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Isolation of a full-length mitotic cyclin cDNA clone *CycIIIMs* from *Medicago sativa*: Chromosomal mapping and expression

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Abstract

Cyclins in association with the protein kinase $p34^{cdc2}$ and related cyclin-dependent protein kinases (*cdk*s) are key regulatory elements in controlling the cell division cycle. Here, we describe the identification and characterization of a full-length cDNA clone of alfalfa mitotic cyclin, termed *CycIIIMs*. Computer analysis of known plant cyclin gene sequences revealed that this cyclin belongs to the same structural group as the other known partial alfalfa cyclin sequences. Genetic segregation analysis based on DNA-DNA hybridization data showed that the *CycIIIMs* gene(s) locates in a single chromosomal region on linkage group 5 of the alfalfa genetic map between RFLP markers UO89A and CG13. The assignment of this cyclin to the mitotic cyclin class was based on its cDNA-derived sequence and its differential expression during G2/M cell cycle phase transition of a partially synchronized alfalfa cell culture. Sequence analysis indicated common motifs with both the A- and B-types of mitotic cyclins similarly to the newly described B3-type of animal cyclins.

Introduction

Cyclins are regulatory subunits of protein kinases encoded by *cdc2* or related cyclin-dependent protein kinase (*cdk*) genes that play a central role in the eukaryotic cell cycle machinery. They were first identified in eggs of marine invertebrates [9] but have now been described in a wide variety of organisms from yeast to mammals (for reviews, see [23,24]). The two best characterized groups of cyclins are the G1 cyclins that regulate progression through the G1 restriction point in animals or START in budding yeast (for review, see [36]) and the mitotic cyclins that drive passage through mitosis (for review, see [27]). Mitotic cyclins can associate with the catalytic domain of

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X78504.

the p34^{cdc2} protein kinase forming the maturation-promoting factor (MPF). Dephosphorylation of specific amino acids in the ATP-binding site of this protein complex by the phosphatase *cdc25* is required for the mitosis of eukaryotic cells [14,29,37]. Vertebrates and insects contain at least two distinct classes of mitotic cyclins, the A- and B-types [27, 38] which can be distinguished by sequence motifs and the timing of their destruction in the cell cycle. The degradation of both cyclins is required for exit from mitosis, however, the A-type cyclins are expressed and degraded earlier during the cell cycle than the B-type cyclins [23] and seem to carry out distinct functions during the S or the G2 phases [16,39]. At the N-terminus, mitotic cyclins contain a short sequence motif of 10 amino acids known as the destruction box which may be recognized by some component of the ubiquitin-conjugating system [17]. The cyclin box, another highly conserved region of the mitotic cyclins, comprises the P box domain which is required for the activation of *cdc25* [40].

Recent progress in the understanding of the mitotic control in animal cells has provided powerful tools to analyze the regulation of the plant cell division cycle. Between the animal and the plant kingdom, the basic elements of the cell cycle machinery seem to be conserved through evolution [8,24]. Functional homologs of the *cdc2* gene encoding the p34^{cdc2} protein kinase have been isolated from different plant species [2,7,10,22] as well as cyclin genes from soybean [19], *Arabidopsis*[20], *Antirrhinum* [11] and maize [32] which were classified as mitotic cyclins. The products of the *Arabidopsis* and maize cyclin genes were shown to induce meiotic maturation of *Xenopus* oocytes indicating that these genes could functionally replace the endogenous cyclin genes. In addition, partial cDNAs of putative cyclin genes were isolated from carrot [19] and from alfalfa [21]. Based on sequence and expression analyses of these partial alfalfa cDNAs, it was suggested that one of the two alfalfa cyclins, *cycMs2*, belongs to the B-type cyclins whereas the other, *cycMs1*, was proposed to represent a novel type of cyclin [21]. However, on the basis of sequence data it is very difficult to classify plant cyclins as A- or B-type cyclins as they generally share common features of both types. Recently, from the available amino acid sequences of plant cyclins and their divergence from the animal and the yeast cyclins, a new nomenclature based on phylogenetic tree analysis was proposed for plant mitotic cyclins forming three groups (I,II,III) [32].

In this paper, we report the isolation and characterization of the first full-length cyclin cDNA clone from *M. sativa* which is expressed predominantly during the G2 and M phases of the cell cycle. Based on the sequence analysis, we propose to classify this cyclin gene, designated *CycIIIMs*, within the plant cyclin group III. We show that the two known partial alfalfa cyclin cDNAs reported previously [21] are closely related to *CycIIIMs* and we determined the chromosomal location of this gene in alfalfa. Moreover, we hypothetise an evolutionary relationship between plant and animal B3 type cyclins.

Materials and methods

Plant growth conditions

Medicago sativa ssp. *sativa* cv. Sitel plants were grown under greenhouse conditions with 16 h light period at 22 °C. Nitrogen-fixing nodules were induced on the alfalfa roots by inoculation with the wild-type *Rhizobium meliloti* strain Rm41 and nodules of two different ages (7 and 12 days) were harvested as described [1]. Plant materials, after harvesting, were immediately frozen in liquid nitrogen and stored at −70 °C.

Construction and screening of a cDNA library

A cDNA library was constructed in the ZAPII vector (Stratagene) from poly(A)$^+$ RNA purified from 3 to 4-day-old non-nitrogen-fixing nodules of *Medicago sativa* ssp. *varia* A2 plants multiplied *in vitro* by cuttings. About 1×10^6 plaques were screened for hybridization with the 750 bp *cycMs2*

probe [21]. Fourteen positive clones were selected and rescreened. The insert of one clone with strong hybridization signal was excised using the *in vivo* excision protocol supplied by the manufacturer (Stratagene) to generate a subclone in the pBluescript SK(-) phagemid. The plasmid purified on CsCl gradient was used as sequencing template. The complete sequence of both strands was determined by the dideoxy-chain termination method [34] using T7 and universal as well as custom primers. Reactions were carried out following the protocol supplied with the used sequenase enzyme (version 2.0, USB).

Computer analysis

Amino acid sequence comparisons were made using the program from the GCG software package (Genetic Computer Group, Madison,WI) and phylogenetic tree analysis was performed with the program of the Computational Biochemistry Research Group (Zurich).

DNA analysis

Total genomic DNA of diploid *M. truncatula* cv. Ghor and tetraploid *M. sativa* ssp. *sativa* cvs. Nagyszénàsi and Cardinal was isolated from leaves using the CTAB method [33] and purified through a CsCl gradient. For Southern blot, total genomic DNA (10 μg) was digested with *Eco* RI restriction enzyme. The DNA fragments were separated by electrophoresis on a 0.8% agarose gel in 1 × TAE buffer and transferred onto nylon membrane (Hybond-N, Amersham) as described [33]. DNA blots were hybridized at 65 °C as discribed [6] with the 420 bp *Bsm* I-*Xho* I fragment (3′ part) and with the full-length *CycIIIMs* gene labelled by random priming (Boehringer). Membranes were washed at the final stringency of 0.2 × SSC, 0.1% SDS at 55 °C for 20 min according to the manufacturer's protocol.

Molecular markers

RFLP markers designated UO are cDNA clones originating from a library described before [3]. The clone denoted by CG is a genomic clone from a *Pst* I library as described before [28]. Southern transfer and hybridization were carried out as described [28]. Segregation pattern of the cyclin gene was detected using the MultiBlotter System MB-96 (Labimap, Plaisir, France). RAPD markers were generated by PCR amplification using 10 base oligonucleotide primers purchased from Operon Technologies (Alameda, CA) as described elsewhere in detail [28].

Calculation of linkage

Scoring the genotypes of the loci was as follows: 1, Mqk93 homozygote for cycq; 3, Mcw2 homozygote cycw; 2, heterozygote; 4, not homozygote for the Mcw2 cycq; 5, not homozygote for the Mcw2. Scoring of genotypes for other loci was as described [25]. The genotypes of the individual loci were used to calculate the linkage and the map distances using the MAPMAKER V2 program [30]. The map distances were calculated according to the Haldane function.

Cell synchronization, mitotic and flow cytometric analysis

Medicago sativa ssp. *varia* A2 cell suspension cultures were synchronized with 15 mM hydroxyurea (Sigma) for 36 h, rinsed thoroughly and then released in fresh medium as described [26]. Samples for mitotic index determination were treated with colchicine (0.5 mg/ml) for 2 h. After Carnoy I fixation the cells were washed in Galbraith's buffer [12], stained with 1 μg/ml DAPI and around 1000 cells per sample were analyzed for mitosis with fluorescent microscopy.

For the flow cytometric analysis, eight drops of the cell culture (around 0.2 ml) were directly put into 1 ml cold enzyme medium (0.5% Cellulase Onozuka RS, 0.5% Macerozyme Onozuka R10,

0.05% Pectolyase Y23 dissolved in B5 culture medium (Sigma) supplemented with 1 M sorbitol and incubated for 30 min at 4 °C with continuous shaking in order to partially remove the cell wall. After washing out the enzyme solution with excess B5 medium, the nuclei were liberated by vigorous pipetting in Galbraith's buffer supplemented to 0.6% Triton X-100. The flow cytometric analysis of 20 000 nuclei per sample was carried out as described earlier [35].

RNA extraction and expression studies

Total RNA from about 1.0 g frozen plant material was purified by the acid guanidium thiocyanate-phenol-chloroform extraction method [5] modified by using volume 0.3 of cold absolute ethanol instead of 1 volume of isopropanol for the first precipitation of RNA and dissolving the final RNA precipitate in 50% formamide instead of 0.5% SDS. 15 μg of total RNA was separated in a formaldehyde agarose gel and transferred to Hybond-N membrane (Amersham) by the alkaline downward capillary transfer method [4]. As a control for loading artefacts, rRNA molecules bound to the filter were stained with 0.04% methylene blue in 0.5 M sodium acetate (pH 5.2) [33]. As a hybridization probe, a 216 bp long PCR fragment was generated (primers: 5′-TGGCCATAGCTTAGCTACTA-3′ forward and 5′-GTAAATGTTGACATAAAGAC3-′reverse; 90 °C 30 s, 40 °C 1 min, 72 °C 1 min, 40 cycles; Amersham *Taq* polymerase) representing a 3′ region (from 1431 to 1647) of the *CycIIIMs* gene exhibiting only 67% homology to the appropriate 3′ part of the known *cycMs2* sequence [21]. [^{32}P]-labelled probes were prepared with one tenth of the PCR reaction (5 μl) using a random hexamer kit (Boehringer) following the manufacturer's recommendations. Membranes were hybridized at 65 °C as described [18]. The final washing solution was 0.2× SSC, 0.1% SDS at 62 °C for 30 min. The hybridized filters were exposed to Kodak X-Omat film using an intensifying screen at −70 °C. The autoradiogram of the cell cycle-specific expression was analyzed with Millipore Bio Image equipment connected to an Unix workstation and a Kodak CCD camera producing an image of 1024 × 1024 pixels.

Results

Isolation and characterization of the CycIIIMs *clone*

A partial alfalfa (*M. sativa* ssp. *sativa*) cyclin cDNA clone *cycMs2* isolated by Hirt *et al.* [21] was used to screen a cDNA library constructed in the λ ZAPII vector and prepared from mRNA of young non-nitrogen-fixing root nodules 4 days after the inoculation of alfalfa roots with the symbiotic *Rhizobium meliloti* bacteria. About 1×10^6 plaques were screened and 14 positive clones were selected on the basis of their hybridization signal. After one round of plaque purification, one clone hybridizing strongly was selected for further analysis. The cDNA insert was subcloned into the pBluescript vector SK(-) using the *in vivo* excision protocol provided by Stratagene and sequenced on both strands. The cDNA consists of 1706 nucleotides (Fig. 1) including a putative polyadenylation signal (AATAA) 25 nucleotides before a 19 residue long poly(A) tail. The largest open reading frame extends from nucleotide 137 through 1421 encoding a predicted protein of 428 amino acids with a molecular mass of 48.81 kDa and an isoelectric point of 5.37. The putative protein harbours all the consensus amino acid sequences characteristic of mitotic cyclins (Fig. 1). It contains the 9 amino acid long destruction box motif RxxLxxxx conserved in all A- and B-type cyclins [17] starting at position 29. It also contains the cyclin box motif, a highly conserved central region of cyclin proteins [31] from the Met-204 to Leu-237 which includes the cyclin P box domain required for the activation of the protein tyrosine phosphatase *Cdc25* and consequently for the activation of the cyclin B-p34^{cdc2} complex at the end of the G2 phase which is a prerequisite of G2-M transition [40].

Comparison of the predicted alfalfa CycIIIMs protein sequence with the primary structure of 13

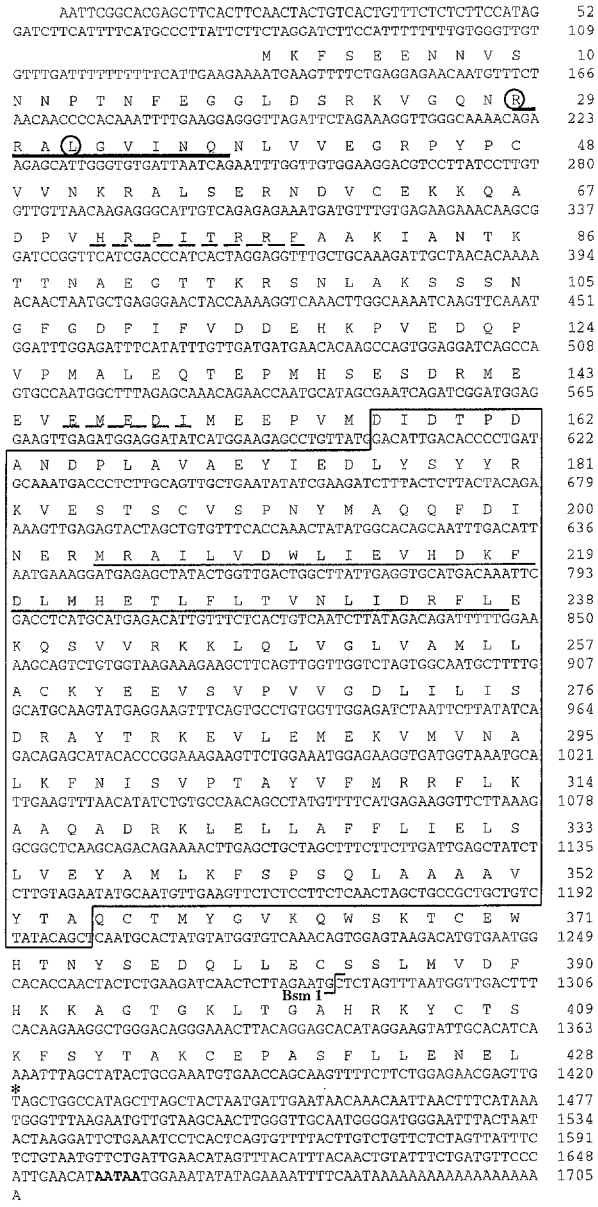

Fig. 1. cDNA and deduced amino acid sequences (single-letter code) of the alfalfa cyclin *CycIIIMs* cDNA clone. The destruction motif is underlined in bold and the highly conserved amino acids of this motif are circled. The motifs characteristic of the group III of plant cyclins [32] are underlined with dashed lines. The cyclin box region displaying the highest similarity to other mitotic cyclins is framed. Within the cyclin box, the P box domain required for the activation of *cdc25* [40] is underlined. A putative polyadenylation signal is indicated in bold. The stop codon is denoted by an asterisk. The *Bsm* I site used to create the 3′ specific probe for Southern analysis is shown.

other known plant cyclins revealed the highest similarities with the partial alfalfa cyclin sequences CycMs1 lacking the C-terminus (97% identity) and CycMs2 lacking the N-terminus (77% identity, 85% similarity) [21] and with a full-length maize cyclin CycIIIZm (55% identity, 71% similarity) [32]. Computer analysis estimating the relatedness of the 13 known plant cyclin sequences revealed that plant cyclins belong to three groups (Fig. 2), in concordance with the results of Renaudin *et al.* [32]. All the three alfalfa cyclins as well as the maize cyclin mentioned above fall into the same group and carry the conserved HRPITRS/RF and EMEDI amino acid sequence motifs at their N-terminal region (Fig. 1) characteristic of the group III cyclins [32]. According to the classification and nomenclature of Renaudin *et al.* [32] we designated the alfalfa full-length cDNA clone *CycIIIMs*.

The CycIIIMs alfalfa cyclin, similarly to other plant cyclins [32], shows homology to both A- and B-type cyclins within the cyclin box region (Fig. 3). However, it is not a unique property of plant cyclins since recently a novel type of verte-

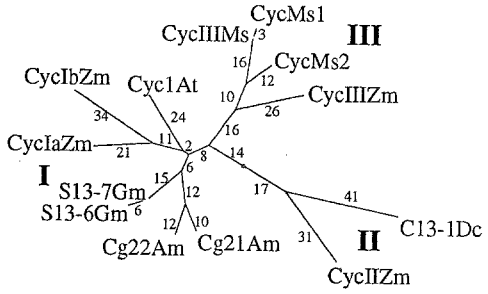

Fig. 2. Unrooted distance tree of amino acid sequences of plant cyclin members obtained by using the Phylotree program (Computational Biochemistry Research Group, Zurich). Distances are drawn to scale and represent the estimate of the Point Accepted Mutation (PAM) distances between each pair of sequences and their variances which are used to weight the distance in the final tree. A circle indicates the weighted centroid of the tree. The sequences of the following proteins were included: CycIIIMs and the two partial cycMs1 and cycMs2 cyclin sequences from alfalfa [21], cyc1At from *Arabidopsis* [20], CycIaZm, CycIbZm, CycIIZm and CycIIIZm from maize [32], C13–1Dc from carrot, S13–6Gm and S13–7Gm from soybean [19] and Cg21Am and Cg22Am from *Antirrhinum* [11].

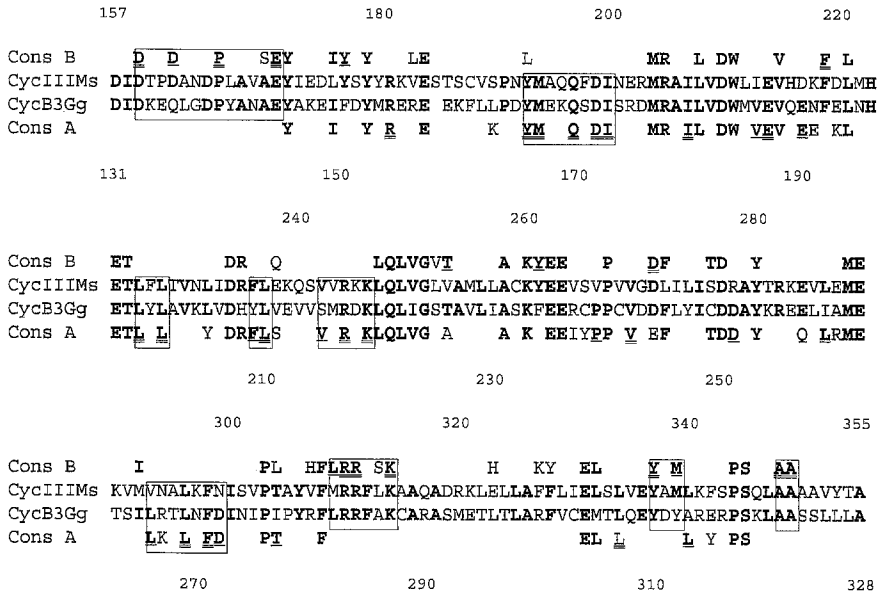

Fig. 3. Alignment of the cyclin box region of the CycIIIMs protein with the corresponding region of the B3-type chicken (*Gallus gallus*) mitotic cyclin CycB3Gg [13] and with the cyclin A and B consensus sequences [13,19]. The residues identical between at least two sequences are printed in bold. Amino acids in the consensus sequences which are present in one or both of the aligned cyclin proteins are underlined with single or double lines, respectively. Longer regions of the alfalfa and chicken cyclins which might represent differences between A- or B-type cyclins are boxed together with the corresponding consensus sequence.

brate cyclins (CycB3Gg) has been identified from chicken (*Gallus gallus*), which is structurally related to and shares functional properties of both A- and B-type cyclins [13]. On the basis of these features CycB3Gg was suggested to represent an evolutionarily early member of the cyclin family. Alignment of the cyclin box region of the alfalfa CycIIIMs and chicken CycB3Gg cyclins and their comparison to the cyclin A- and B-consensus sequences [13,19] showed that both the alfalfa and the chicken cyclin had similar degree of homology to the cyclin A- and B-consensus, respectively. Moreover, in many cases the cyclin A- and B-type specific motifs and residues were similarly arranged in both proteins (Fig. 3).

The CycIIIMs *gene locates at a single chromosomal locus in the diploid alfalfa genome*

High-molecular-weight genomic DNA was isolated from the autogamous diploid species *M. truncatula* cv. Ghor and from the two cultivars of the tetraploid heterogamous *M. sativa* ssp. *sativa*. *Eco* RI digested DNA fragments were hybridized to the full-length *CycIIIMs* (Fig. 4a) and to the 420 bp *Bsm* I-*Eco* RI fragment (Fig. 4b) corresponding to the 3′ part of the cDNA (see Fig. 1). Though *CycIIIMs* cDNA has no internal *Eco* RI site, in the diploid genome two bands hybridized strongly with the full-length probe whereas only one with the 3′ part of the cDNA (lane 1 in Figs. 4a and b). This may suggest that the gene is present in a single chromosomal region in *M. truncatula*, and the existence of the two hybridizing bands with the full-length clone might be due to the presence of intron(s) containing an *Eco* RI cleavage site. In both cultivars of *M. sativa*, 3 bands hybridized strongly with the entire probe and 2 with the 3′ part of the cDNA (lanes 2 and 3 in Figs. 4a and b) indicating that the tetraploid *M. sativa* species contains either an additional *Eco* RI site in an intron region or more likely two different RFLP alleles. Fainter bands indicated by the arrows in Fig. 4 suggest the existence of further gene(s) homologous to *CycIIIMs* both in the diploid and the tetraploid genomes.

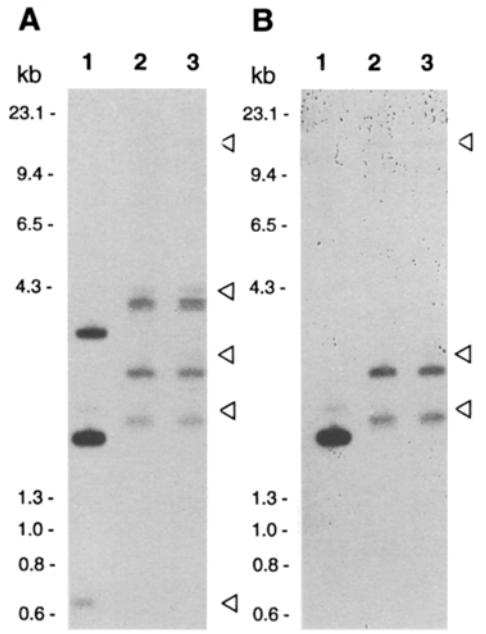

Fig. 4. Southern hybridization of *CycIIIMs* to genomic DNA of diploid *M. truncatula* cv. Ghor (lane 1) and tetraploid *M. sativa* ssp. *sativa* cvs. Nagyszénàsi (lane 2) and Cardinal (lane 3). *Eco* RI-digested DNA fragments were hybridized with the ^{32}P-labelled 1706 bp full-length (A) and the 420 bp 3′ *Bsm* I-*Eco* RI fragment (B) of the *CycIIIMs* cDNA. DNA size markers are shown on the left side of the slots. Arrowheads indicate faint hybridizing bands.

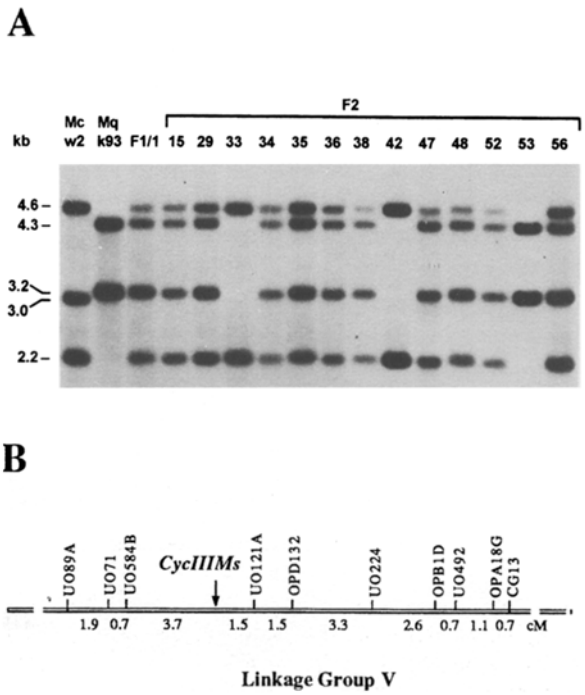

Fig. 5. Chromosomal mapping of *CycIIIMs*. A. The hybridization pattern of the *CycIIIMs* locus in the parents, *Medicago sativa* ssp. *coerulea* (Mcw2) and *M. sativa* ssp. *quasifalcata*-(Mqk93), F1/1 hybrid and selected individuals of the F2 progenies. Lambda DNA digested by *Pst* I was used as molecular mass marker. B. Location of the *CycIIIMs* locus on the genetic map of alfalfa. The region flanked by markers UO89A and CG13 on linkage group 5 is shown. Details of the mapping data for the other loci are described elsewhere [25]. Genetic distances are Haldane distances in centimorgans (cM).

Characterization of the CycIIIMs *locus and its position on the alfalfa genetic map*

To determine the location of the cyclin *CyclIIMs* locus on the genetic map of *Medicago*, the inheritance of the parental genotypes was analyzed in a segregating population which is the self-mated progeny of an F1 hybrid plant (F1/1) derived from a cross between the diploid ($2n = 2x = 16$) yellow-flowered *M. sativa* ssp. *quasifalcata* (Mqk93) and the diploid ($2n = 2x = 16$) purple-flowered *M. sativa* ssp. *coerulea* (Mcw2) [28]. The genotypes of the individual plants in the mapping population for the cyclin gene were determined by DNA-DNA blot hybridization. The segregation of the cyclin alleles as hybridizing bands using the full-length clone as probe is shown in Fig. 5a. Using the cyclin probe for hybridization, three and two hybridization bands were detected in the *Eco* RI-digested total DNA of Mcw2 and Mqk93, respectively. The sizes of the *CycIIIMs*-specific hybridizing bands of Mcw2 and Mqk93 are 4.6, 3.0, 2.2 kb and 4.3, 3.2 kb, respectively. These bands were designated cycw4.6, cycw3.0, cycw2.2 and cycq4.3, cycq3.2. In the F1/1 progeny, two bands from the Mcw2 parent (cycw4.6, cycw2.2) and two bands from the Mqk93 parent (cycq4.3, cycq3.2) were inherited. It seems that the Mcw2 parent is heterozygote for the cyclin locus and the cycw3.0 hybridizing band represents the other allele, while Mqk93 is homozygote for the *CycIIIMs* locus according to the *Eco* RI restriction and hybridization patterns. From the segregation pattern of the cyclin alleles it is evident that the two bands of the appropriate par-

ents were always inherited together, and consequently they are genetically linked. Since there might be an *Eco* RI restriction site in the middle of the structural gene of *CycIIIMs*, it is proposed that the two hybridizing *Eco* RI bands are two fragments of the same structural gene. On this basis, the *CycIIIMs* allele of Mcw2 and Mqk92 were designated cycw and cycq, respectively.

From the hybridization pattern, the segregation of the *CycIIIMs* alleles inherited by the F2 indi-

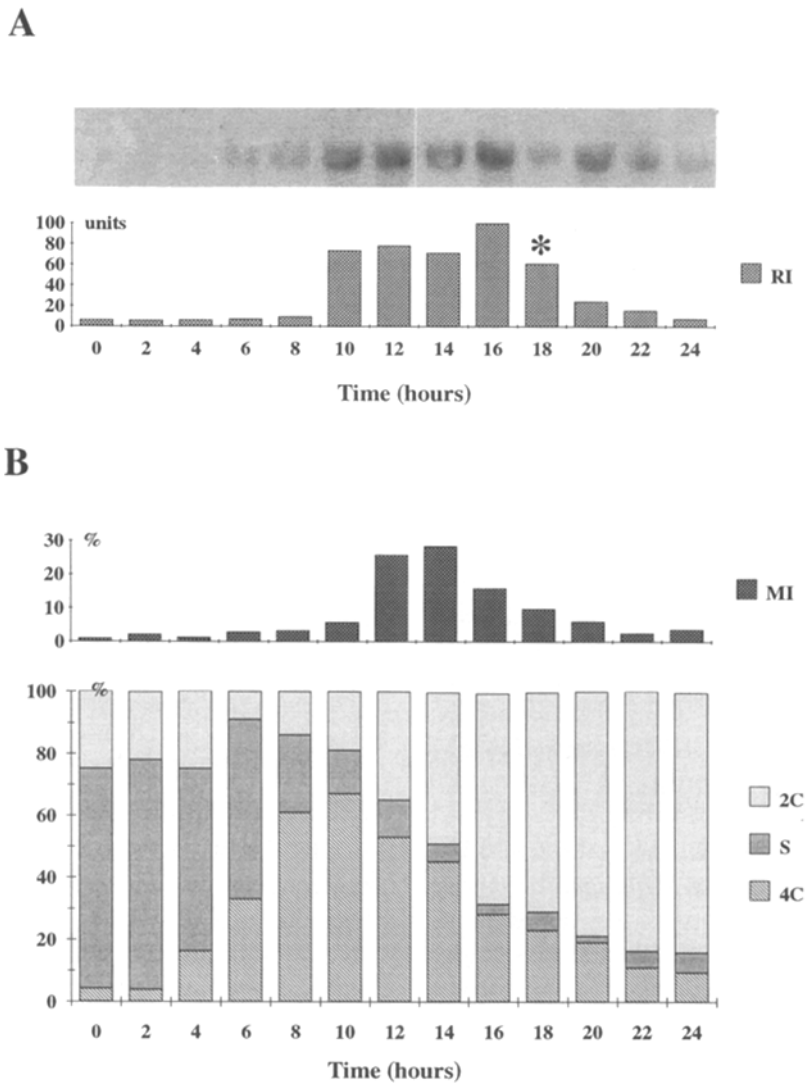

Fig. 6. CycIIIMs expression during the cell cycle progression of partially synchronized alfalfa cell culture. A. 15 μg of total RNA samples isolated from alfalfa *Medicago sativa* ssp.*varia* A2 cells collected at two hour intervals after the release of the cells from a hydroxyurea block were analyzed by northern hybridization using a 216 bp long probe of the 3′-untranslated region of the *CycIIIMs* cDNA. The autoradiogram was analyzed by a Millipore image analyzer, and the relative integrated intensity (RI) of the bands is also shown in arbitrary units. The strongest intensity (at 16 h) was considered as 100 units. The intensity of the 18 h band (*) was multiplied by a factor of two, on the basis of the staining intensity of rRNA molecules bound to the filter as an RNA quantity control (data not shown, see Materials and methods). B. Flow cytometric analysis of nuclear DNA content (2C < S < 4C) of the partially synchronized alfalfa cell culture and the mitotic index (MI) analysis of the same colchicine-treated cell samples were carried out as described in Materials and methods, at the same time intervals as above. The frequencies of the cells in the different cell cycle phases at these time periods are shown.

viduals was calculated. Theoretically the alleles segregate according to the 1:2:1 ratio. The observed segregation of cycw and cycq was cycw/cycw:cycw/cycq:cycq/cycq = 1.0:5.5:1.2. This segregation is comparable to the degree of distortion described before [28].

The genotype of the *CycIIIMs* locus carried by the individuals in the F2 population was used to find its location on the alfalfa genetic map by calculating the linkage to other loci using the maximum likelihood equation of the computer program MAPMAKER V2 [30]. This analysis revealed that the *CycIIIMs* locus mapped to linkage group 5 between loci UO89A and CG13 [28]. Using more genetic markers (RFLP as well as RAPD markers) the *CycIIIMs* locus was mapped more precisely in this region between markers UO584B and UO121A. The map location of *CycIIIMs* is shown on Fig. 5b.

Expression of CycIIIMs *in partially synchronized cell culture and plant tissues*

To determine the pattern of expression of *CycIIIMs* gene, northern blot analysis was performed with total RNA isolated at 2 h intervals from a partially synchronized cell suspension culture after release from a hydroxyurea block. Figure 6b shows that during the 36 hour hydroxyurea treatment 71% of the cells accumulated in the early S phase. After the removal of the drug these cells passed synchronously to the G2 phase, reaching the maximum G2 frequency (67%) at 10 h followed with a mitotic peak between the 12th and 14th hours (28% mitotic index). Most of the G2 cells passed to the G1 phase (79%) at 22 h after the release. The RNA message of the *CycIIIMs* gene, as revealed by using a 3′ specific probe for northern analysis, increased in parallel with the increase of the proportion of G2/M cells (Fig. 6a). It reached its maximum between the 10th and 16th hours after the block, which is the period of the highest mitotic activity, and then decreased. It was hardly detectable when the percentage of G2/M cells was below 5%. This result indicates that this cyclin gene may play a role in G2/M transition and thus belongs to the mitotic cyclin family.

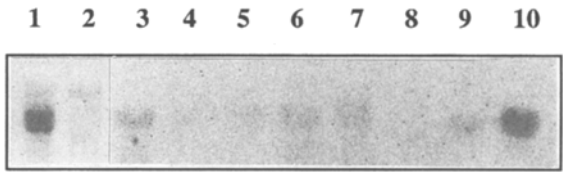

Fig. 7. Organ-specific expression of the *CycIIIMs* alfalfa cyclin gene. 15 μg of total RNA samples has been used for northern analysis of *CycIIIMs* mRNA levels in 7- and 12-day-old alfalfa root nodules developed after the inoculation of alfalfa roots with *R. meliloti* (lanes 1 and 2, respectively), in exponentially but asynchronously growing suspension cultured A2 cells (lane 3), in flower buds (lane 4), flowers (lane 5), stems (lane 6), shoot tips (lane 7) and leaves (lane 8), in roots after the removal of their apex (lane 9) and in root tips (line 10). As hybridization probe a 216 bp long PCR fragment representing the 3′-untranslated region of the cDNA sequence was used. The uniform loading of the RNA samples was confirmed by methylene blue staining (data not shown).

The expression of the alfalfa *CycIIIMs* gene was also investigated in different plant tissues by northern analysis of total RNA samples. Gene expression was detected only in tissues with cell division activity (Fig. 7). The gene was strongly expressed in young root nodules harboring an active apical meristem (lane 1) as expected since the screened cDNA bank was prepared from this organ. The expression was similarly strong in the root apex (lane 10) and relatively high in the exponentially but asynchronously growing suspension cultured alfalfa cells (lane 3). The mRNA level was much lower in stems and roots (lanes 6 and 9, respectively), still detectable in flower buds and flowers (lanes 4 and 5, respectively), but not in leaves (lane 8) and mature nitrogen-fixing nodules (lane 2) where the meristematic activity had become low.

Discussion

We report here on the isolation and the characterization of a full-length cyclin cDNA clone from alfalfa. Sequence analysis with the Swissprot protein data base revealed extensive homology of the primary protein sequence deduced from the

cDNA sequence to cyclin proteins from other plant species, most notably to the partial alfalfa cDNA sequences *cycMs1* and *cycMs2*[21]. Sequence comparison analysis indicated that *CycIIIMs* and *cycMs1*and *cycMs2* are close homologues, exhibiting 97% and 77% identity, respectively, at the amino acid level. Our analysis on the divergence of the known plant mitotic cyclins concords with their classification recently suggested [32]. The analysis indicated that CycIIIMs belongs to the group III, which consists of the two partial alfalfa cyclins, cycMs1 and cycMs2 [21], and the CycIIIZm cyclin isolated from maize [32]. Based on the genetic heterogeneity of heterogamous perennial alfalfa species and subspecies, it is likely that the partial *cycMs1* cDNA from *M. sativa* ssp. *sativa* RA3, differing only in five amino acid residues in the sequenced region, represents an allelic variant of the *M. sativa* ssp. *varia CycIIIMs* gene. Therefore, the *cycMs1* gene does not belong to a new class of cyclins as has been proposed [21]. It is not clear from the available sequence data whether *cycMs2* is an allelic form of *CycIIIMs* or whether it is a closely related gene. The analysis of the segregation of the *CycIIIMs* gene among the diploid *Medicago* indicates that this cyclin gene represents a single genetic region mapped between the UO89A and CG13 RFLP marker loci in the linkage group 5. At present, we do not know whether this single chromosomal region contains one or more structural genes in close proximity to each other. The additional weak bands on Southern blots may correspond to distinct cyclin genes hybridizing to the highly conserved motifs of plant cyclins.

Sequence analysis of the *CycIIIMs* gene revealed the presence of specific mitotic cyclin motifs such as the destruction motif as well as the P box domain present in all A- and B-type cyclins. Moreover, we have shown that the expression of the *CycIIIMs* gene during cell cycle progression of a partially synchronized alfalfa cell culture was tightly linked to the presence of cells in the G2/M cell cycle phase, as revealed by flow cytometric, mitotic and northern analyses of cell samples obtained at two hourly intervals after release of the cells from a hydroxyurea block. Likewise, predominantly G2/M phase specific expression was reported for the two other known alfalfa cyclin sequences *cycMs1* and *cycMs2*, although a considerable message level was observed in all cell cycle phases using the whole *cycMs1* sequence as a probe [21]. This difference might be due to the use of different hybridization probes. Therefore, both the sequence data and the expression pattern of *CycIIIMs* were typical of a mitotic type of cyclin.

On the basis of sequence analysis it is not possible to assign clearly CycIIIMs nor any other plant mitotic cyclin to either A- or B-type as they share characteristic sequence motifs of both types [19,32]. It seems from phylogenetic studies including yeast, animal and plant cyclins that groups II and III of plant mitotic cyclins form a separate class which is more homologous to B-type cyclins (data not shown) while the group I is more closely related to the A-type cyclins as has already been proposed [19,32]. However, the CycIIIMs protein (but not the other plant cyclins) has a destruction motif RXALGVIXX characteristic of A-type cyclins [17]. Mitotic cyclins sharing not only structural characteristics but also the functional properties of A- and B-type cyclins have recently been described from animal species as well [13]. Amino acid sequence comparison of the cyclin box region of one of these so called B3-type cyclins and CycIIIMs with the cyclin A and B consensus sequences revealed remarkable similarity between the two cyclins in respect of the distribution of A- and B-type specific motifs or residues. B3-type mitotic cyclins were proposed to represent an evolutionary early class of animal cyclins that might be the closest relatives of the plant cyclins. It remains to be elucidated whether single plant cyclins simultaneously assure functional properties of A- and B-type mitotic cyclins or whether different molecules with separate functions have also evolved in plants.

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References

1. Allison LA, Kiss GB, Bauer P, Poiret M, Pierre M, Savouré A, Kondorosi E, Kondorosi A: Identification of two alfalfa early nodulin genes with homology to members of the pea *Enod12* gene family. Plant Mol Biol 21: 375–380 (1993).
2. Bergounioux C, Perennes C, Hemerly AS, Qin LX, Sarda C, Inzé D, Gadal P: A *cdc2* gene of *Petunia hybrida* is differentially expressed in leaves, protoplasts and during various cell cycle phases. Plant Mol Biol 20: 1121–1130 (1992).
3. Brummer EC, Kochert G, Bouton JH: RFLP variation in diploid and tetraploid alfalfa. Theor Appl Genet 83: 89–96 (1991).
4. Chomczynski P: One-hour downward alkaline capillary transfer for blotting of DNA and RNA. Anal Biochem 201: 134–139 (1992).
5. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159 (1987).
6. Church GM, Gilbert W: Genomic sequencing. Proc Natl Acad Sci USA 81: 1991–1995 (1984).
7. Colasanti J, Tyers M, Sundaresan V: Isolation and characterisation of cDNAs encoding a functional $p34^{cdc2}$ homologue from *Zea mays*. Proc Natl Acad Sci USA 88: 3377–3381 (1991).
8. Dudits D, Bögre L, Bakó L, Dedeoglu D, Magyar Z, Kapros T, Felföldi F, Györgyey J: Key components of cell cycle control during auxin-induced cell division. In: Ormrod JC, Francis D (eds) Molecular and Cell Biology of the Plant Cell Cycle, pp. 111–131. Kluwer Academic Publishers, Dordrecht (1993).
9. Evans T, Rosenthal ET, Youngblom J, Distel D, Hunt T: Cyclin: A protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. Cell 33: 389–396 (1983).
10. Ferreira P, Hemerly A, Villarroel R, van Montagu M, Inzé D: The *Arabidopsis* functional homolog of the $p34^{cdc2}$ protein kinase. Plant Cell 3: 531–540 (1991).
11. Fobert PR, Coen ES, Murphy GJP, Doonan JH: Patterns of cell division revealed by transcriptional regulation of genes during the cell cycle in plants. EMBO J 13: 616–624 (1994).
12. Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E: Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220: 1049–1051 (1983).
13. Gallant P, Nigg EA: Identification of a novel vertebrate cyclin: cyclin B3 shares properties with both A- and B-type cyclins. EMBO J 13: 595–605 (1994).
14. Gautier J, Solomon MJ, Booher RN, Bazan JF, Kirschner MW: *cdc25* is a specific tyrosine phosphatase that directly activates $p34^{cdc2}$. Cell 67: 197–211 (1991).
15. Gibson AH: Methods for legumes in glasshouses and controlled environment cabinets. In: Bergersen FJ (ed) Methods for Evaluating Biological Nitrogen Fixation, pp. 139–184. Wiley, London (1980).
16. Girard F, Strausfeld U, Fernandez A, Lamb NJC: Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. Cell 67: 1169–1179 (1991).
17. Glotzer M, Murray AW, Kirschner MW: Cyclin is degraded by the ubiquitin pathway. Nature 349: 132–138 (1991).
18. Györgyey J, Gartner A, Németh K, Magyar Z, Hirt H, Heberle-Bors E, Dudits D: Alfalfa heat shock genes are differentially expressed during somatic embryogenesis. Plant Mol Biol 16: 999–1007 (1991).
19. Hata S, Kouchi H, Suzuka I, Ishii T: Isolation and characterization of cDNA clones for plant cyclins. EMBO J 10: 2681–2688 (1991).
20. Hemerly A, Bergounioux C, van Montagu M, Inzé D, Ferreira P: Genes regulating the plant cell cycle: isolation of a mitotic-like cyclin from *Arabidopsis thaliana*. Proc Natl Acad Sci USA 89: 3295–3299 (1992).
21. Hirt H, Mink M, Pfosser M, Bögre L, Györgyey J, Jonak C, Gartner A, Dudits D, Heberle-Bors E: Alfalfa cyclins: differential expression during the cell cycle and in plant organs. Plant Cell 4: 1531–1538 (1992).
22. Hirt H, Páy A, Györgyey J, Bakó L, Németh K, Bögre L, Schweyen RJ, Heberle-Bors E, Dudits D: Complementation of a yeast cell cycle mutant by an alfalfa cDNA encoding a protein kinase homologous to $p34^{cdc2}$. Proc Natl Acad Sci USA 88: 1636–1640 (1991).
23. Hunt T: Cyclins and their partners: from a simple idea to complicated reality. Semin Cell Biol 2: 219–222 (1991).
24. Jacobs T: Control of the cell cycle. Devel Biol 153: 1–15 (1992).

25. Kaló P, Csanádi G, Endre G, Felföldi K, Kiss GB: Improved genetic linkage map of alfalfa and its comparison to other *Medicago* maps. Submitted (1994).
26. Kapros T, Stefanov I, Magyar Z, Ocsovszky I, Dudits D: A short histone H3 promoter from alfalfa specifies expression in S-phase cells and meristems. In Vitro 29: 27–32 (1993).
27. King RW, Jackson PK, Kirschner MW: Mitosis in transition. Cell 79: 563–571 (1994).
28. Kiss GB, Csanádi G, Kálmán K, Kaló P, Ökrész L: Construction of a basic genetic map for alfalfa using RFLP, RAPD, isozyme and morphological markers. Mol Gen Genet 238: 129–137 (1993).
29. Kumagai A, Dunphy WG: The *cdc25* protein controls tyrosine dephosphorylation of *cdc2* protein in a cell-free system. Cell 64: 903–914 (1991).
30. Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L: MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1: 174–181 (1987).
31. Nugent JHA, Alfa CE, Young T, Hyams JS: Conserved structural motifs in cyclins identified by sequence analysis. J Cell Sci 99: 669–674 (1991).
32. Renaudin J-P, Colasanti J, Rime H, Yuan Z, Sundaresan V: Cloning of four cyclins from maize indicates that higher plants have three structurally distinct groups of mitotic cyclins. Proc Natl Acad Sci USA 91: 7375–7379 (1994).
33. Sambrook J, Fritsch EF, Maniatis T: Molecular cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).
34. Sanger F, Nicklen S, Coulson AR: DNA sequencing with the chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467 (1977).
35. Savouré A, Magyar Z, Pierre M, Brown S, Schultze M, Dudits D, Kondorosi A, Kondorosi E: Activation of the cell cycle machinery and the isoflavonoid biosynthesis pathway by active *Rhizobium meliloti* Nod signal molecules in *Medicago* microcallus suspensions. EMBO J 13: 1093–1102 (1994).
36. Sherr CJ: G1 phase progression: cycling on cue. Cell 79: 551–555 (1994).
37. Strausfeld U, Labbé J-C, Fesquet D, Cavadore JC, Picard A, Sadhus K, Russel P, Dorée M: Dephosphorylation and activation of $p34^{cdc2}$/cyclin B complex *in vitro* by human *cdc25* protein. Nature 351: 242–245 (1991).
38. Swenson KI, Farrell KM, Ruderman JV: The clam embryo protein cyclin A induces entry into M phase and the resumption of meiosis in *Xenopus* oocytes. Cell 47: 861–870 (1986).
39. Walker DH, Maller JL: Role for cyclin A in the dependence of mitosis on completion of DNA replication. Nature 354: 314–317 (1991).
40. Zheng X-F, Ruderman JV: Functional analysis of the P box, a domain in cyclin B required for the activation of *cdc25*. Cell 75: 155–164 (1993).