDKOANV	54
ZmRoa2	MEICEEAMAAHKSAFLDFLDQDVGK	VYMQAURD VONKRHR I	44
ZmRoa3	MEINEEAMAAHKSAFLDFLDQDVGKS	VWMQANRD VONKRHR I	44
ZmPr 1	IDIID FHYKDVDEEFLQR/TENTRI IGITAL	EADDIN EPT-EAYTV-DEDODIL TORVDEGPDGG	112
ALPE1	DEPENDENT DEPENDENT OF TENTRE VS FS.	A ADDING EPT-EAFPDEDHDILWTONADDGTDN	127
SpMem7	THE PLOYON FEEL OCTOADD VSALOONANIE TE PC	DEADATHS PPT-VEINTRNEVINVINVINOPLINEPMISDET	158
HaMcm7	MONDO AEDDPELVDSUCENARIMAKI FAL	AUOUL DOVK-EREVVNK V DVY EHELMMEOR	112
MmMcm7	AEDDPEVDS CENAKS SRING	VOOLEEYK-EKEVVNK V DVY EHELMMEOR	112
X1Mcm7	TOLDD AEEDPCLVDA CENTRE TNEFA	DAVQULLPQYK-EREVVHKLAUDVYLEHELMMEQR	111
DmMcm7	IDLIDIAEFNESLAEAUVDNCREVISIES	VIAELLPSYK-QQEVHAKTALDVYTEHRLMMESR	112
CeMcm7	WNVER KESDIPDALELSEARTSNTKEVEVER	TISDM QDYLGDKQAPVILA DAY FORLHMDRNEG	124
ZmRoa2	GHID RNHNLDLARR IRTPGE MQPAS	DAVSEVARNLD	84
ZmRoa3	HGHIDERNHNLDEARREIRTPGEEMQPAS	Sat ARNLD	84
ZmPr 1	LOKMPPINK	FILM I RAFSKVTPLT BOUSSIN COL	156
ALPE1	HOOTESLUK	Y KAPSKGRPST BEUL SHICO	154
SpMcm7	EHKGOPPDI T	GVDLYFRPVTRNKKPFSVRDLEGENLCSL	181
ScMcm7	NEIRSENLMDTTMD PSSMNDALREVVEDETEL PPNLT	RVFLVFKPLSQNCARRYRKKAISSKPLSVRQTFGDFLGQL 2	238
HsMcm7	VRSPQNOVPALLM	RFELVFQGPSSSKPRVIREVRADSVGKF	160
MmMcm7	VRNPQNQ PSEIM	REPLYERGPSSSKPRVEREVEADSVCKL	160
X1Mcm7	MRDPHNQ PPIM	R KAPSSSKARV RD DS CK	159
DmMcm7	QRDERNSIPSIT M	AVETCOMMUNICATION	173
ZmRoa2	PKULKUGE	VMVG SGPFGFHRVTPBD MSSF CT	120
ZmRoa3	PK LK GE	VMVG SGPFGFHRVTPSD MSSF GT	120
	* *	* *	
ZmPr1	OKISGIVIECSDVEPLIQVAVYTCEECGFEIVCEUTARV	MPLIECESQRCKLEKAKGNLIELRASKELEFQEVKLQEL 2	236
AtPr1	VRISCIVIECEDVEPLEAVAVYTCEDCCHEIVCEVTSRV	FMPLFKCPSSRCRLUSKAGNPILCLRASH LHFQEAKHQEL 2	234
SpMcm7	T RG VIT T DV PS TVNAVIC RCGYEV CE ROKT	LEMSEC SDECKKODAKCOLF STRAS LPFOR K OEL 2	261
Schem7	T DE LEVER D	THESE TREES OF OT A SOLL THE STRAST SAFELCK THE S	240
MmMcm7	TUDC UTDU FU DD WUATYTCH OCCAPTUCPIOSPT	MPLINCESOFCOT PSCCPLVL TPCSE WEFOFULLOFH 2	240
X1Mcm7	TURE UTEV EWERMUWATVTCLOCCART/CPLOSPT	MPLIMCISRECOTARSCORDUCTROSIS INFORMATION 2	239
DmMcm7	VTVRG VTRCTEVEPM VVATYTCERCGSETVCPVNSLS	TPVHDCPSDDCRVNKAGCRLYL TRGSFFVFFQEVKNQEH 2	240
CeMcm7	VSHKGVVIRAHEVEPCVEVMTYTCDTCAAEVYCPUKGMQ	TEPVNCENKECVEAKANCRLHHOLRGSHEVHEOELKLOEL 2	253
ZmRoa2	CVEGIVTEC LVEPKVVKSVHFCPVTGDFLSREYRDITS	SFVGLPTGSVYPTREDNGNLEVTEYGMCE KDHCT S QEV 2	200
ZmRoa3	CHECHATECELVEPKEVKSVHFCPVTCDFLSREYRDIT:	SFVGLPTGSVYPTREDNGNLEVTEYGHCEEKDHCTES	200
ZmBr 1		DYVER AND AND A DELLA DE	116
ATPEL	AFRVPRCHTPRATTIN DOLLTPRVSDCVV FF CITLP	PYTCERAL DAGLA ADT IL ATS THERE YEEVEEN DE A	314
SpMcm7	TN VPICH PR TH YCAI REVNED IND C LP	TPYTGERAMRAGE TOU IN CHY SOT INNYTNIEKTPOSE	341
ScMcm7	SOUVPVGHIPRSLNIHVNGTLVRSLSPGDIVDVTGIFLP	APYTGFRALKAGL TETYL AQFVRQHKKKFASFSLTSDVE 3	398
HsMcm7	SDOVPVGNIPRSITVLVEGENTRIAOPGCHVSVTGIFLP	LRTGFRQVVQGL SET L AHR VKMNKSEDDESGAGELT 3	320
MmMcm7	SDOVPVGNIPRSITVVLEGENTRIAOPGDHVSVTGIFLP	LRTGFQQHAQGLESETYLEAHWIVKHTKSDDDVSGAGELS 3	320
X1Mcm7	SDOVPVGNIPRCHSVYTRGENTRLACPGEHVGITGVFLP	LRTGFROVVOGL SET L SHR VKMNKTDDDELGTOELS 3	319
DmMcm7	SD VPVGH PR T MCRG V RMAOPG H V G LP	MRTGFAQMIQGL SET L MHR ICINNNDEISDKDAELT 3	320
ZmRoe2	DENSARGO PRIDU DE EDDIVIDE CERCI DE STUCIERA	PVSVGSVSC_FDTVL_TANNSSI_LNEFANAPVVTDEDL	278
ZmRoa3	PENSAPCO PR DI L'EDDLVDCCKPG RIS VG KA	PCKSKGSVSGFRUVLIENN SLLNBEANAPVYTREDL 2	278
ZmPr1	EQIDRLAEDGEISKLARSLAPEIFGHEDVKKALLLLUV	SAPHERLADGMETEGDLHICHHGDPGVAKSQLLEHIINVAP	396
AtPr1	EQIARLAEDGEIVNKLSRSLAPEIVGHEDIKKALLLLUV	GAPHRQLKDGMKIRGDVHICLNGDPGVAKSQLLKHIINVAP	394
SpMcm7	AAIAELNQGGOVMERLARSIAPEIYGHEDVKKALLLLUV	3GVTKELGDGMRIRGDINICLTGDPGVAKSQLLKYISKVAP	421
ScMcm7	ERVMELITSG V NRL KSTAPEI GNLDVKKALLLLV	GUDKRVGDGMUTRGDINUCH GDPGVAKSQLLKATCKTSP	478
MmMcm7	SEEL NOT AFFORT FULL AND AFFORT OFFORT VERY AND	CODOSP-ROMETRONINICLICOPOVARSQLUSYIDRIAP 3	399
X1Mcm7	EEELROITDE FVERLASIAPEINGHEDVKKALLLLV	GUDHSP-RGMKIRGNINVCLEGDPGVAKSOLLSYHDRIAP	398
DmMcm7	PEELEELAQDOFVERLATSLAPEINGHLDVKKALLLLLVG	GUDERP-DGMETERGNINICLEGDPGVAKSQLLGYISELAV	399
CeMcm7	AELEVERREGENVETLAASTAPEIFGHVDVERCLEMALVO	3GNDNSS-NGMKIRGCINVLMMGDPGVAKSQLLGYVNRLAP	408
ZmRoa2	KRMKEISRRNDTEDLLGNSLAPSIYGHLWIKKAVVLLHLG	3GVEKNLKNCTHLRCDINEMHVGDPSVAKSQLLRAVMNIAP 3	358
ZmRoa3	KRMKEISRRN TEDLLGNS APSIGHLW KKAVVLLIL	3CVEKNLKNGTHLRGDINHMHVGDPSVAKSQLLRAVMNIAP 3	358
ZmPr 1	ROVYTTOROSSOVOLTA AVOUDDI INFOUT FOCALLY AD	CALDEEDEMEESDETATHEWNEGOTIETAKAGT THE	476
AtPri	RGVYTTGEGSSGVGLTAAVMEDOUTMENVLEGGALVLAD	GICAIDEFDKMDESDRTAIHEVMEOOTVSTAKAGITTSLN	474
SpMcm7	RGVYTTGRGSSGVGLTAAVMRDPVTDEMVLEGGALVLAD	GICCIDEFDKMDESDRTAIHEVMEOOTISISKAGITTTLM	501
ScHcm7	RGVYTTGRGSSGVGLTAAVMEDPUTDEMILEGGALVLAD	GICCIDEFDKMDESDRTAIHEVMEQQTISISKAGINTTLN	558
HsMcm7	RSCYTTGRGSSGVGLTAAVLEDSVSGELTLEGGALVLAD	GVCCIDEFDKMAEADRTAIHEVMEQQTISIAKAGILTILN	479
MmMcm7	RSCYTTGRGSSGVGLTAAVERDSVSGELTLEGGALVLAD	GVCCIDEFDKMAEADRTAIHEVMEQQTISIAKAGILTTLN 4	479
X1Mcm7	RSCYTTGRGSSGVGLTAAVHEDPVTGEMTLEGGALVLAD	GWCCIDEFDKMMDSDRTAIHEVMEQQTISIAKAGIMTILN 4	478
DmMcm7	RSCYTTORGSSGVGLTAAVMEDPLTGENTLEGGALVLAD	GUCCIDEFDKMADODRTATHEVMEQOTISTAKAGIMTTLN	479
ZmRog2	LAISTICEGSSCUCLTANTSDOFTEERSLEGGALVLAD	CIDEFORMAD BORTATHEVMEQUITETAKAGIMTTLN	138
ZmRoa3	LATSTTCEGSSCUCLTAAVTSDOFTSERREEAGAINLAD	REAL TO THE THE AND OD RUATHEVALOOT TAKAGTHASLN	438

Fig. 1. Analysis of the ZmPRL sequence. Alignment of the deduced amino acid sequence with MCM7 proteins from various species. Two sequences corresponding to maize MCM3 (ROA proteins) are also included. Homology search was performed using the BLASTP search program [52] and sequences were aligned using the CLUSTALW program (version 1.5; [30]). Identical residues are shaded in black and similar in grey. Dashes indicate gaps in the alignment. Numbers on the right correspond to the last amino acid position. The Gene Bank accession number for the ZmPRL gene is AJ313531. The Gene Bank accession numbers for the homologous are P43299 (AtPRL), O75001 (SpMCM7), P38132 (ScMCM7), P33993 (HsMCM7), NP032594 (MmMCM7), Q91876 (XIMCM7), AA032857 (DmMCM7), T03920 (CeMCM7), AF073330 (ZmROA2) and AF073331 (ZmROA3). The region between T^{141} and Y^{640} corresponds to the well conserved MCM domain. Within this domain, between G^{370} and D^{523} , is the ATPase motif. The black bar above the sequence corresponds to the A–D motifs that are a modified version of the Walker-type NTP-binding domain, conserved in a number of APTases [39]. Labeled with asterisks are the four cysteine residues of the zinc finger type motif.

AtPr1	ARTAVIAAANPAAGRYDLRRIPAENINLPPALLSPFDIII0IIILDRADHDSDLEIARHALHCHOTEESP	542
SpMcm7	ARTSILAAANPLYGRYNEKVAPIHNINLEAALLSREDILELILDTESEETDEH ACHVTYVHMINEOP	569
ScMcm7	ARTSILAAANPLYGRYNPRLEPLDNINLPAALLSRFDILFLELDIPSEDDDEKLAEHVTYVHMENKOP	62.6
HaMcm7	ARCSTLAAANPAYGPYNPRRSLEONTOLPAALLSPFDLLWLTODRPDRDNDLRLAOHTTYVHOHSROP	547
MmMcm7	A BOSTLA A AND A VOR VINEDRS LEONING PAALLS REFUL MUTOD PDD DNDL PLACHT TVAHOUS POR	547
VINCTO7	A DO STELA A AND A VOD WE WE WE ON THE DATA SEPTEMENT OF POPULATION OF SMORT	546
DmMcm7	A PARTI A A AND A REPAY DE VICAL CONTRACTOR DE ALLES DE DE LA CONTRACTOR DE AVAILANTE A VICAL DE A	547
Dimitem?	ANALY ANALY CONTRACT AND	547
Cenem /	ARTATTAAANPATGRYNENRSTEONYDD PAADLSFYDD TELEODRAD ENDRITAEHTTYVHOEGCEPN	557
ZmRoa2	ARCSVIAAANPIYGTYDRSLIPTRNIGLPDSLLSFFDLLFIVLDONDPEIDRO SEHVARHRYCTDDGGARSLDREGYJ	518
ZmRoa3	ARCEVIAAANFINGTYDRSLIPTKNIGLEDSLLSFFDIIITILLDQNDPEIDRQUSEHJARHRYCTDDGGARSLDKEGYJ	518
ZmPr1	RVIPSVERELEEVIATAYSSIPOEEAKS	592
AtPr1	ALGEPLEPNILEAYISAAD-RLSPYDAELEEYIATAYSSIRQEEAKS	590
SpMcm7	KMDFEPLDPNMIRHYISSAR-QYR <mark>P</mark> VVFKDVCDYVTG <mark>AYVOLR</mark> ONOKRDEAN	1 620
ScMcm7		677
H=Mcm7	PSOFEPLDMKLMERY LAMCE-EKOPMULESI ADVITAAYVENERAU	594
MmMcm7	PAGEEDIDMELMERY JAMCH-EROPTVEESLADVITAA WENEREAR	594
X1Mcm7	PSOLOCUDARY AND ATTACK SYCHATTES ADVITAD WEEKFAD	593
Dm Mem 7	DTDUYA DWILLION CLEVENT DEL TAVIACAVUL DEAD	504
Callen 7	PERVER STATE TO A DEPARTMENT OF A DEPARTMENTA D DEPARTMENT OF A DEPARTMENTA D D DEPART	594
Cenem?		504
2mRoa2	EEDDGDANAAIFVKYDRMLHGQDRRRGKKSKODRI TVKFI SKYTHYASNLIQPRI TDEASDH ATSYAEI RDGSAN-AKS	597
ZmRoa3	EEDDGDANAAIFVKYDRMLHGQDRRRGKKSKQDREITVKFIERKVIHYAENLIGEREITDEASEHEATSVAEIEDGSAN-AKS	597
ZmPr1	NAPTSYT IRTLSILRISIALARLEFSETVAQSDVDEALRLIGHSKYSL-YSD-DRQRSGLDAISDIYSILRDEAAR	668
AtPr1	NTPHSYT VPTLLSILRISAALARLEFSESVAQSDVDEALRLOMSKISL-YAD-DRQKAGLDAISDT SILEDEAAR	666
SpMcm7	EROFAHT PRTLLAILREGOALARLEFSNRVEIGUVDEALRESVSKSSL-YDDLDPSSHDTTITSKI KIIRDMLNSIF	699
ScMcm7	KFSFGOATPFTLLGITE SOALAKLELADMADID VEEA PLVRVSKESL YOETNKSKEDESPTTKIFTITEKMLO	753
HaMem7	SKDATYT ARTILATER STALATER WVDVWEKE VNEA REFERENDELEM BOSL-LGDKGOTARTORPADVITATVELVS	670
MmMcm7	SKDATYTSARTILATIRI STALARI SMUDIWEKEIWNEATRI MEM MDSI-LGEKGOTARTORPADVITATIEFLYS	670
X1Mcm7	NUTHER ART LOT DISTALATED TERVIDER WATA DITEN BOT OGDEGHASPTORDADEL STUERWEG-	670
DmWem7	OPDITE ADVIDE TO STATE TO STATE ADVIDE ADVID	671
C-Ward		671
Cenem?	SSDP IF V PREILEGTOR A TARAKTELST INDESIVEETA RE OF ARDSE-RPEONRIEKRAPVDAA AVIILETA	680
ZmRoa2	GGGTLP I AFTIETT IS STAHA SHELRHEVLKSOVEAA OVINFAIYHKELTEMEEREQREMEMRQQADHDAGATGG	675
ZmRoa3	GGGTLPIMARTETTER SHAHAMERHEVLKSUVEAA QUINFAIYHKELTEMEEREQREMEMKQQADHDAGATGG	675
ZmPr1	TSSMDVRYAHALNLISR GYSEA LKECLEE AS NO O HPSTFD HF DV	720
AtPr1	SKKTHUSYANALNUISR GYSEA LKEC EE AA NWO OF PHTFD RE	716
SpMcm7	DVEGNERS TLRAIRERVLA GTEDHLINT QE TDEGULTTNNGQT ME DPDLHMEN	760
ScMcm7	ETGKNT SYENIVKTVRL GFTML LSNC OF SYLWY H INEGNT KE DDGTMDTDQEDSLVSTPKLAPOTTASA	831
HaMcm7	GGRSWRFSEAEORCVSWGFTPACFOAALDEVEELNVMOONASRTRITEV	719
MmMcm7	RGRSOHFSEAFORCISICFTPA FOAA DEVEFEANDOWNTSRTR TE	719
X1Mcm7		720
DmMcm7		720
CoMercia 7	- ADNA BARD TOPCTI THE REPORT OF THE CLUMBER IN ANY	720
Cenem/		730
2mRoa2	IVDGGGSSGNDPHDVDVGSNDQNVSAER EA EABLGQHTLANHIDQMS DDIEQMVNRESTAPYTRSQVEFILERF	752
2mRoa3	IVDGHGSSGNDPMDVDVGSNDQNVSAERUQAUEAULGQHULANHIDQMSUDEIEQMVNRESTAPYTRSQVEFILER	752
ZmPr1		
AtPr1		
SpMcm7		
ScMcm7	NVSAQDSDIDLQDA 845	
HsMcm7		
MmMcm7		
X1Mcm7		
DmMcm7		
CeMcm7		
ZmPos2	ODANDUWIDDCUUDII 200	
2mRod2	QUANKVRIKDOVVRII (65	

Fig. 1. (Continued)

3.2. Genomic structure and mRNA accumulation of the ZmPRL gene

Southern analysis was performed on digested maize DNA with a probe corresponding to the central region of the MCM domain, which is the most conserved region of the protein. The bands obtained indicate that this gene belongs to a small multigene family and that there exist one or two copies of the gene in the maize genome (Fig. 3). This result is further confirmed by physical mapping of the MCM7 gene in maize (carried out by Dr Alain Murigneux, Biogemma). This experiment was carried with an EcoRI digestion, showing that in the maize genome there are two loci where the ZmPRL probe maps. One of them maps to chromosome 1 and the other one, to chromosome 3. A similar situation has been reported for the MCM3 gene of maize [19], with two to four copies of closely related sequences being present in the genome of this species. In the case of Arabidopsis, additional genomic DNA fragments were detected by low stringent Southern hybridization when using a probe corresponding to the PRL gene [17].

ZmPRL has been cloned as a gene involved in the embryogenesis of maize and with a special accumulation in the apical region of the embryo. To confirm this fact, an RNA blot analysis was performed with RNA extracted from embryos at different stages of development. As it can be observed in Fig. 4A ZmPRL is expressed during early and mid embryogenesis, from the very begging of this process, 0 DAP, until 30-40 DAP. It is during this period that the embryo grows and the different organs and structures are established. It is, therefore, a period in which the embryo is very active in cell division and cell proliferation, being also active in cell differentiation. Around 45 DAP the embryo starts desiccation, dormancy, and it prepares for germination. At this stage, the embryo is not active in cell proliferation anymore.

Taking advantage of the relatively large size of the maize embryo, we were able to analyze *ZmPRL* expression in the apical, basal and central regions of maize embryos of 20 DAP by dissection of each region. As it can be seen in Fig. 4B, *ZmPRL* mRNA accumulates preferentially in the upper region of the 20 DAP em-

544

bryo, the area that contains the shoot apical meristem. In the basal region, which contains the root, and in the central region, ZmPRL has a lower level of expression.

Analysis of ZmPRL mRNA accumulation during the early stages of germination (Fig. 4C) reveals that this gene is expressed in some tissues, although the level of expression is lower than during the embryogenesis stages. ZmPRL mRNA during germination mainly accumulates in the region that contains the apical meristem. A low level of mRNA can also be detected in young leaves and in the root tip. It is neither detected in older leaves, in the coleoptile, in the node region (including the scutellum) nor it is in the upper region of the root. In general, ZmPRL mRNA accumulation correlates with regions of the plant active in proliferation with an increased level in the apical meristematic regions.

3.3. Accumulation pattern of the ZmPRL mRNA by in situ hybridization

To determine the spatial and temporal pattern of ZmPRL mRNA accumulation during embryogenesis, sections of immature embryos at different stages of development were hybridized in situ with a probe corresponding to the ZmPRL gene. The results are shown in Fig. 5. At the transition stage, around 8 DAP, the embryo consists of the embryo proper in the upper region, with smaller and more cytoplasmatic cells, and

the suspensor, in the basal region, which is formed by larger and more vacuolated cells. At this stage, ZmPRLhybridization can be observed in the upper region of the embryo, corresponding to the embryo proper. It can also be seen some ZmPRL accumulation in the endosperm, mainly in the transfer cells (Fig. 5A). Around 13 DAP, at a stage in which the coleoptile, the scutellum and the shoot and root primordia are already formed, ZmPRL accumulation is restricted to the meristematic apical region of the embryo; in the shoot apical meristem, and in the leaf primordia. It also has a low level of expression in the scutellum (Fig. 5C and D).

Around 20 DAP it can be observed that the presence of ZmPRL mRNA is restricted to the regions that are active in cell proliferation, such as the shoot meristem, leaf primordia, around the provascular cells of the coleoptile procambium and in the provascular cells of the embryo axis (Fig. 5E). At the root level, ZmPRL is expressed in the pericyle and in the provascular elements of the primordial root (Fig. 5H). In the scutellum, ZmPRL mRNA is detected in the provascular strands and in the subepidermal layers, forming a gradient towards the embryo axis (Fig. 5F). It is worth noting that there is a region in the meristem tip where ZmPrl mRNA is not detected. Our results are in accordance with the idea that the shoot meristem in maize also contains a central region in its tip where cells



Fig. 2. Phylogenetic analysis performed with different members of the MCM2-7 family from various organisms. Bootstrap values supporting major clusters are shown.



Fig. 3. Southern blot analysis of genomic DNA from Z. mays. A ZmPRL cDNA fragment corresponding to the conserved MCM domain (from nt. 1068 to 1740, corresponding to the region between amino acid residues 306 and 580) was used to probe the DNA digested with BamHI, EcoRI and HindIII. Molecular weights are indicated on the left.

divide at a very low rate as it has already been proposed [41].

The patchy expression that can be observed in more detail in Fig. 5G is characteristic of cell-cycle regulated genes, such as histone H4 [42], or different cyclins [43,44]. This patchy pattern of accumulation has also been described for the maize MCM3 homolog [19] and for *PRL* in *Arabidopsis* [18]. On the other hand, the global pattern observed here for the *ZmPRL* gene is very coincident with accumulation patterns obtained for other genes related to proliferation, such as *TM20* [45].

4. Discussion

ZmPRL is a member of the MCM family of proteins, which have an important role in licencing DNA for replication and in ensuring that only one round of replication will take place in each cell cycle [38]. The family is formed by six proteins that are highly conserved between all eukaryotes, from yeast to mammals, insects and plants. All six members of MCM family are required to form an heterohexameric complex that allows DNA synthesis by binding to chromatin at the multiple origins of replication. in regulating DNA replication in plant cells. The importance and general function of the protein is reflected in the similarity of the ZmPRL protein sequence to the other proteins of the MCM7 family that ranges from 77 to 43% and it is specially high in the domains that may have a functional meaning such as the zinc finger or the ATP binding domain (Fig. 1). If the maize members of the MCM3 family of proteins are included in the analysis (Fig. 2) it appears that ZmPRL and MCM3 sequences cluster in separate branches indicating the independent evolution of these two subfamilies and pointing to their important function in the complex of proteins taking part in the regulation of the cell cycle throughout the evolution of eukaryotes.

Analyzing the accumulation pattern of the ZmPRL it can be concluded that, in general terms, the expression pattern obtained is in agreement with the pattern expected for a gene related to cell cycle [44] and proliferation [45]. Maize ZmPRL, as shown in this work, is highly expressed during early embryogenesis, during the period in which the apical-basal pattern and the basic structures of the adult plant are established. Later in embryogenesis and during germination, the expression becomes restricted to the meristematic regions, that have high mitotic activity during all plant life, and to organ primordia (leaf and root). These results are consistent with the expression pattern observed with At-PRL and maize MCM3 mRNAs, both of them showing



Fig. 4. mRNA accumulation of *ZmPRL* analyzed by RNA blot. RNA from different stages of embryogenesis (A), from three regions in which a 20 DAP embryo had been dissected (B) and from different organs during germination (C), was isolated and similar amounts of it were loaded on a denaturing gel and probed with a *ZmPRL* cDNA fragment corresponding to a non-conserved region of the sequence (from nt. 1890 to the end of the sequence, corresponding to the region between Aa. 630 and 720). The approximate length of the *ZmPRL* mRNA is indicated. Ethidium bromide staining of the different total RNAs is shown in the lower panels. Lanes are labeled as follows K0–K10, kernels from 0 to 10 DAP, E18–E60, embryos from 18 to 60 DAP, B (basal region), C (central region), A (apical region), N, (node), S (shoot meristem), C (coleoptile), YL (young leaf), OL (old leaf), R (root apex).



Fig. 5. mRNA accumulation pattern of the ZmPRL gene in histological sections of maize embryos by in situ hybridization. Longitudinal sections of a 8 DAP embryo (A) and a 10 DAP embryo (B). Transversal section of a 13 DAP embryo (C). Magnification of the apical region of a 13 DAP embryo (D). Transversal sections of a 20 DAP embryo, at the apical level (E), and at the root level (H). Magnification of the scutellum, showing the gradient of the signal going from the outer layers towards the embryo axes, there is no accumulation of ZmPRL in the protoderm, transversal section (F). Detail of the shoot region of a 20 DAP embryo, showing the patchy accumulation characteristic of the cell cycle regulated genes in a transversal section (G). The control was obtained using a sense probe on a transversal section of a 20 DAP embryo (I). Sections are labeled as follows, ep (embryo proper), s (suspensor), tc (transfer cells of the endosperm), sam (shoot apical meristem), ram (root apical meristem), sc (scutellum), c (coleoptile), lp (leaf primordia), cp (coleoptilar procambium), pc (provascular cells), ps (provascular strands of scutellum) and p (pericycle). Bars in (A), (C) and (I) = $200 \ \mu m$, in (B), (E) and (H) = 500 μ m, in (D), (F) and (G) = 160 μ m.

high levels of expression in those cells active in proliferation [17–19]. A more detailed analysis of the pattern of mRNA accumulation by in situ hybridization has allowed us to see what cell types and what regions of the embryo are expressing ZmPRL mRNA. The patchy appearance of the hybridization signal is a feature of cell cycle regulated genes, like histone or cyclin genes, [42–44] and it has also been observed for maize MCM3and AtPRL. It is reasonable to propose that ZmPRL is expressed during G1 as it has been described for *At*-*PRL* [18].

Little is known about the mechanisms controlling cell cycle in plants, but it has been shown that in plants there are D-type cyclins, [46–48] and maize contains two Rb related proteins [26,49,50], suggesting that cell cycle regulation may be more similar to animals than to yeast which do not contain D-type cyclins neither Rb [25]. The interaction between MCM7 and Rb in humans or Xenopus has been reported recently [24]. It could be interesting to know if ZmPRL in maize is interacting with the maize RRB1 or RRB2 proteins in order to have a more complete information on the control of cell cycle progression and DNA replication in plant cells. Maize *RRB* mRNA has been described as having an ubiquitous expression with higher levels of accumulation in the shoot apical meristem [50]. This is in agreement with the pattern of expression described for ZmPRL. A correlation in the patterns of mRNA expression for ZmRRB and ZmPRL can be observed pointing towards a related function or even an interaction of the two proteins in maize.

Recent studies propose an important role for cell cycle in controlling apical-basal pattern formation during embryogenesis in plants [27]. It has been shown by mutant studies that the distinct developmental processes taking place during embryogenesis, such as cell differentiation, apical-basal organization and morphogenesis, are uncoupled and studies on embryo patterning of dominant negative *cdc2aAt* mutants of Arabidopsis indicate that only the pattern formation is affected by the mutation, but not the differentiation of cells [51]. Although many mutants have been described that affect pattern formation during embryogenesis, none of them corresponds to a mutation in a gene related to the cell cycle. This is probably because a mutation in such a gene would be lethal in an haploid state. Nevertheless, although cell divisions are uncoupled from vegetative development, they are instrumental in elaborating embryo structures and modulating embryo and seedling morphogenesis [27].

Here, we show that a gene involved in cell division events specifically accumulates in the cells taking part in the construction of the apical-basal pattern and morphogenesis of embryos in general. The homolog of ZmPRL in Arabidopsis, AtPRL, is accumulated in the adaxial side of the leaf, with a similar pattern of expression of other genes, such as AP1 and AP3 [18]. AP1 and AP3 are related to the yeast MCM1 transcription factor that regulates the transcription of MCM7 in yeast. In this case, it has been proposed [18] that these genes could be regulating the balance between cell division and differentiation along the radial axis of the shoot, and influence leaf and floral organ shape. The specific accumulation of genes that take part in regulating the cell cycle, such as ZmPRL in the shoot meristem, may be an indication of this fact.

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