

**REGULATION OF CELL CYCLE SPECIFIC GENES
THROUGH PLANT GROWTH
AND DEVELOPMENT**

Ph.D. Theses

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INTRODUCTION

In the past decade attention has particularly increased for plant cell division, since novel cell-cycle characteristics were discovered apart from the conserved basic mechanisms, which can be found only in plants. Cell division research requires knowledge in more details and for such researches plants give an exceptional opportunity. In contrast to animals, growth of plants is considerably postembryonic. The new organs, such as the root, the stem, the leaves and flowers are from the division of the so-called „life-long” cells, which is followed by plant growth and differentiation. This specific cell zone is the meristem. The leaves and flowers develop from the shoot meristem, while the root meristem provides new cells in the growing root. Based on these characteristics plants are very suitable targets to simultaneously study cell cycle, growth and development of multicellular living beings.

The regulation of the cell cycle is a very complicated and complex process, in which both inner and outer cell factors play part. Inside the cell the most important regulators of the cell cycle are protein-complexes, which consist of catalytic and regulating subunits. Both cyclins and cyclin dependent kinases have many forms. Present of cyclin-CDK complexes, having the most important role in the cell cycle coordination, regulates different checkpoints of the cell cycle. Formation of protein complex is preceded by the increasing level of cyclin, which is in turn usually the outcome of some other indicating factors (for example growth factors) (Dymlacht, 1997).

To study the biochemistry and molecular biology of cell division it is essential to have a cell system, where the division of cells within a population can be synchronized. The most suitable system to examine the cell cycle proved to be the *in vitro* plant cell culture, because most of the cells are active and dividing, therefore it passes through certain phases of the cell cycle. In this system the cell division can be influenced in many ways. Through the use of reversible inhibitors, that can stop cell cycle at certain points, cells will get into the same phase, to a so-called synchron. With these experimental systems we can fully analyse the regulating units of cell cycle.

The cyclin dependent kinases (CDK) are one of the most important coordinators of the eukaryotic cell cycle regulation. These serin-treonin protein kinases – considering their function regulating the cell cycle – bind cyclins (by forming complexes) and determine cell division steps. The plant CDK proteins (in harmony with the animal systems) are

functionally grouped and distinguished with letters (CDKA, B, D, E, F). In plants the CDKA proteins contain a PSTAIRE sequence motive in their cyclin-binding domain and these kinases regulate the G1/S and G2/M transitions. Four B-Type CDKs are known, which are plant-specific CDKs harbouring the typical PPTALRE or PPTTLRE motif, which accumulate in the G2 and M phase and regulate the G2/M transition.

Cell division is a determining mechanism in the development of the plant, although the full regulating process is not known yet, so it is presumed that the different CDKs have a key role. We can assess the role of these proteins in the regulation of cell cycle, in the plant growth by the nowadays well-known transgenic plant lines overproducing or depleting expression of the target gene.

The different CDK-cyclin complexes achieve their regulatory function throughout the cell cycle process by forming connection with other proteins. The retinoblastoma proteins present substrates of CDK cyclin complexes, which have an important role in the regulating steps of the cell cycle. During the G1/S phase transition the CycD/CDK complex phosphorylates the RBR protein, in this way the E2F transcription factor is released from the inhibition and directs the cells into S phase. Viruses use also this regulating step to multiply their genetic information. RBR regulates processes outside and within the cell cycle through formation of different protein-protein interactions, as for example with the FVE protein, which is part of a protein-complex, that represses transcription of FLC (Flowering Locus C), thereby regulating flowering. Examinations of plant RBR proteins help us to obtain further insight in the regulating function of RBR.

AIMS

In the case of monocotyledonous plants cell cycle studies need a well established synchronization system, as known published data, at the tobacco BY-2 cell-line, with that we could widen our knowledge in the field of examining the cell cycle of the monocotyledons, at the same time it could provide to get to know the evolutionary differences between monocotyledonous and dicotyledonous plants.

For this reason we choose to optimize synchronization technique and we developed this method for our experimental model organism, the rice. With our experiments, we tried to increase further our knowledge, to help to understand the cell cycle regulation.

The central role of cyclin dependent kinases (CDK) was already proved in cell cycle regulation. The aim of our work was to get further insight on the effects of CDK kinases on cell division and on plant growth and development. For this study we chose to create and examine transgenic plants overproducing the chosen kinases.

It is known, that the basic mechanisms of living organisms are similar, so the regulating elements of cell cycle are preserved as well. From these basic regulating components we chose the plant RBR genes, as one of the most interesting modulator of cell cycle. Our aim was especially to identify, compare and examine rice RBR proteins.

MATERIALS AND METHODS

The model plants of our experiments were rice *Oryza sativa* L. *Japonica* cv. Taipei-309 and Unggi9 species. The cell-lines derived from Taipei-309 genotype and *Medicago sativa* L. *ssp. varia* A2 genotype cell culture (Magyar et al., 1997).

We performed flow cytometric analysis on various nuclei isolated with „chopping” method from our synchronization experiments, and we analyzed quantitative changes of transcript level of some known genes by quantitative RT-PCR.

In our kinase overproducing experiments we created binary vectors (pCHD2A, pCHD2D, pCHD2F) and based on the method of Hiei et al., 1994, we infected calluses with *Agrobacterium tumefaciens*. We proved the presence of transgene with PCR technique, by development of antibiotic resistance, and we proved the amount of overproduced protein by Western analysis.

RESULTS

Synchronization of rice cells

I. I performed synchronization experiments to study the plant cell cycle through measuring the differences in DNA content (resulted from changes of the nucleus) during the division cycle using flow cytometry. First of all I determined the suitable blocking-material and optimised the circumstances of its use. First, I treated the cell cultures with hidroxyurea and after removing the impending agent I detected the DNA content changes of the nucleus of the two rice cultures by flow cytometer. I can state based on the synchronization experiments that hidroxyurea stopped most of the cells in the G1/S phase, while after the washing step cells continued their cell cycle, soon they proceeded into the G2/M phase.

II. From the synchronization experiment of the Unggi9 cell culture I determined the duration of the cell cycle, where the G2 phase and the mitosis were detected at around 21 hours.

III. At the experiments on the Taipei-309 cell-line I observed, that the blocked and then reactivated cells of the cell culture were in phase G2 at 12-15 hours. During the experiments with the two different cell cultures I observed significant differences between the varieties, which are very important in the case of comparative analyses.

IV. I proved that the synchronization experiment on the Taipei-309 cell culture was successful by examining the expression of cell cycle specific genes with quantitative RT-PCR, which in the following experiments is an important technique of studying the cell cycle.

Studying the overproduction of Medicago CDK proteins in rice plants

V. During my work I created transgenic rice plants overproducing *Medicago* CDK proteins to study the effects of these proteins on cell cycle, plant growth and development.

VI. In the Medsa;CDKA1;1 protein overproducing transgenic plants I detected a change in the number of rice seeds, which suggests that the CDKA1 kinases have a role in regulating the cell division, and they also influence the development of the generative organs.

VII. During my studies I observed a phenotype change in the Medsa;CDKB1;1 kinase overproducing rice plants, which means that the size of these transgenic plants was smaller than the control plants.

VIII. From the mitotic kinases I carried out experiments with the Medsa;CDKB2;1 by creating these transgenic kinase overproducing plants. I observed that these modified plants flowering earlier than the control plants.

Studying the plant RBR genes

IX. During my work I described the rice *RBR* genes and the *Medicago* *RBR* gene. These plant genes are new representatives of the RBR family, as the proteins coded by them contains A and B pocket domains which are the characteristics of RBR proteins, similarly to animal RBRs.

X. Based on the available sequence database I determined the *TaRBR1* total and the *TaRBR2* partial nucleotide sequence of the wheat using PCR and sequencing. These genes present also new members of plant RBR family.

XI. I examined the genomic organization of newly isolated *RBR* genes, determining that these genes are organized into 18 exons in plant genomes and they can be differentiated through their specific intron organization.

XII. I examined the *RBR* genes coded protein sequences with a comparative analysis using CLUSTALW. Based on the alignment I created a filogenetic tree, that helped to group the plant RBR proteins. Subfamily I. includes the dicotyledonous RBR proteins, subfamily II. contains monocotyledonous type one, while subfamily III. includes monocotyledonous type two RBR proteins. I determined, that these subfamilies have typical possible phosphorylating pattern, which might distinguish the functional specification of the proteins in each subfamily.

XIII. By quantitative RT-PCR I examined the expression of identified genes and proved, that the rice *RBR* genes are not only differ in their structure, but in their tissue-specific expression as well, which means, that the mRNA level of the rice *RBR2* gene is high in the differentiated cells, while I detected larger amount of *OsRBR1* gene transcript in the actively dividing cell suspension and embryos.

XIV. I examined the localization of the rice *RBR* genes expression in plant with *in situ* hybridization experiments. I determined, in harmony with the quantitative PCR results that the *OsRBR2* gene is expressed in the differentiated cells of the plant.

XV. I examined the differences between the RBR proteins with yeast two-hybrid method. I determined similarities and differences in the protein interactions. Every examined plant RBRs interacted with the RepA protein of the MSV virus, while on the other hand a newly isolated protein, the OsRBR12 interacted only with the MsRBR and the OsRBR1 proteins. With these results I also proved, that the monocotyledonous RBR subfamilies are functionally differentiated, while in dicotyledons only one RBR performs these functions.

Publications related to the Theses:

Lendvai Á., Csordás-Tóth É., Pettkó-Szandtner A., Horváth H.V., Györgyey J., Dudits D. (2007) Dicot and monocot plants differ in Retinoblastoma-related protein subfamilies. *Journal of Experimental Botany* 58(7):1663-1675

Lendvai Á., Pettkó-Szandtner, Horváth V.G., Csordás-Tóth É., Györgyey J., Dudits D. (2004) The number of retinoblastoma genes may reflect phylogenetic divergence of dicot and monocot plants. Annual Main Meeting of the Society for Experimental Biology, *Comparative Biochemistry and Physiology* 137(3):S248

Lendvai Á., Nikovics K., Bakó L., Dudits D., Györgyey, J. (2002) Synchronization of *Oryza sativa* L. cv. Taipei-309 cell suspension culture Proceedings of the 7th Hungarian Congress of Plant Physiology *Acta Biologica Szegediensis* 46(3-4): 39-41

Other publications:

Miskolczi P., Lendvai Á., Horváth G. V., Pettkó-Szandtner A., Dudits D. (2007) Conserved functions of retinoblastoma proteins: From purple retina to green plant cells. *Plant Science* 172 (4): 671-683

Csordás-Tóth É., Kiss A., Lendvai Á., Dudits D., Vandepoele K., Inzé D. (2004) Transcript profiling in synchronized rice cells Annual Main Meeting of the Society for Experimental Biology, *Comparative Biochemistry and Physiology* 137(3):S247

Dorjgotov D., Szűcs A., Ötvös K. Szakonyi D., Kelemen Zs., Lendvai Á., Pónya Zs., Barnabás B., Brown S., Dudits D., Fehér A. (2003) Specific features of RHO GTPase-dependent signalling in plants. *Cell Biology International* 27(3):191-2

Göntér I., Szarka B., Lendvai Á., Molnár-Láng M., Mórocz S., Dudits D. (2002) Problems and possibilities of wheat-maize somatic hybridization Proceedings of the 7th Hungarian Congress of Plant Physiology *Acta Biologica Szegediensis* 46(3-4): 11-12