Summary of PhD Thesis

ANALYSIS OF TWO PLANT PROTEIN COMPLEXES ASSOCIATED WITH TRANSCRIPTION AND CELL CYCLE PROGRESSION

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LIST OF PUBLICATIONS

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Fülöp, K., Pettkó-Szandtner, A., Magyar, Z., Miskolczi, P., Kondorosi, É., Dudits, D. and Bakó, L. (2005). The *Medicago* CDKC;1-CYCLINT;1 kinase complex phosphorylates the carboxy-terminal domain of RNA polymerase II and promotes transcription. Plant J, in press.

Fülöp, K. (2003). Characterization of the plant anaphase-promoting complex: gene expression and protein-protein interaction studies. Acta Biol Szeged, 47:59. (abstract)

INTRODUCTION AND OBJECTIVES

Regulation of basic cellular functions is conserved among plants and animals reflecting the common evolutionary origins of the two kingdoms. However, during the long independent evolution of these two lineages, the original sets of genes have progressively undergone independent diversification and today, besides serving conserved cellular mechanisms, they contribute to kingdom-specific functions as well. The results presented in this thesis can be included in such large chapters as transcription and cell cycle regulation, where the basic molecular elements show clear conservation across kingdoms.

In the **first part** of the thesis, experiments aiming at characterizing an alfalfa cyclin-dependent kinase (CDK) are described. The family of cyclin-dependent kinases is well known in animals as fundamental members of the cell cycle machinery and, more recently, as basic regulators of transcription. CDKs are serine-threonine specific kinases that are activated by a cyclin regulatory subunit. In yeasts, a single kinase, Cdc28/Cdc2 associated with phase-specific cyclins is sufficient to drive the cell division cycle, while higher eukaryotes have several CDKs to promote cell cycle progression.

In yeasts and animals, certain CDKs are implicated in the control of transcription by altering the phosphorylation pattern of the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II. This domain is hypophosphorylated in the preinitiation complex but gets heavily phosphorylated during initiation and transcript elongation. CDK-cyclin complexes known to be involved in the phosphorylation of the CTD are Cdk7-cyclin H, Cdk8-cyclin C and Cdk9-cyclin T/K complexes in metazoans. These kinase-cyclin pairs have distinct biochemical properties and their

kinase activity is required at different phases of the transcription cycle. While Cdk7 acts positively on early steps of transcription, the kinase activity of Cdk8 inhibits the recruitment of RNA polymerase II to the preinitiation complex. Cdk9 is the catalytic subunit of positive transcription elongation factor b and promotes efficient transcript elongation.

Plants also have multiple CDKs classified into six types form A to F. The A-type CDKs are the functional homologues of the Cdc2-type kinases, while B-type CDKs are unique to plants. The classes D and F include kinases with CDK-activating kinase activity, while the CDKC and E groups have been poorly investigated as of yet. D-, E- and C-type CDKs are homologous to metazoan Cdk7, Cdk8 and Cdk9 kinases, respectively.

The aim of our work was to characterize the alfalfa CDKC;1 kinase.

The **second part** of the results contributes to our current understanding on the plant anaphase-promoting complex and its activators.

Controlled degradation of regulatory proteins via the ubiquitin-proteasome pathway is an important mechanism in cell cycle regulation. A large multiprotein complex, the anaphase-promoting complex (APC) is responsible for the destruction of cyclins and other regulatory proteins by the exit from mitosis. Compared to other known ubiquitin ligases, APC is unusually complex with at least 11 subunits in vertebrates and 13 in yeast. Mapping of protein-protein interactions within the APC of these organisms has begun, but the rationale of this high complexity is still elusive as well as the role of many subunits. It has been hypothesized that this complexity might allow intricate temporal and spatial regulation of APC activity.

3. To assess the eventual substrate-specificity of AtCcs52 proteins, their interaction with several mitotic cyclins, two A- and three B-type, was investigated. In these *in vitro* binding assays, the activators displayed distinct interaction patterns with the cyclins indicating that they might interact with partially different subsets of substrates.

4. In *Arabidopsis* cell suspension culture synchronized to G1/S phase by aphidicolin treatment, expression of most APC subunits proved to be constitutive, whereas *cdc27a* and *cdc27b*, the three *Atccs52* genes, *ubc19* and *ubc20* encoding E2 enzymes displayed differences in their cell cycle regulation. These data indicate the existence of numerous $APC^{Cdc20/Ccs52/Cdc27}$ forms in *Arabidopsis*, which, in conjunction with different E2 enzymes might have distinct or complementary functions at distinct stages of the cell cycle.

positive transcription elongation factor b, which consists of Cdk9-cyclin T.

Part II. Anaphase-promoting complexes in *Arabidopsis*

1. In order to define whether the three *Arabidopsis* proteins have distinct or redundant functions, their corresponding genes were over-expressed from an inducible promoter in fission yeast. In addition to proliferation arrest, expression of the *Atccs52* genes provoked also drastic morphological changes that were characteristic for each gene. The binding of the *Arabidopsis* activators to the fission yeast APC was demonstrated by co-immunoprecipitation. The distinct phenotypes elicited by the expression of *Atccs52* genes in fission yeast indicated that all the AtCcs52 proteins may activate the yeast APC but have different functions.

2. The recruitment of AtCcs52 proteins to the plant APC was demonstrated *in planta* by immunoprecipitating the endogenous complex with anti-Cdc27A antibodies from transgenic plants expressing epitope-tagged versions of the AtCcs52 proteins. Pairwise yeast two-hybrid analyses and co-immunoprecipitation experiments using *Arabidopsis* APC subunits indicated that the basic molecular organization of the complex is conserved in plants as well. It was possible to identify the Apc2-Apc11 heterodimer, the minimal ubiquitin ligase module of the human APC, and the binding of E2 enzymes Ubc19/20 to the cullin domain of Apc2. Further co-immunoprecipitation experiments demonstrated the direct binding of AtCcs52 activators to the Apc2 and Apc10 subunits.

Stage-specific activation and substrate selection of the APC are defined by the binding of either of two activator proteins, Cdc20 or Cdh1. Plants have two classes of Cdh1-type activators, Ccs52A and Ccs52B proteins, that display different cell cycle and developmental regulation in *Medicago*. Ccs52A proteins are the functional orthologues of the yeast Cdh1 APC activator, whereas B-type Ccs52 proteins might represent plant-specific activators. The *Arabidopsis* genome contains three *ccs52* genes: *Atccs52A1*, *Atccs52A2* and *Atccs52B*. Moreover, the cyclin family is highly complex in plants, thus control of cyclin stability during the cell cycle might be intricately regulated. In this work we aimed at characterizing the *Arabidopsis ccs52* genes, cell cycle regulation of APC activators, APC components, E2 enzymes and mitotic cyclins.

METHODS

Recombinant DNA work (PCR, standard cloning techniques)
Yeast manipulations
- yeast two-hybrid interaction analysis
- complementation assay
- heterologous expression in fission yeast

Protein techniques
- bacterial protein expression and protein purification
- antibody purification
- immunoprecipitation, immunoblot
- kinase assay
- subcellular fractionation of proteins

Plant manipulations

synchronization of cell cycle in suspension cultured alfalfa and *Arabidopsis* cells

transfection of *Arabidopsis* protoplasts

RNA isolation and RT-PCR analysis

Immunolocalization

In vitro transcription reactions

RESULTS

Part I. Characterization of a C-type cyclin-dependent kinase from alfalfa

1. *Medicago* proteins were screened for interaction with CDKC;1 by the yeast two-hybrid system, which allowed the identification of a cyclin that was similar to human and plant T-type cyclins. The two proteins showed a high interaction specificity since *Medicago* A-, B- and D-type cyclins as well as A-, B- and E-class CDKs were unable to form complexes with the CDKC;1 and cyclin T proteins, respectively.

2. Specific antibodies were produced against the CDKC;1 kinase in order to characterize it biochemically. The CDKC;1 complex immunoprecipitated from alfalfa cells was found to possess protein kinase activity against proteins such as the myelin basic protein, the C-terminal domain of RNA polymerase II and the retinoblastoma protein *in vitro*. Unlike complexes of the cell cycle kinases CDKA and CDKB, the CDKC;1 complex failed to phosphorylate the typical CDK substrate histone H1. Co-expression of epitope-tagged CDKC;1 and cyclin T in *Arabidopsis* protoplasts resulted in the formation of a

CDK-cyclin complex that displayed a high CTD kinase activity indicating functional interaction *in vivo*.

3. In a cellular fractionation experiment, CDKC;1 was found in the nuclear protein fraction and its associated CTD kinase activity was detected therein. Nuclear localization of the complex was further confirmed by immunostaining of *Arabidopsis* protoplasts expressing the epitope-tagged CDKC;1 and cyclin T proteins. However, size-fractionation of alfalfa nuclear proteins showed that CDKC;1 was not included in the RNA polymerase II holoenzyme.

4. In an alfalfa cell culture synchronized to G1/S phase by hydroxyurea treatment, both the protein level of CDKC;1 and its CTD-kinase activity were constant throughout the cell cycle arguing against a direct involvement of the kinase in cell cycle control.

5. To assess the role of the CDKC;1 complex in RNA polymerase II-mediated transcription, its site specificity within the heptapeptide repeat of CTD was determined first. The mutation of serine at position 2 in the repeat did not prevent substrate phosphorylation. In contrast, when serine at position 5 was replaced by nonphosphorylatable alanine, phosphorylation by CDKC;1 was completely abolished indicating that the kinase complex targets Ser^5 within the CTD *in vitro*.

6. Finally, in promoter-specific *in vitro* transcription assays, the CDKC;1-cyclin T kinase complex was able to replace human Cdk9 function in depleted HeLa nuclear extract and efficiently promoted transcription.

As the *Medicago* CDKC;1-cyclin T complex mirrors the most important features of metazoan Cdk9-cyclin T complexes, we conclude, that CDKC;1-cyclin T is a plant orthologue of metazoan