cdc2MsB, a cognate cdc2 gene from alfalfa, complements the G1/S but not the G2/M transition of budding yeast cdc28 mutants

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Summary
The product of the cdc2 gene encodes the p34cdc2 protein kinase that controls entry of yeast cells into S phase and mitosis. In higher eukaryotes, at least two cdc2-like genes appear to be involved in these processes. A cdc2 homologous gene has previously been isolated from alfalfa and shown to complement a fission yeast cdc28* mutant. Here the isolation of cdc2MsB, a cognate cdc2 gene from alfalfa (Medicago sativa) is reported. Southern blot analysis shows that cdc2MsA and cdc2MsB are present as single copy genes in different tetraploid Medicago species. cdc2MsB encodes a slightly larger mRNA (1.5 kb) than cdc2MsA (1.4 kb). Both genes were found to be expressed at similar steady state levels in different alfalfa organs. Expression levels of both cdc2Ms genes correlate with the proliferative state of the organs. Complementation studies revealed that in contrast to cdc2MsA, cdc2MsB was not able to rescue a cdc28* fission yeast mutant. cdc2MsB was also unable to rescue a G2/M-arrested cdc28* budding yeast mutant which could be rescued by expression of the cdc2MsA gene. Conversely, cdc2MsB but not cdc2MsA was found to complement the G1/S block of another cdc28* budding yeast mutant. These results suggest that cdc2MsA and cdc2MsB function at different control points in the cell cycle.

Introduction
All eukaryotes have been shown to contain a 34 kDa protein kinase which plays a central role in the regulation of cell division. The kinase has been shown to be the product of the cdc2+/CDC28 genes in yeasts and animals (for review, see Nurse, 1990) and in various plant species (for review, see Doonan, 1991) and is denoted p34cdc2.

The p34cdc2 kinase is part of the mitosis promoting factor (MPF). MPF activation involves physical association of p34cdc2 with cyclin proteins. In the absence of cyclins, cells cannot enter mitosis and p34cdc2 kinase activity is absent (Booher and Beach, 1987, 1988; Minshull et al., 1990; Murray and Kirschner, 1989; Murray et al., 1989). At metaphase, cyclins are destroyed and protein kinase activity vanishes (Murray et al., 1989). Degradation of cyclins has been shown to be required for completion of mitosis and might be necessary for inactivation of p34cdc2 kinase (Murray et al., 1989).

In contrast to yeast, several cdc2-like kinases have been identified in higher eukaryotes (Dunphy and Newport, 1989; Eldledge and Spottswood, 1991; Fang and Newport, 1991; Lehner and O’Farrell, 1990; Paris et al., 1991).

Also in higher plants, several cdc2-like genes have been reported (Colasanti et al., 1991; Feiler and Jacobs, 1990, 1991; Ferreira et al., 1991; Hata, 1991; Hirayama et al., 1991). We have previously isolated a homologue of the fission yeast cdc2 gene from alfalfa (Hirt et al., 1991). In Northern blot analyses, several distinct transcripts have been observed. We therefore decided to search for other cdc2-related genes.

We report here the isolation of cdc2MsB, a cognate cdc2 gene from Medicago sativa. In transcript analysis of vegetative organs, expression of both genes was observed at similar steady state levels. While cdc2MsA was shown to complement a cdc21* fission yeast mutant (Hirt et al., 1991), cdc2MsB was unable to perform the same function. In conditional budding yeast mutants, cdc2MsA but not cdc2MsB was able to complement a G2/M cdc28* mutant, and conversely cdc2MsB but not cdc2MsA was able to rescue a G1/S cdc28* mutant.

Results
Isolation and sequencing of cdc2MsB cDNA from M. sativa
To isolate cdc2-related genes, we hybridized 300 000 colonies of an alfalfa cDNA library with a labelled alfalfa cdc2 fragment. Positive colonies were isolated and sequenced. Three clones were found to have identical nucleotide sequences to cdc2MsA, the originally identified cDNA sequence (Hirt et al., 1991).

One clone showed differences at four positions in the ORF (data not shown). Three of these changes had occurred in third codon positions and did not change the protein sequence. Only one nucleotide difference is predic-
ted to cause an amino acid exchange at position 203 (see Figure 2). This resulted in the exchange of a serine in cdc2MsA by a phenylalanine which is the commonly found amino acid in most cdc2 proteins. Since the 3' nontranslated nucleotide sequence is also highly similar to cdc2MsA, this clone is assumed to be an allele of cdc2MsA.

One clone showed marked differences in the restriction pattern and the subsequently determined nucleotide sequence. This clone was denoted cdc2MsB. As shown in Figure 1, the cDNA sequence shows a nontranslated region of 69 nucleotides before the first ATG. An ORF of 879 nucleotides putatively encodes a protein of 293 amino acids. The cDNA has a 345 nucleotide 3' end and contains a poly(A)⁺ tail of 12 nucleotides. Interestingly, all cdc2MsA clones were truncated at both the 5' and 3' ends (data not shown). This is surprising since four clones were found of the cdc2MsA type and only one for cdc2MsB. This possibly reflects different mRNA stabilities of the two cdc2Ms transcripts.

Sequence comparison of alfalfa cdc2MsA and cdc2MsB

Alignment of the predicted alfalfa cdc2MsA and cdc2MsB protein sequences as shown in Figure 2 revealed 89% identity. The amino acid exchanges did not appear to be particularly clustered in any one region, except possibly in the carboxy-terminal domain. cdc2MsB contains all the motifs of a functional protein kinase and has a perfect copy of the PSTAIR sequence, which is a trademark of the cdc2 proteins. Comparison of cdc2MsB with the predicted protein sequences of the recently reported cdc2 cDNAs from Arabidopsis thaliana, maize and pea (Colasanti et al., 1991; Feiler and Jacobs, 1991; Ferreira et al., 1991) resulted in scores similar to cdc2MsA. Alignment with the predicted yeast and animal cdc2 protein sequences yielded identity scores between 55 and 65%. Interestingly, homology to the two cdc2 proteins from Drosophila melanogaster, which are only 65% identical to each other, was 57 and 61% (Lehner and O'Farrell, 1990). Furthermore, 67% identity was observed to Eg1 from Xenopus (Paris et al., 1991). This cdc2-like gene was unable to complement budding and fission yeast cdc28/cdc2 mutants and is speculated to be involved in the control of the G1/S transition (Paris et al., 1991). In summary, on the basis of protein sequence homology, cdc2MsB cannot be classified to the mitotic or interphase subtypes.

cdc2MsA and B are not allelic with each other

Since M. sativa is an autotetraploid organism, it was important to determine whether cdc2MsA and cdc2MsB might represent alleles of the cdc2Ms gene. Therefore, lines of two different alfalfa species and a species hybrid were analysed by Southern blotting. The genomic DNAs were digested with HindIII and hybridized with 3'-specific probes of the cdc2MsA and cdc2MsB genes (Figure 3b and a, respectively). The restriction patterns for cdc2MsA and cdc2MsB were very different. All Medicago varia lines tested (Figure 3a and b, lanes 1–3) and also M. sativa (Figure 3a and b, lane 4) contained the cdc2MsA and the cdc2MsB genes. Since none of the probes contained HindIII sites within their nucleotide sequences, this indicates that four copies of the cdc2MsA, as well as of the cdc2MsB gene, are present per genome. In conclusion, cdc2MsA and cdc2MsB represent different genes and are present as single copy genes per haploid set of chromosomes.

Expression of cdc2MsA and cdc2MsB in alfalfa suspension-cultured cells and organs

To determine the transcript sizes of the cdc2MsA and cdc2MsB genes, total RNA was isolated from M. varia suspension-cultured cells and separated on a 1% agarose gel containing 1% formaldehyde. Hybridization with the gene-specific 3' probes revealed mRNA sizes of 1.5 kb for cdc2MsB and 1.4 kb for cdc2MsA (data not shown).

To investigate whether cdc2MsA and cdc2MsB are expressed in an organ-specific manner, we analysed alfalfa root, young leaf (3 mm long), adult leaf, stem, vegetative meristem, flower bud and suspension-cultured cells for steady state transcript levels of the two genes as shown in Figure 4, lanes 1–7, respectively. Since the use of total RNA resulted in hardly detectable hybridization signals, poly(A)⁺ RNA was isolated from each organ and analysed by Northern hybridization with cdc2MsA- and cdc2MsB-specific probes (Figure 4a and b, respectively). Considerable transcript levels of cdc2MsA and cdc2MsB were detected in most organs. However, in adult leaf hardly any mRNA of either cdc2Ms genes was observed (Figure 4a and b, lane 3). This transcriptional pattern correlates with the proliferative state of the organs. Comparison of transcript levels for cdc2MsA and cdc2MsB in each organ revealed no specific differences.

As control, the same filter was reprobed with a radiolabelled Arabidopsis thaliana 25S rDNA fragment as shown in Figure 4c (although undetectable by ethidium bromide staining, poly(A)⁺ RNA still contains sufficient amounts of rRNA to allow detection with radiolabelled probes). With the exception of flower bud (Figure 4c, lane 5) all organs showed similar 25S rRNA levels. This indicates that the lower cdc2MsA and cdc2MsB transcript levels in flower bud were due to the lower amounts of poly(A)⁺ in this organ. On the other hand, the 25S rRNA level in adult leaf was comparable with those of other organs and cannot explain the very low cdc2Ms mRNA levels.
Figure 1. Nucleotide sequence of the alfalfa cdc2MsB cDNA and its predicted amino acid sequence.
We have previously shown that cdc2MsA was able to complement a cdc2\textsuperscript{−} fission yeast strain at the restrictive temperature (Hirt et al., 1991). To test whether the same is true for the cdc2MsB gene, we cloned the cdc2MsA and cdc2MsB genes into the yeast expression vector pSM-1. The plasmid contains the SV-40 promoter which was shown to be efficiently used in Schizosaccharomyces pombe (Jones et al., 1988). After transformation of the

![](image)

**Figure 2.** Sequence alignment of the predicted alfalfa cdc2MsB and cdc2MsA proteins. Identical amino acid residues are indicated by dots, differences are shown by single letter code.

![](image)

**Figure 3.** Southern blot analysis of cdc2MsA and cdc2MsB.

DNA from different *M. varia* lines (lanes 1–3), *M. sativa* (lane 4) and a *M. varia × M. sativa* hybrid (lane 5) were hybridized with gene-specific \textsuperscript{32}P probes of cdc2MsB (a) and with cdc2MsA (b).

![](image)

**Figure 4.** Steady state mRNA levels of cdc2MsA and cdc2MsB in alfalfa.

25SAt

1 2 3 4 5 6 7

(a) cdc2MsA

(b) cdc2MsB

(c) 25SAt

We used Pog9(+), a DNA isolated from *Arabidopsis thaliana* root tip, young war; Pog9(−) stem, vegetative meristem, flower bud and suspension cultured cells (lanes 1–7, respectively). Northern hybridization was performed with gene-specific \textsuperscript{32}P probes of cdc2MsA (a), cdc2MsB (b), or *Arabidopsis* 25S rDNA (c) as a control.

25SAt

1 2 3 4 5 6 7

(a) cdc2MsA

(b) cdc2MsB

(c) 25SAt

**cdc2MsB cannot complement a cdc2\textsuperscript{−} fission yeast strain**

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25SAt

1 2 3 4 5 6 7

(a) cdc2MsA

(b) cdc2MsB

(c) 25SAt

**cdc2MsB cannot complement a cdc2\textsuperscript{−} fission yeast strain**
found to grow on plates at 28°C as shown in Figure 5 (a–c, respectively). However, at temperatures above 30°C, only cdc2MsA/pSM-1-transformed cells were able to form colonies (Figure 5c). We conclude that cdc2MsB is not able to complement the fission yeast cdc2-33 mutant.

cdc2MsB can complement the G1/S transition but not the G2/M transition of cdc28 budding yeast mutants

To test whether cdc2MsB can complement the CDC28 function in budding yeast, we cloned cdc2MsB into the yeast expression vector pYES2 to give pYES2/cdc2MsB. Also, the cdc2MsA gene was cloned into the same vector to give pYES2/cdc2MsA. In the pYES2 plasmid, expression of the cdc2Ms genes can be induced by the GAL1 promoter. Two different cdc28 budding yeast mutants were transformed with pYES2/cdc2MsA, pYES2/cdc2MsB, and the empty pYES2 plasmid as a control. The one Saccharomyces cerevisiae strain was the cdc28-4ts mutant which is known to block the cells in 'START', i.e. when cells are about to enter the S phase. The other S. cerevisiae strain was the cdc28-1Nts mutant, which was shown to block cells in G2/M (Surana et al., 1991). Transforms were first selected for uracil prototrophy. Then, for induction of the GAL1 promoter, colonies were plated on to fresh medium plates containing galactose and incubated for 2 days at 23, 25, 28, 30, 33, 35 and 37°C. cdc28-1N cells which were transformed with either the pYES2/cdc2MsB construct (Figure 6c) or with pYES2 alone (Figure 6a) were able to grow only at 23, 25 and 28°C (Figure 6, 28°C) but not at higher temperatures (Figure 6, 36°C). In contrast, pYES2/cdc2MsA-transformed cdc28-1Nts cells were able to grow at 25, 28, 30, 33 and 36°C (Figure 6b). This shows that alalfa cdc2MsA but not cdc2MsB is able to substitute for the mutated CDC28 protein at the G2/M transition.

When cdc28-4ts budding yeast cells were transformed with pYES2/cdc2MsB (Figure 6f), they were able to grow at 23, 25, 28, 30, 33 and 36°C. In contrast, cdc28-4ts cells which were transformed with pYES2/cdc2MsA (Figure 6e) or pYES2 (Figure 6d) did not grow at temperatures above 28°C. This shows that the alalfa cdc2MsB is able to substitute for the CDC28 function in G1/S.

To analyse the complementing effect of the alalfa cdc2 genes on cell division activity of budding yeast cells in a different way, the cell numbers of S. cerevisiae cdc28-4 and S. cerevisiae cdc28-1N cells transformed with pYES2, pYES2/cdc2MsA or pYES2/cdc2MsB, were determined at 28°C and 33°C as shown in Figure 7 (a and b, respectively). At the permissive temperature of 28°C, S. cerevisiae cdc28-1N cells which were transformed with pYES2, pYES2/cdc2MsA or pYES2/cdc2MsB grew equally well (Figure 7a). Under the restrictive conditions at 33°C, only pYES2/cdc2MsA-transformed cells showed an increase in cell number (Figure 7a). When the same analysis was performed with S. cerevisiae cdc28-4 cells that were transformed with the three constructs, incubation at the restrictive temperature resulted in a cell number increase only in pYES2/cdc2MsB-transformed yeasts (Figure 7b) but not in those that expressed the cdc2MsB gene (Figure 7b).

Discussion

In budding and fission yeasts, the induction of both DNA replication and mitosis requires the action of the p34cdc2 kinase (Hartwell, 1974; Lörincz and Reed, 1984; Nurse and Bissett, 1991; Reed, 1980). While yeasts contain only one cdc2 gene, several cdc2-like kinases have been cloned
Since alfalfa is an autotetraploid plant, the possibility existed that the two cdc2Ms genes might be alleles. Considerable evidence argues against this possibility. First, we found a cDNA clone which is nearly identical to cdc2MsA. Although several nucleotide differences were observed, only one produced an amino acid substitution. Furthermore, the 3' noncoding region was also highly similar to cdc2MsA. Therefore, this clone probably represents a true allele. In contrast, the cdc2MsB gene is only 89% identical to cdc2MsA at the amino acid level, and the 3' nontranslated sequences of cdc2MsA and cdc2MsB differ considerably. Secondly, Southern blot analysis with the cdc2MsA and cdc2MsB genes showed that each of the two genes is present in four copies, i.e., one copy per haploid genome. Two different alfalfa species, *M. varia* and *M. sativa*, both contained cdc2MsA as well as cdc2MsB, therefore, we consider cdc2MsA and cdc2MsB to be different genes.

The presence of two different cdc2-like genes implies that the encoded kinases should perform different functions. A crucial test for the function of a cdc2 homologous gene is the complementation of temperature-sensitive yeast mutants that are defective in the function of the cdc2 kinase and cannot pass through the cell cycle at the restrictive temperature. When the two alfalfa cdc2 genes were tested for complementation of a fission yeast cdc2Ts mutant, only the cdc2MsA gene was able to substitute for the fission yeast cdc2 gene, suggesting that the cdc2MsB might have a different function in the cell cycle. When the same genes were tested in two budding yeast mutants, one of which arrests at the G1/S, the other at the G2/M transition point (Surana et al., 1991), cdc2MsB could only rescue the G1/S arrest but not the G2/M block while cdc2MsA was able to complement the G1/S but not the G2/M arrest.

Figure 7. Complementation studies of budding yeast cdc2Ts mutants with alfalfa cdc2MsA and cdc2MsB. cdc28-1N and cdc28-4 budding yeast mutants were transformed with pYES2, pYES2/cdc2MsA and pYES2/cdc2MsB. Ura+ prototrophic transformants were grown in a medium containing medium at 28°C (●) and 33°C (○). (a) cdc28-1N cells transformed with pYES2 at 28°C (●) and 33°C (○), or with pYES2/cdc2MsA at 28°C (○) and 33°C (●) or with pYES2/cdc2MsB at 28°C (●) and 33°C (○). (b) cdc28-4 cells transformed with pYES2 at 28°C (●) and 33°C (○) or with pYES2/cdc2MsA at 28°C (●) and 33°C (○) or with pYES2/cdc2MsB at 28°C (●) and 33°C (○).

from higher animals (Elledge and Spottswood, 1991; Fang and Newport, 1991; Lehner and O'Farrell, 1990; Paris et al., 1991). The function of these additional cdc2-like kinases has yet to be defined, but some evidence implies them to be involved in the onset of DNA replication (Dunphy and Newport, 1989; Fang and Newport, 1991; Pines and Hunter, 1990).

Also in higher plants, several cdc2-like genes have been reported (Colasanti et al., 1991; Feiler and Jacobs, 1991; Ferreria et al., 1991; Hata, 1991; Hirayama et al., 1991), but their functions are unclear. We have previously isolated a homologue of the fission yeast cdc2 gene from alfalfa (Hirt et al., 1991). Here, we report the isolation and characterization of cdc2MsB, a cognate cdc2 gene from *M. sativa*.
primes. Therefore, it appears possible that the two alfalfa cdc2Ms genes which we have identified are the only cdc2-like genes in this species. The high similarity of the predicted amino acid sequences appears to be puzzling on first sight. However, it should be noted that yeast cells only possess one cdc2 kinase which performs both the G1/S and the G2/M transition steps in the cell cycle. Inspection of the amino acid differences in the cdc2Ms genes with the mapped sites of S. pombe cdc2 mutants (Ayscough et al., 1992; Carr et al., 1989), revealed two sites where amino acid changes could contribute to functional differences of the encoded kinases. In one case the S. pombe cdc2wee1 mutant 1w/2w maps to a region where both cdc2Ms genes show several differences to each other (position 138-142 in Figure 2). In the other case, the S. pombe cdc2 mutant L210 has an exchange from a phenylalanine to a leucine. Interestingly, the cdc2MsB sequence has the wild-type phenylalanine at this position whereas the cdc2MsA has a serine (position 203 in Figure 2). Experiments to verify the different roles of the cdc2Ms genes face the problem of the identification and quantification of the different protein kinase activities. The presently used p13sucent affinity columns are known to bind a multitude of proteins, including at least some of the cdc2-like kinases (Hunter, personal communication). Antibodies raised against the cdc2 proteins or the highly conserved PSTAIR peptide are commonly used to identify these kinases on Western blots. Owing to the high similarity of the cdc2Ms genes, these antibodies cross-react with the different cdc2-like kinases (Fang and Newport, 1991). At present, we are synthesizing peptide antibodies against these regions of the cdc2 proteins which show a high degree of divergence to prevent cross-reaction. With the help of these antibodies, we hope to identify specific substrate regions for the two kinases and to study the regulatory pathways acting upon them.

Apart from different roles during the cell cycle, some of the cdc2-like genes appear to be expressed in a tissue-specific manner as reported for other yeast subclones (Meyerson et al., 1992). In Northern blots of various alfalfa organs, we found only minor differences in expression of the two genes. However, comparison of young and adult leaf showed a marked change in transcript levels of both cdc2Ms genes. This suggests a correlation of cdc2Ms transcripts with the proliferative state of the organs. Flow cytometric analysis of these organs (data not shown) and cdc2 in situ hybridization data in Arabidopsis (Martinez et al., 1992) corroborate this finding. To determine more precisely which cells in the different organs express cdc2MsA or cdc2MsB, in situ hybridization with gene-specific probes will be necessary. On the other hand, the cdc2 protein kinase is known to be subjected to transcriptional, translational and post-translational controls in animals and yeast (for review, see Nurse, 1990). Therefore, the finding of similar steady state transcript levels of the cdc2Ms genes in different vegetative organs does not preclude that the encoded kinases have different activities and perform distinct roles in particular tissues or specialized cells.

**Experimental procedures**

**Plant tissue culture**

A suspension culture of M. varia (line A2) was used (Böge et al., 1988). The suspension, which originated from Murashige and Skoog medium, was supplemented with 0.2 mg l⁻¹ 2,4 dichlorophenoxyacetic acid and 1 mg l⁻¹ thiamine. The cultures were grown at 28°C under a 16 h photoperiod (Philips TLD 80 W/865). Poly(A)⁺ RNA was isolated from a alfalfa suspension-cultured cell line (Stratagene, Calif.) which was hybridized with the cdc2MsA cDNA. Sequences of the gene fragment (Hirt et al., 1991) were isolated and sequenced using a sequencing kit (Pharmacia, Sweden). The fragment was found to encode cdc2MsA.

**Library screening**

The cdc2MsA cDNA was identical to the cdc2MsB cDNA which had been isolated from somatic embryos from induced cultured cells (Györgey et al., 1991). A ZAP X library was prepared according to the manufacturer's instructions (Stratagene, Calif., USA). Approximately 300 000 colonies were screened with a 3²P-labelled alfalfa 0.75 kb PstI cdc2MsA (Györgey et al., 1991). Five positive clones were isolated. These clones were sequenced in both directions with a T7 polymerase (Pharmacia, Sweden). Three clones identical to the cdc2MsA gene described earlier were isolated. One clone was found to be an allelic form of the other two. The cloning was found to encode cdc2MsB.

**Analysis**

Tissue samples from different lines of M. varia cv. Rumbler and cvs RAL were used. DNA (10 μg) from each sample was digested with HindIII and separated on a 1.5% agarose gel. The nylon filters were hybridized with the two cdc2 gene-specific probes.

The same probes, primers of the two alfalfa cdc2 genes CCAACTGAAGGCTGCT and ATCTGGCAGTGCAGACCG were used in PCRs with primers CGAACATCGTCTC and CGAACATCGTCTC, respectively. The fragments were digested with HindIII, primers were obtained by enzymatic fragment labelling according to the conditions as described in the section on FNA extraction of transcript levels. The 226 and 170 bp fragments with HindIII and PstI and cloned into Bluescript KS+ (Stratagene). Gene-specific probes were isolated according to the standard protocols described in the section on FNA extraction of transcript levels. The 226 and 170 bp fragments with HindIII and PstI and cloned into Bluescript KS+ (Stratagene). Gene-specific probes were isolated using a cleavage kit (Stratagene). DNA (10 μg) from each sample was digested with HindIII and separated on a 1.5% agarose gel. The nylon filters were hybridized with the two cdc2 gene-specific probes. The DNA (10 μg) from each sample was digested with HindIII and separated on a 1.5% agarose gel. The nylon filters were hybridized with the two cdc2 gene-specific probes.
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Acknowledgements

We appreciate the kind gift of the S. pombe cdc2-33, leu\(^+\), his\(^+\) strain from P. Nurse and the S. cerevisiae W303 strains carrying the cdc28-4 or the cdc28-1N mutant alleles from K. Nasmuth. We thank P. Gründler for the A. thaliana 2SS cDNA gene and M. Ptok for technical assistance. This work was supported by grant S 6004 BIO from the Österreichischer Fonds zur Förderung der Wissenschaftlichen Forschung.

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