Multiple cyclin-dependent kinase complexes and phosphatases control G2/M progression in alfalfa cells

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Abstract
Reversible phosphorylation of proteins by kinases and phosphatases plays a key regulatory role in several eukaryotic cellular functions including the control of the division cycle. Increasing numbers of sequence and biochemical data show the involvement of cyclin-dependent kinases (CDKs) and cyclins in regulation of the cell cycle progression in higher plants. The complexity represented by different types of CDKs and cyclins in a single species such as alfalfa, indicates that multicomponent regulatory pathways control G2/M transition. A set of cdc2-related genes (cdc2Ms A, B, D and F) was expressed in G2 and M cells. Phosphorylation assays also revealed that at least three kinase complexes (Cdc2Ms A/B, D and F) were successively active in G2/M cells after synchronization. Interaction between alfalfa mitotic cyclin (Medsa:CycB2:1) and a kinase partner has been reported previously. The present yeast two-hybrid analyses showed differential interaction between defined D-type cyclins and Cdc2Ms kinases functioning in G2/M phases. Localization of Cdc2Ms F kinase to the preprophase band (PPB), the perinuclear ring in early prophase, the mitotic spindle and the phragmoplast indicated a pivotal role for this kinase in mitotic plant cells. So far limited research efforts have been devoted to the functions of phosphatases in the control of plant cell division. A homologue of dual phosphatase, cdc25, has not been cloned yet from alfalfa; however tyrosine phosphorylation was indicated in the case of Cdc2Ms A kinase and the p13^suc1-bound kinase activity was increased by treatment of this complex with recombinant Drosophila Cdc25. The potential role of serine/threonine phosphatases can be concluded from inhibitor studies based on okadaic acid or endothall. Endothall elevated the kinase activity of p13^suc1-bound fractions in G2-phase alfalfa cells. These biochemical data are in accordance with observed cytological abnormalities. The present overview with selected original data outlines a conclusion that emphasizes the complexity of G2/M regulatory events in flowering plants.

Introduction
Control of the division cycle and the cell number is critically important for plant growth and morphogenesis. Present concepts outlining the molecular regulatory mechanisms of progression through the cell cycle are primarily based on results from yeast and Drosophila genetics, animal cell biology and biochemistry (Okayama et al., 1996; Neufeld et al., 1998). During duplication, cells undergo several discrete transitions representing unidirectional changes of cellular state. Current cell cycle research focuses on how these transitions are coordinated to occur with precise timing and in a defined order. It is especially significant to discover how environmental, hormonal or developmental signals influence cell cycle events in higher plants. Activation or arrest of the cell cycle are critical regulatory steps in completion of developmental programmes. The ongoing cell cycle research with different plant species largely relies on knowledge of evolutionary conserved elements in cell division con-
control that have been first described from yeast, insect or animal cells. An increasing number of experimental data support a view that plants share a considerable number of regulatory pathways known in other multicellular higher eukaryotes. These include a variety of cyclin-dependent kinase complexes, classes of cyclins, CDK inhibitors, homologues of the retinoblastoma protein and the E2F transcriptional factor (for review, see Mironov et al., 1999). Given the plant-specific features in cytoskeleton structure, fertilization, endosperm formation, resting stage of embryo, organization of meristems, totipotency of somatic cells, we have to postulate plant-specific regulatory elements in cell division control. Particularly, the hormonal regulation of plant growth and development can be dependent on unique pathways. Alfalfa as an experimental system can offer advantages for cell cycle research in understanding the molecular basis of symbiotic nodule development, somatic embryogenesis or environmental adaptation.

**Timing of kinase activities reflects differential function of three CDK complexes during G2/M progression**

At the centre of the cell cycle control machinery, the cyclin-dependent kinase (CDK) complexes with kinase and cyclin components play a key role in regulation of the cell division. The catalytic kinase and regulatory cyclin subunits can form active or inactive complexes depending on the phosphorylation status of the partners (Fisher, 1997). A single, major CDK has been identified in fission and budding yeast: CDC2/CDC28 (Hindley and Phear, 1984; Lorincz and Reed, 1984). In mammalian cells, several distinct Cdc2-related kinases and various types of cyclins provide more complex regulation of division at multiple levels (for review, see Yang and Kornbluth, 1999). Plants, like higher animals, have multiple CDKs (Dudits et al., 1998) and cyclins (Renaudin et al., 1996).

In contrast to the constitutive expression of yeast and animal CDK genes in all cell cycle phases, several plant cdc2 homologous genes exhibit different mRNA levels in various phases of the cycle. Accumulation of certain CDK transcripts in G2/M cells was an unexpected finding in the case of alfalfa (cdc2Ms D and F) and *Arabidopsis* (cdc2bAt) (Segers et al., 1996; Magyar et al., 1997). These kinases also deviated from the typical Cdc2-related kinases in the amino acid sequence of the cyclin-binding PSTAIRE region. Their PPTALRE or PPTTLRE motifs have only been found so far in plant CDKs (for reviews, see Burssens et al., 1998; Dudits et al., 1998). Since the genes encoding the cognate PSTAIRE CDKs (Cdc2Ms A, B) were also active in G2/M cells, it is likely that these kinases can also participate in regulatory mechanisms of G2/M progression. Immunoprecipitation with specific antibodies clearly showed histone H1 phosphorylation activities of the alfalfa cdc2Ms A/B and F kinases in synchronized G2/M cells (Magyar et al., 1997). Additional kinase assays provided further evidences for the potential role of yet another CDK, Cdc2MsD (Mészáros et al., manuscript). These three kinases differ also in their substrate preferences and the time of activity peak during G2/M transition. The Cdc2Ms A/B and F exhibited a broader substrate specificity and the alfalfa D kinase preferentially phosphorylated topoisomerase II *in vitro*. The functional significance of this modification might be related to the role of this enzyme in chromosome condensation (Warburton and Earnshaw, 1997). Several phosphorylation experiments indicated that first the Cdc2Ms A/B kinase activity was increased in G2 cells (Figure I). Although the D kinase was active during the whole cycle, an elevated activity at the G2/M boundary could be recognized parallel with the decline of Cdc2Ms A/B kinase peak. The F kinase activity coincided with the mitotic phase of the cycle.

Immunolocalization studies also suggest a different function for these CDKs that are active in G2/M cells. The previous immunofluorescence analysis of alfalfa cells or pea nuclei with anti-CT-Cdc2Ms A/B or PSTAIRE antibodies revealed the nuclear localization of these kinases in interphase cells (Bögre et al., 1997; Citterio et al., 1997). This is in agreement with the early findings of Colosanti et al. (1993); however, the maize kinase was also detected on the preprophase band. This difference might originate from the low specificity of antibodies used in these experiments. Histone H1 phosphorylation studies presented in Figure 1 indicate that the Cdc2Ms F kinase is a mitotic kinase. The present fluorescence confocal microscopic studies further strengthen this suggestion. Figure 2 presents a series of images which show the localization of this kinase on the preprophase band, prophase spindle, metaphase spindle and phragmoplast. The localization of this kinase on these cellular structures can be an indication for the specific role of PPTTLRE kinases in karyokinesis and cytokinesis. Determination of cell plate formation is a crucial step in cellular de-