Key proteins involved in the reactivation and control of the cell division checkpoints in alfalfa

Ph.D. Thesis

by

Pál Miskolczi

Supervisors

Dr. Gábor V. Horváth

Prof. Dr. Dénes Dudits

Institute of Plant Biology

Laboratory of Cell Division Cycle and Stress Adaptation

Biological Research Center

Hungarian Academy of Science, Szeged

University of Szeged

2007

INTRODUCTION AND AIMS

The power of the cell to hand on their nature their descendents is a fundamental aspect of the life of the cellular built organisms in our world. Not surprisingly the cell division cycle is one of the most intensively studied biological processes yet, particularly given its importance for many human cancers. The significance of cell cycle research was confirmed by Nobel Prize awarded by Leland H. Hartwell, R. Timothy (Tim) Hunt and Sir Paul M. Nurse for their discoveries of key regulators of the cell cycle in 2001.

The fundamental processes of the cell division and the molecules that take part in regulation are rather conservative, and operate in a similar way in all eukaryotic organisms. One of the determinant factors of plant adaptiveness is the continuous division of cells, and formation of new organs, the flexibility of their organogenesis has to be in conformity with the environment because of their immovable nature. CDK activating cyclins have been identified in surprisingly high numbers in plants, and the participation of two CDK types at G2/M phase of cell cycle process, one of which is a plant specific CDK kinase, can participate to this plasticity.

In the past two decades our research group has contributed significantly to plant cell cycle research by cloning and analysing several key regulatory genes (CDK-s, cyclins, CDK inhibitors, RB-related genes, E2F genes) from alfalfa and rice. I joined the research work of our group by examination of proteins taking part in alfalfa cell division regulation. To achieve this we applied for two plant experimental approaches. We used the A2 cell suspension synchronization system to study the cell cycle regulation of alfalfa. We examined the reactivation of CDKs in dedifferentiated cells to certain hormone effects, and the first steps of the reactivation of cell division by the established alfalfa leaf protoplast culturing system.

In my thesis I present the results which intend to answer the following questions:

- Which D-type cyclins interact with alfalfa CDK kinases, and in which cell division phase regulation do they have a role?
- Can A- and plant specific B-type kinase complexes phosphorylate the alfalfa retinoblastoma-related protein?
- How does the quantity of MsRBR1 protein change during the process of cell division in alfalfa?

- How do the plant hormones influence the level of alfalfa RBR protein in A2 cell suspension, and in Medicago truncatula R-108 callus capable of direct regeneration?
- In which hormonal conditions do regulating activation of alfalfa CDK-cyclin complexes at G1/S and G2/M transition?
- How can we determine the exact expression and activation of CDK kinases at G2/M transition and mitosis?

Materials and Methods

Plant material and cell cultures

- *Medicago sativa L. ssp. varia*; A2 genotype, plant and cell suspension.
- Cell suspension culture of *Medicago truncatula* cv. R 108

Recombinant DNA work

- Isolation of plazmid DNA, PCR, gene cloning and transformation

Protein techniques

- Protein extraction from plants
- SDS polyacrylamid-gelelectrophoresis (SDS-PAGE)
- Immunoblot analysis
- Coupled *in vitro* transcription and translation (TnT)
- Immunoprecipitation
- CDK kinase assay
- Expression and purification of bacterial expressed proteins
- Making polyclonal antibodies and affinity purification
- Yeast two-hybrid interaction analysis
- p13suc1 affinity purification

Cell biology methods

- Synchronization of A2 cell suspension
- Isolation protoplast of alfalfa
- Immunolocalization
- S-phase detection by BrdU-incorporation
- Flow cytometry
- Determination of Mitotic index

DNA and protein sequence analysis

RESULTS

The accurate process of cell division is the base of life and reproduction of both unicellular and more complex organisms. The detailed cognition of the cell division is important from the aspect of the human genetics and plant biology, because there is a basic need for growing adequate quantity and appropriate quality of plant nutrition. The importance of the plant molecular biology is confirmed by the investigation focusing on discovering the complete plant genome. Due to the continuously broadening instrumental development of molecular biology in the past two decades it became possible to collect considerable amount of knowledge on plant cell cycle regulation.

Compared to molecular regulation processes of the animal model organisms, the complex plasticity of morphology, cell division and differentiation of higher plants, and their immovable nature allows to conclude significant differences. Based on the previous results it can be stated that the main participant molecules of the cell cycle regulation show considerable sequential and functional homology between plants and animals, but unquestionable the component of the plant specific processes.

In our group we managed to isolate proteins known and found the most important in animal cell cycle regulation (cyclin dependent kinases, cyclins, inhibitors) and other important proteins such as homologues of the retinoblastoma tumour suppressor and E2F transcriptional factors from the economically important, nitrogen-binding alfalfa. During our research work we focused on the more detailed cognition of the function of the above mentioned proteins in the alfalfa cell cycle regulation, and we found the following important results:

1. We investigated the role of the new type alfalfa Medtr;CYCD1;1 and Medtr;CYCD6;1 cyclins isolated by yeast two-hybrid technique in the cell cycle regulation and we searched which CDK kinases they can form active complexes with. We managed to confirm the interaction between the alfalfa D-type cyclin and the CDK protein by the yeast two-hybrid assays. Medtr;CYCD1;1 showed stronger interaction with B-type CDK-s, whereas Medtr;CYCD6;1, a cyclin that does not contain an LxCxE RB-binding motif, showed clear interaction with CDKA in the yeast two-hybrid tests. The Medsa;CYCD3;1 cyclin translated by reticulocyte cell lysate system was able to bind to and activate alfalfa CDKA kinase. According to its expression data CYCD3;1 may have a role in activation of CDKA in G1/S phase, it is confirmed by GST-CYCD3;1 fusion protein bound kinase activity in our cell culture

synchronizing experiments. Generally D-type cyclins similarly to Medtr;CYCD6;1 cyclin accumulate mainly in the dividing cell, which reflects to their role in the cell cycle regulation. Although interestingly, the quantity of protein similarly to a few other plant D-type cyclins remains nearly equal during the cell cycle phases.

2. We confirmed by the *in vitro* phosphorylation assays that immunoprecipitated CDKA from whole protein extract of alfalfa cell suspension, and the mitotic CDKB2;1 complexes are able to phosphorylate the alfalfa retinoblastoma-homologue (MsRBR1) protein.

3. RBR analysis gained particular attention in plant molecular research recently. We confirmed by our experiments, that the retinoblastoma-homologue has a role in the regulation of plant cell cycle. The appropriate polyclonal anti-body made it possible to study the role of the alfalfa RBR protein in the cell cycle. Observing the quantity of the alfalfa retinoblastoma homologue (MsRBR1) in synchronized cell cultures, we observed that the quantity of protein is slowly increasing until the S and G2/M phases of the cell cycle, and possibly there is a decrease again from M-phase. Results from both hydroxyurea and double blocked synchronized assays confirm that the presumed hyperphosphorylation form of MsRBR1 changes depending on the cell cycle. The quantity of this MsRBR1 form is getting stronger from the S-phase remains until the next cycle after the late G2/M phase, out of accordance with the normal size, decreasing quantity of MsRBR1 protein. Accordingly we presume that the retinoblastoma-homologue phosphorylation plays a similar role in regulation of the plant G1/S cell cycle transition, as it has been confirmed in the case of pRB.

4. We examined the effect of certain plant hormones on quantity of MsRBR1 protein. Since the amount of alfalfa retinoblastoma-homologue proved to be sufficient only in cell suspension samples, we decided to use this as experimental object. According to the results we can confirm, that discrepant RBR protein can be detected in hormone free and auxin containing medium, which show correspondence with the cell cycle activities of the suspensions.

5. Alfalfa RBR protein allows to conclude an interesting modification in the microcallus cell culture of *Medicago truncatula* R-108 after plated on hormone free medium for around a week. While it is also observable that the protein level of RBR decreased below the detectable amount after a few days in the same experiments. The observed possible modification of alfalfa RBR protein at higher molecular weight range supposedly refers to the role of the RBR protein in the chromosome

remodelling occurring in the process of differentiation in the Mt-108 microcallus population. Further research work is needed to identify the type of this presumed RBR modification. However a heat shock protein was found in the separated complex of anti-RBR immunoprecipitated samples that homologue known to interact and stabilizes the pRB protein.

6. We examined what kind of molecular processes play role in hormone induced cell cycle reactivation in case of alfalfa leaf protoplast cells, more precisely, how and when the CDK-related protein complexes activate within the first division of cells, and what kind of role the examined auxin and cytokinin hormones have in this process. According to our experiments we stated that auxin is essential to the synthesis of alfalfa CDKA kinase, whereas cytokinin addition was essential in every case to promote the entry of the reactivated protoplast cells into mitosis.

7. We confirmed using the double blocked combined synchronized technique of alfalfa cell suspension, that cells need the active CDKA/cyclin complex in the transition at G2/M checkpoint. Expression of CDKB2;1 plant specific kinase occurs right after the transition at G2/M checkpoint, and it also gains considerable kinase activity, which supports the mitotic function of the kinase.

LIST OF PUBLICATIONS

(Present thesis is based on articles marked by an asterisk)

Lendvai, Á., A. Pettkó-Szandtner, É. Csordás-Tóth, **P. Miskolczi,** G.V. Horváth, J. Györgyey, and D. Dudits, (2007) Dicot and monocot plants differ in retinoblkasoma-relatied protein subfamilies. J Exp Bot, 58(7): p. 1663-1675.

*Dudits, D., M. Cserháti, **P. Miskolczi**, and G.V. Horváth, (2007) The growing family of plant cyclin-dependant kinases with multiple functions in cellular and developmental regulation, in Cell Cycle Control and Plant Development, D. Inzé, Editor., Blackwell Publishing: Oxford UK ISBN: 9781405150439: Oxford. p. 1-30.

***Miskolczi, P.**, Á. Lendvai, A. Pettkó-Szandtner, G.V. Horváth, and D. Dudits, (2007) Conserved functions of retinoblastoma proteins: from purple retina to green plant cells. Plant Science, **172**: p. 671–683.

Pettkó-Szandtner, A., T. Mészáros, G.V. Horvath, L. Bakó, E. Csordás-Tóth, A. Blastyak, M. Zhiponova, **P. Miskolczi**, and D. Dudits, (2006) Activation of an alfalfa cyclin-dependent kinase inhibitor by calmodulin-like domain protein kinase. Plant J, **46**(1): p. 111-23.

Fülöp, K., A. Pettko-Szandtner, Z. Magyar, **P. Miskolczi**, E. Kondorosi, D. Dudits, and L. Bakó, (2005) The Medicago CDKC;1-CYCLINT;1 kinase complex phosphorylates the carboxy-terminal domain of RNA polymerase II and promotes transcription. Plant J, **42**(6): p. 810-20.

Ötvös, K., T.P. Pasternak, **P. Miskolczi**, M. Domoki, D. Dorjgotov, A. Szűcs, S. Bottka, D. Dudits, and A. Fehér, (2005) Nitric oxide is required for, and promotes auxin-mediated activation of, cell division and embryogenic cell formation but does not influence cell cycle progression in alfalfa cell cultures. Plant J, **43**(6): p. 849-60.

Espinosa-Ruiz, A., S. Saxena, J. Schmidt, E. Mellerowicz, **P. Miskolczi**, L. Bakó, and R.P. Bhalerao, (2004) Differential stage-specific regulation of cyclin-dependent kinases during cambial dormancy in hybrid aspen. Plant J, **38**(4): p. 603-15.

Fehér, A., T. Pasternak, K. Ötvös, **P. Miskolczi**, and D. Dudits, (2002) Induction of embryogenic competence in somatic plant cells: a review. Biologia, **57**: p. 5-12.

Pasternak, T., E. Prinsen, F. Ayaydin, **P. Miskolczi**, G. Potters, H. Asard, H.A. Van Onckelen, D. Dudits, and A. Fehér, (2002) The Role of auxin, pH, and stress in the activation of embryogenic cell division in leaf protoplast-derived cells of alfalfa. Plant Physiol, **129**(4): p. 1807-19.

Fehér A., T. Pasternak, **P. Miskolczi**, F. Ayaydin, D. Dudits, (2001) Induction of the embryogenic pathway in somatic plant cells. Acta Hortic, **560**: p. 293-298.

Davletova, S., T. Mészáros, **P. Miskolczi**, A. Oberschall, K. Török, Z. Magyar, D. Dudits, and M. Deák, (2001) Auxin and heat shock activation of a novel member of the calmodulin like domain protein kinase gene family in cultured alfalfa cells. J Exp Bot, **52**(355): p. 215-21.

***Miskolczi, P.,** A. Pettkó-Szandtner, G.V. Horváth, D. Dudits, A. Fehér, and J. Györgyey, (2000) A novel plant cyclin /Medicago cyclin D. Patent EP 00870133.6 (16 jun 2000) PCT/EP01/06771

Ayaydin, F., E. Vissi, T. Mészáros, **P. Miskolczi**, I. Kovács, A. Fehér, V. Dombradi, F. Erdödi, P. Gergely, and D. Dudits, (2000) Inhibition of serine/threonine-specific protein phosphatases causes premature activation of cdc2MsF kinase at G2/M

transition and early mitotic microtubule organisation in alfalfa. Plant J, **23**(1): p. 85-96.

*Mészáros, T., **P. Miskolczi**, F. Ayaydin, A. Pettkó-Szandtner, A. Peres, Z. Magyar, G.V. Horváth, L. Bakó, A. Fehér, and D. Dudits, (2000) Multiple cyclin-dependent kinase complexes and phosphatases control G2/M progression in alfalfa cells. Plant Mol Biol, **43**(5-6): p. 595-605.

*Pasternak, T.P., **P. Miskolczi**, F. Ayaydin, T. Mészáros, D. Dudits, and A. Fehér, (2000) Exogenous auxin and cytokinin dependent activation of CDKs and cell division in leaf protoplast-derived cells of alfalfa. Plant Growth Regulation, **32**: p. 129-141.

Fehér, A., T. Pasternak, F. Ayaydin, T. Mészáros, and **P. Miskolczi**, Zs. Kelemen, L. Bögre, F. Laureys, H. Van Onckelen, and D. Dudits, (1999) Hormones and stress in the induction and regulation of cell division in cultured alfalfa cells. Biologia Plantarum, **42**: p. 63.

Dudits, D., Z. Magyar, M. Deák, T. Mészáros, **P. Miskolczi**, A. Fehér, S. Brown, E. Kondorosi, A. Athanasiadis, S. Pongor, L. Bakó, C. Koncz, and J. Györgyey, (1998) Cyclin-dependent and calcium-dependent kinase families:response of cell division cycle to hormone and stress signals. PLANT CELL DIVISION:MOLECULAR AND DEVELOPMENTAL CONTROL, ed. D. Dudits and D. Inzé. London: Portland Press. 21-46.

Magyar, Z., T. Mészáros, **P. Miskolczi**, M. Deák, A. Fehér, S. Brown, E. Kondorosi, A. Athanasiadis, S. Pongor, M. Bilgin, L. Bakó, C. Koncz, and D. Dudits, (1997) Cell cycle phase specificity of putative cyclin-dependent kinase variants in synchronized alfalfa cells. Plant Cell, **9**(2): p. 223-35.