

**Ph.D. thesis**

**Ping YU**

**Doctoral School of Biology**

**Szeged**

**2015**

**Plant RBR proteins are phosphorylated in cell cycle-phase  
dependent manner and the B” regulatory subunit containing  
OsPP2A holoenzyme mediates the dephosphorylation of  
OsRBR1**

Ph.D. thesis

Ping YU

Supervisor: Dr. Horváth V. Gábor

Biological Research Centre, Hungarian Academy of Sciences,  
Institute of Plant Biology

Doctoral School of Biology University of Szeged

Szeged

2015

## TABLE OF CONTENTS

<b>SUMMARY .....</b>	<b>5</b>
<b>LIST OF ABBREVIATIONS .....</b>	<b>6</b>
<b>1. INTRODUCTION.....</b>	<b>10</b>
1.1. pRB and RB-family members, p107 and p130 in mammals .....	10
1.1.1. Similarities between the pocket proteins .....	11
1.1.2. Differences between the pocket proteins .....	12
1.2. Plant retinoblastoma-related proteins (RBRs) .....	13
1.2.1. The plant RBRs can be categorized into three different groups .....	15
1.2.2. Expression profiles of the plant RBR subfamily genes .....	16
1.3. The role of phosphorylation/dephosphorylation in the regulation of the retinoblastoma protein .....	18
1.3.1. The phosphorylation of pRB by CDKs.....	19
1.3.2. Dephosphorylation of pRB by protein phosphatase 1 (PP1) in mammalian cells .....	20
1.3.3. PP2A phosphatases can dephosphorylate the mammalian pocket proteins under stress conditions .....	21
1.3.4. Phosphorylation of plant RBR proteins by the cyclin D/cyclin-dependent kinase complex.....	22
1.3.5. Dephosphorylation of plant phospho-RBRs .....	23
1.4. The PP2A protein phosphatase .....	23
1.4.1. The structure of PP2A.....	24
1.4.2. The role of the PP2A regulatory subunits.....	25
1.4.3. Post-translational regulation of B regulatory subunit .....	27
<b>2. AIMS OF STUDY .....</b>	<b>28</b>
<b>3. RESOURCES AND METHODS.....</b>	<b>29</b>
3.1. The yeast two-hybrid system .....	29

3.1.1. Construction of <i>Oryza sativa</i> var. japonica cv. UNGGI-9 cDNA libraries for yeast two hybrid screening .....	29
3.1.2. Plasmid construction for yeast two-hybrid experiments and for recombinant protein production .....	30
3.1.3. Yeast two-hybrid screens and pairwise interaction analysis.....	30
3.1.4. Quantitation of $\beta$ -galactosidase activity in yeast extracts.....	31
3.2. Plant suspension culture synchronization (alfalfa and rice).....	32
3.3. Flow cytometry .....	32
3.4. Recombinant protein purification .....	33
3.4.1. GST- tagged fusion protein expression and purification .....	33
3.4.2. His <sub>6</sub> -tagged protein expression and purification.....	33
3.5. Pull down assays .....	34
3.6. Antibodies and Western blotting.....	34
3.7. Protein phosphorylation .....	35
3.8. Immunoprecipitation.....	36
3.9. Protein phosphatase activity.....	37
<b>4. RESULTS .....</b>	<b>38</b>
4.1. Immunodetection of MsRBR1 and phospho-MsRBR1 proteins in cultured alfalfa cells...	38
4.2. Cell cycle phase-dependent variations in MsRBR1 and phospho-MsRBR1 protein levels	40
4.3. Cell cycle phase-dependent phosphorylation and dephosphorylation of OsRBR1 .....	42
4.4. Retinoblastoma-related protein OsRBR1 interacts with the rice phosphatase OsPP2A B'' regulatory subunit .....	44
4.5. The B pocket domain in OsRBR1 is sufficient, and the C-terminal region in OsPP2A B'' is indispensable for the interaction between those two proteins.....	46
4.6. OsPP2A B'' interacts with and is phosphorylated by cyclin-dependent kinase .....	48
4.7. Confirmation of the putative phosphorylation sites of OsPP2A B'' regulatory subunit via site-directed mutagenesis and proteomic analysis .....	50
4.8. Phosphorylation of OsPP2A B'' may promote its association with OsRBR1.....	52

4.9. Phosphorylation of OsPP2A B'' regulatory subunit Effects on the phosphatase activity of holoenzyme .....	54
<b>5. DISCUSSION .....</b>	<b>57</b>
5.1. Specific antibodies used in the detection of MsRBR1/OsRBR1 and phospho-MsRBR1/OsRBRs .....	58
5.2. Variation in cell-cycle dependent RBR and phospho-RBR protein levels in alfalfa and rice cell cultures .....	59
5.3. PP2A phosphatase B'' regulatory subunit interacts with OsRBR1 specifically and preferentially associates with members of the subfamily II of OsPP2A catalytic subunits .....	61
5.4. The B pocket domain in OsRBR1 is sufficient, and the C-terminal region in OsPP2A B'' is obligato for the interaction between these two proteins.....	64
5.5. Phosphorylation of the OsPP2A B'' regulatory subunit and identification of its putative phosphorylation sites.....	66
5.6. Non-phosphorylation mutant of OsPP2A B'' regulatory subunit influences PP2A phosphatase activity, but has no affect on the formation of the PP2A heterotrimer.....	68
<b>6. ACKNOWLEDGEMENT.....</b>	<b>70</b>
<b>7. REFERENCES.....</b>	<b>71</b>
<b>8. SUMMARY .....</b>	<b>83</b>
<b>9. ÖSSZEFOGLALÁS.....</b>	<b>89</b>
<b>10. APPENDICES .....</b>	<b>94</b>

## SUMMARY

The thesis work started with the test of specific antibodies used for MsRBR1/OsRBR1 and phospho-MsRBR1/OsRBRs detection. With these antibodies, cell-cycle dependent RBR and phospho- RBR proteins variation in alfalfa and rice cultured cells were checked. Total amount of MsRBR1/OsRBR1 barely changed throughout the cell cycle, while phosphorylated forms of MsRBR1/OsRBR1 proteins showed clear cell cycle dependent changes.

In my study, I found that the OsPP2A B'' regulatory subunit associates with OsRBR1 but not with OsRBR2. The B pocket of OsRBR1 is essential and sufficient for the interaction between OsPP2A B''. As for B'', none of the truncated version can associate with OsRBR1; the C-terminal of B'' is essential but not sufficient for the interacting between these two proteins. Three putative CDK phosphorylation sites of the OsPP2A B'' regulatory subunit were verified through LC-MS/MS analysis and the set up of a series of site-directed mutagenesis. The B'' regulatory subunit interact with PSTAIRE-motif containing kinases directly and phosphorylate by them, the conclusion comes from the results of co-immunoprecipitation.

Elimination of phosphorylation sites in B'' did not affect the binding to the PP2A catalytic subunit but did weaken the binding to OsRBR1; the effect was more significant in *in vivo* yeast two-hybrid system. The phosphorylation elimination of B'' regulatory subunit down-regulated the activity of B'' containing PP2A heterotrimeric holoenzyme. In contrast, phosphorylation mimicking of B'' regulatory subunit up-regulated the activity of B'' containing PP2A heterotrimeric holoenzyme. Neither eliminated nor mimicked the phosphorylation of B'' regulatory subunit changed the binding strength to PP2A catalytic subunit. The phosphorylation of B'' subunit just stimulated the activity of the PP2A complex.

It can be postulated that the free  $\text{Ca}^{2+}$  ions has the role in regulate the activity of B'' subunit since it contains two EF-hand domain. The postulation was verified by the experiments which indicated that the presence of  $\text{Ca}^{2+}$  increased the activity of the PP2A holoenzyme and conversely, the absence of  $\text{Ca}^{2+}$  (with the chelator, EGTA) inhibited the phosphatase activity of PP2A complex.

## LIST OF ABBREVIATIONS

AD	Active Domain
Ade	Adenine
AL1	Viral replication factor
A-subunit	Scaffolding subunit of protein phosphatase 2A
ATP	Adenosine 5'-triphosphate
BD	Binding Domain
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
Cdc6	Cell division cycle mutant 6
CDK	Cyclin Dependent Kinase
cDNA	Complimentary Deoxyribonucleic Acid
CDPK	Calcium Dependent Protein Kinase
CHX	Cycloheximide
CIAP	Calf Intestinal Alkaline Phosphatase
CKS1	CMP-KDO Synthetase
C-subunit	Catalytic subunit of protein phosphatase 2A
DMSO	Dimethyl Sulfoxide
DTT	1, 4-Dithiothreitol
E2F	Adenovirus E2 binding transcription factor moter-binding Factor
EDTA	Ethylenediamine Tetraacedtic Acid
EdU	Ethynyl deoxyuridine

EF-hand	EF hand domain
EGTA	Ehtylene Glycol Tetraacetic Acid
EST	Expressed Sequence Tag
FGF	Fibroblast Growth Factor
G1 and G2	Gap1 and Gap2
GST	Glutathione-S-transferase
Gwl	Greatwall Kinase
HDAC	Histone Deacetylase
HEAT	Huntington-elongation-A subunit-Tor
HMGR	3-Hydroxy-3-Methylglutaryl-CoA Reductase
HPV E7	Human Papillomaviruses E7
HU	Hydroxyurea
IP	Immunoprecipitation
IPTG	Isopropyl- $\beta$ -D-1-thiogalactopyranoside
LB	Luria Bertani
M	Mitosis
MALDI-TOF	Matrix Assisted Laser Desorption Ionization-Time of Flight
MAPK	Mitogen Activated Protein Kinase
MBP	Myelin Binding Protein
MDM2	Murine Double Minute 2
MSV	Maize Streak Virus
NCBI	National Center for Biotechnology Information
NP40	Nonyl Phenoxy polyethoxylethanol



OA	Okadaic Acid
OD	Optical Density
PAGE	Polyacrylamide-gel electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PKA	Protein Kinase A
PKC	Protein Kinase C
PMSF	Phenyl Methane Sulfonyl Fluoride
PP2A	Protein Phosphatase 2A
PP2A <sub>D</sub> and PP2A <sub>T</sub>	Dimeric and Trimeric forms of PP2A
PR59/PR48/PR72/G5PR	PP2A regulatory subunits, belongs to B'' subfamily
pRB	Retinoblastoma Protein
PRC2	Polycomb Repressive Complex 2
RBR	Retinoblastoma-Related
Rep A	Protein product of Rep gene, replication initiator protein
RNA	Ribonucleic Acid
RNAi	RNA interference
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SD	Synthesis Defect
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
S-phase	Synthesis phase
SUC1	Sucrose transport protein

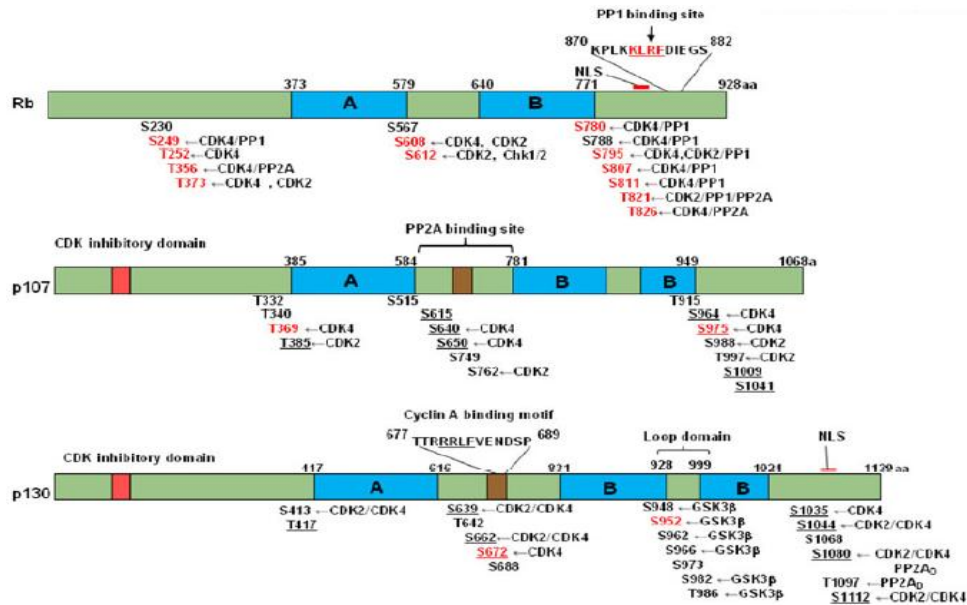
SV40	Simian Virus 40
TGMV	Tomato Golden Mosaic Virus
TH	Tyrosine Hydroxylase
TIGR	The Institute for Genome Research
TPD1	Tapetum Determinant 1
WDV	Wheat Dwarf Virus

# 1. INTRODUCTION

## 1.1. pRB and RB-family members, p107 and p130 in mammals

The retinoblastoma protein (pRB), a 928 amino acid nucleophosphoprotein, is the product of the retinoblastoma (*RB*) gene, the first cloned and the best known tumor-suppressor gene (Friends et al., 1986). Since its discovery, pRB has been a focal point of cancer research. Up till now, the pRB has been implicated in many cellular processes, such as regulation of the cell cycle, DNA-damage responses, DNA repair, DNA replication, protection against apoptosis and differentiation; all of which could contribute to its function as a tumor suppressor (Burkhart and Sage, 2008). The *RB* gene is closely related to two other genes in humans: *p107* and *p130* (Ewen et al., 1991; Baldi et al., 1996). These three proteins form the RB family.

The three RB-family proteins are all composed of three regions: the amino-terminal region, the pocket region and the carboxy-terminal region. Homologues of the three human RB-family proteins have also been found in mice, *Drosophila*, chicken and other organisms. The pocket region consists of an A and B domain separated by a spacer (**Fig. 1**). The presence of the spacer region allows the assembly of the two subdomains into a pocket-like structure. For this reason, the three members of the RB family are known as pocket proteins (Hannon et al., 1993; Mayol et al., 1993). The pocket region is important for the binding of other proteins. The exons of the *RB* gene encoding domain A, domain B and the spacer region between domains A and B are similar in all members of the RB-family. It is interesting that highly conserved amino-acid residues can be found in the same positions between p107 and p130. This feature is not shared by pRB/p105, which seems to suggest that the p107 and p130 genes are in evolutionary terms closer to each other than to p105 (Ichimura et al., 2000). An additional proof for this closer relationship is provided by the higher similarity of the spacer regions between p130 and p107 compared to pRB (Mayol et al., 1993).



**Figure 1. Functional domains and phosphorylation sites of RB-family proteins.**

The pocket region of RB-family proteins consists of two conserved functional domains identified as A and B pockets. This figure also shows the identified phosphorylation sites and respective kinases/phosphatases for pocket proteins. (Kolupaeva V, and Janssens V. 2013)

### 1.1.1. Similarities between the pocket proteins

The gene structure of all the three pocket proteins is similar to other housekeeping genes, and three proteins also share high similarities in the primary amino acid sequences of the pocket region (Claudio et al., 2002). Due to the similarities, p107 and p130 show the same ability as pRB to bind to viral oncoproteins, such as the human papillomavirus (HPV16) *E7* gene product, SV40 large T antigen and the adenovirus *E1A* gene product. These viral proteins possess a LXCXE motif and interact with the B domain of the pocket proteins (Mayol et al., 1993; Ewen et al., 1991; Hannon et al., 1993). Significantly, the regions of these oncoproteins interacting with the pocket proteins were found to overlap with the regions needed for oncogenic transformation (Classon and Dyson, 2001).

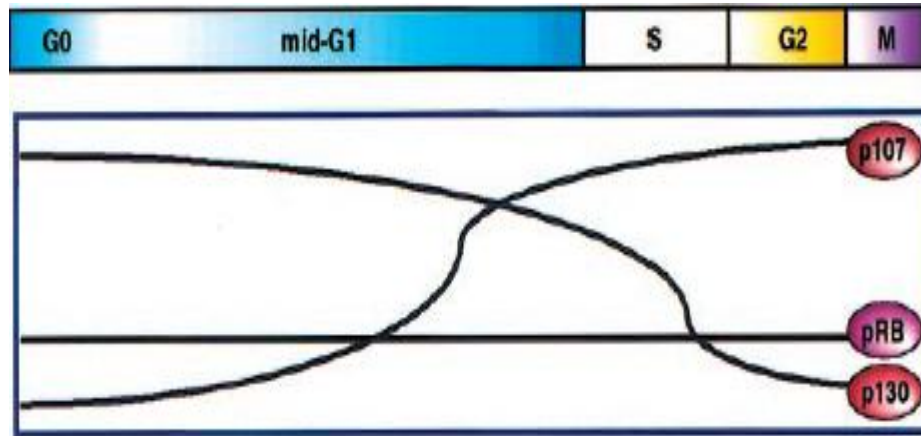
In addition to their structural and primary amino acid sequence similarities, pRB, p107 and p130 share quite similar biological properties. For instance, when overexpressed, pRB, p107 and p130 have been found to arrest cells in the G1 phase of the cell cycle (Goodrich et al., 1991; Zhu et al., 1993; Claudio et al., 1994). pRB overexpression can arrest the cell cycle of all cell types,

whereas the effects of p107 and p130 expression on the cell cycle are seen only in a limited number of cell types (Classon and Dyson, 2001). Concomitant ablation of all the three *RB*-related genes in embryonic stem cell causes a deregulated G1/S transition, abolition of G1 arrest and the absence of senescence in cell cultures. As a consequence, the differentiation capacity of the stem cells is strongly impaired (Dannenberg et al., 2000; Galderisi et al., 2006). These data confirm the essential role of the pocket protein family in cell cycle control, G1/S transition and in tumorigenesis. The p107 and p130 proteins, like pRB, contain multiple sites for phosphorylation by cyclin-dependent serine/threonine kinases (CDKs), including a large cluster of sites in the extreme C-terminus of the proteins (Ewen et al., 1991; Hannon et al., 1993; Li et al., 1993) and their phosphorylation patterns are cell cycle-dependent (Mihara et al., 1989; Shirodkar et al., 1992; Baldi et al., 1995) (summarized in **Fig. 1**).

### 1.1.2. Differences between the pocket proteins

The expression patterns of the three pocket protein encoding genes during the cell cycle are different (**Fig. 2**). The levels of pRB are quite constant during the cell cycle and in quiescent cells (Grana et al., 1998). *p130* is highly expressed in quiescent and differentiated cells; its level is rapidly down-regulated when quiescent cells enter the cell cycle via stimulation. In contrast, the *p107* level is generally quite low in differentiated cells and its level rises when quiescent cells re-enter the cell cycle (Classon and Dyson, 2001). The three pocket proteins are excellent substrates for CDKs under *in vitro* conditions and in the cell the pRB, p107 and p130 proteins are phosphorylated in a cell-cycle-dependent manner. The pattern of pRB phosphorylation will be described in details below. The pattern of p107 phosphorylation closely resembles that of pRB: p107 is in a non- or underphosphorylated state in quiescent cells, when cells progress towards the S phase, it becomes phosphorylated. The phosphorylation initiation coincides exactly with the activation of cyclin D-dependent kinase (Beijersbergen et al., 1995; Xiao et al., 1996). In contrast, p130 phosphorylation in quiescent cells is not due to CDK-activity. As Mayol et al. (1996) reported, amino acids of p130 that are phosphorylated in quiescent cells have been mapped to a short loop in the B-domain of the pocket and are unique to p130. On the other hand, the phosphorylation of p130 that occurs during cell cycle

progression appears to be initiated also by cyclin D-associated kinases (Mayol et al., 1996; Classon and Dyson, 2001).



**Figure 2. Expression patterns of three pocket proteins.**

Relative changes in the protein levels of each pocket protein throughout the cell cycle. (Grana et al., 1998)

The pocket proteins are best known for their roles in restraining the G1/S transition through the regulation of E2F transcription factors (Cobrinik, 2005). Members of the pocket protein family show a clear difference in their ability to interact with various E2F transcription factors. pRB can bind *in vivo* to activator-type E2Fs (E2F1, 2, 3a) as well as to E2F3b, which functions mainly as a repressor. In contrast, p107 and p130 preferentially bind to repressor-type E2Fs (E2F-4 and E2F-5) (Nevins, 1998; Classon and Dyson, 2001). Consequently, the pocket proteins not only inhibit E2F mediated transactivation but also work together with another set of E2Fs to repress transcription and inhibit G1-S progression. In general terms, pRB regulates E2Fs that are strong activators while p107 and p130 interact with E2Fs that are primarily considered to be co-repressors. Up till now, the basis for this specificity has not been resolved (Dimova and Dyson, 2005; Cobrinik, 2005).

## 1.2. Plant retinoblastoma-related proteins (RBRs)

Since the discovery of the first maize RB-related homologue (Grafi et al., 1996), plant cDNAs encoding RB-related proteins (RBRs) have been reported in several species, including *Zea mays*, *Nicotiana tabacum*, *Chenopodium rubrum*, *Arabidopsis thaliana*, *Populus tremula* × *Populus tremuloides*, *Cocos nucifera*, *Oryza sativa* and *Pisum sativum* (Shimizu-Sato et al., 2008). The

conservation of amino acids between animal and plant RB-related proteins suggests that the proteins have similar biochemical properties. There are several common points between mammalian RB family members and plant RB-related proteins. First, the RBRs are composed of an amino-terminal region, a pocket region consisting of an A and B domain and a C-terminal domain; second, RBRs contain several potential CDK phosphorylation sites; third, plant RBRs interact with mammalian viral oncoproteins, SV40 T-antigen, adenovirus E1a, and HPV E7, as well as plant virus proteins, the RepA polypeptide of wheat dwarf virus (WDV), the maize streak virus (MSV); and the tomato golden mosaic virus (TGMV) replication factor, AL1. They also associate with other cellular proteins, such as E2F family proteins, D-type cyclins (Ach et al., 1997; Horvath et al., 1998; Durfee et al., 2000; Boniotti and Gutierrez, 2001; Lendvai et al., 2007; Miskolczi et al., 2007; Shimizu-Sato et al., 2008).

To date, the role of RBR proteins can be separated into four groups (Gutzat et al., 2012): 1) proliferation. Ebel et al. (2004) provided the first clue about this point. They reported that in heterozygous *Arabidopsis* mutants with a loss-of-function *rbr* allele 50% of the ovules are aborted when the mutant allele is maternally inherited, the mature unfertilized mutant showed supernumerary nuclear division and misexpression of cellular markers in the embryo sac. RBR deletion will cause hyperproliferation during plant male gametogenesis (Chen et al., 2009) and lead to a reduction of crossover formation during meiosis (Chen et al., 2011). RBR also showed a dosage-dependent pleiotropic effect on sporophytic development, trichome differentiation, and regulation of PRC2 subunit genes in leaves (Johnston et al., 2010); 2) developmental transitions. In RBR-deficient mutants, development of seedlings was arrested after germination because late embryonic genes induced by low amount of sucrose and resulted in abnormal cell division. The results indicated that RBR is a positive regulator of the developmental switch from embryonic heterotrophic growth to autotrophic growth (Gutzat et al., 2011); 3) stem cell maintenance. Wildwater et al. (2005) reported that if they decrease the expression level of RBR via RNAi in *Arabidopsis* roots, the quantity of stem cells raised because of less differentiation; while overexpression of RBR increase the assumption of stem cells. The similar results also showed in the reporter of Borghi et al. (2010). Conditional reduction or loss of RBR function disrupted cell division patterns, promoted context-dependent cell proliferation, and negatively influenced establishment of cell differentiation; 4) differentiation.

Huntley et al. (1998) reported the maize RB homologue ZmRb-1 temporally and spatially regulated during maize leaf development. The protein is highly expressed in differentiating cells, but almost undetectable in proliferating cells.

### **1.2.1. The plant RBRs can be categorized into three different groups**

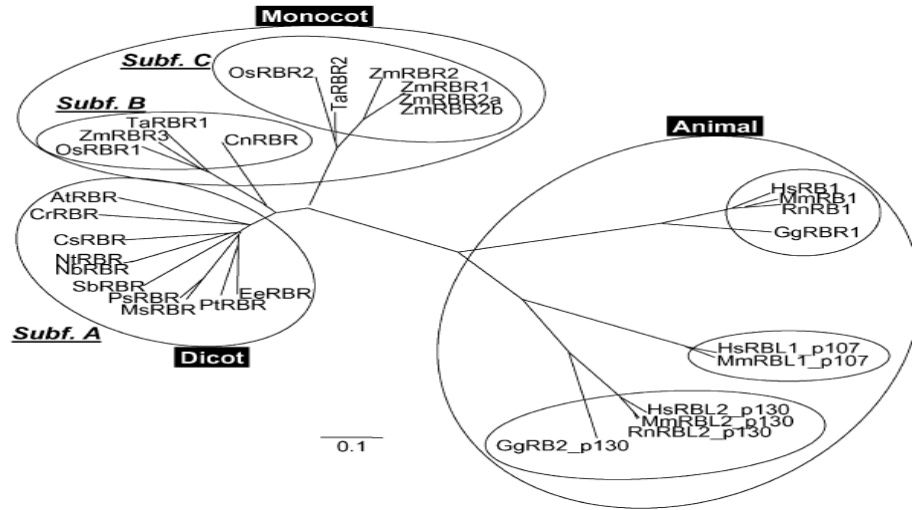
At least two RBR homologues can be found in monocot grasses whereas only a single representative RB homologue sequence could be detected in the genome of dicot species from all the plants for which complete genomic or near-to-complete genomic and EST sequence data are available (Lendvai et al., 2007; Miskolczi et al., 2007). This observation highlights an interesting difference between dicot and monocot species.

The phylogenetic tree of known plant and a few typical animal retinoblastoma family proteins (**Fig. 3**) clearly demonstrates that the plant RBR proteins can be categorized into three distinct subfamilies on the basis of amino acid sequence similarities. The dicot RBR proteins formed subfamily A, whereas the monocot RBR proteins formed the subfamilies B and C. The dicot RBR proteins share 66-91% amino acid identity. Within subfamilies B and C, the sequences of monocot RBR proteins share 69-85% and 74-90% identity, respectively. When comparing the amino acid identity between subfamilies, subfamilies A and B share 54-67% identity, whereas subfamilies A and C share 48-60% identity. The identity between the two distinct monocot subfamilies B and C is 48-59%. The data also suggest that the relationship between the dicot RBR proteins (subfamily A) and monocot subfamily B proteins is closer than between monocot subfamily C and the dicot subfamily A. As a conclusion, the phylogenetic tree reveals a clear phylogenetic separation between the known monocot RBR proteins, and hints at a more complex RB pathway in the monocot cereal species than in dicot plants (Lendvai et al., 2007; Miskolczi et al., 2007).

In recent published article (Gutzat et al., 2012), more complicated phylogenetic relationships of RBR proteins in the plant lineage were showed. Same as the older version, most sequenced dicot genomes contain one *RBR* gene which cluster in one group (There has one exception on *M.esculenta*, which contains three different RBRs. Two of them share 85% identity to each other, while the third one, RBR3, shares 51% and 52% identity with the RBR1 and RBR2. The amino acids alignment



shows around 200 amino acids less in the N-terminus of the RBR3 compare to the other two RBRs.); all sequenced monocots contain more than one *RBR* gene that cluster together in two different clades. RBR protein has been identified in unicellular green algae. Another interesting thing is in *Volvox carteri*, the male and female contain different *RBR* genes.



**Figure 3. Comparison of plant and animal RB family members.**

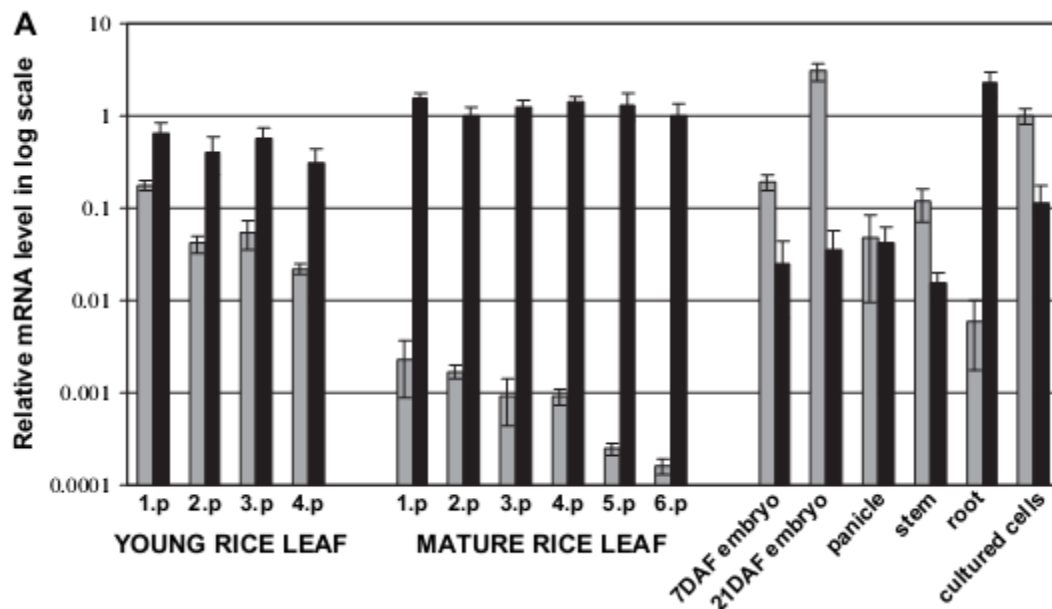
Phylogenetic tree of RB homologue sequences from both plants and animals. The multiple alignment was made by the ClustalW program and displayed by the Phylodendron program (<http://iubio.bio.indiana.edu/treeapp/>). The scale beneath the tree measures the evolutionary distance between sequences (Miskolczi et al., 2007).

### 1.2.2. Expression profiles of the plant RBR subfamily genes

The study of Lendvai et al. (2007) explicitly revealed the characteristic differences between the two rice *RBR* genes by comparing the expression patterns in the leaves in various developmental stages (**Fig. 4**). The transcription level of the rice *OsRBR2* gene can be detected in all leaf tissues (young leaves, mature leaves, and embryos) evenly, while the expression of *OsRBR1* exhibits different distribution pattern. In general, 10 times more *OsRBR1* mRNA is accumulated in the younger leaves than in old leaves. While in old leaves, there is a gradual reduction of the level of *OsRBR1* mRNA from the basal section of the leaves towards the leaf tip. This may be due to the fact that the basal meristem generates lines of cells that remain aligned from the base to the tip of the leaf in monocots and these cell files show gradually decreasing activity of cell cycle specific genes (Lendvai et al., 2007). However, in the differentiated tissues, such as roots and leaves (especially old

leaves), relatively high amounts of *OsRBR2* mRNAs were accumulated. The *OsRBR1* transcripts were found to be more abundant than the *OsRBR2* gene transcripts in developing embryos and cultured cells. These experimental data suggest that the *OsRBR1* gene may play a role in cell division or cell cycle progression while the *OsRBR2* gene plays a role similar to that of p130, which is highly expressed in quiescent and differentiated cells.

In maize, the 110kDa form of ZmRBR1 is associated with the differentiation process in leaf cells (Huntley et al., 1998), while expression of the RBR3 protein is more related to active proliferation (Sabelli et al., 2005). RBR3 has an essential and positive role in regulating MCM gene expression, DNA replication, and cell transformation, while ZmRBR1 has a clear cell cycle inhibitory function (Sabelli et al., 2009).



**Figure 4. The relative mRNA levels of the *OsRBR1* and *OsRBR2* genes in rice tissues.**

Gray columns represents *OsRBR1*, black columns represents *OsRBR2* expression levels. Young leaves were sectioned into 4 pieces, mature leaves were sectioned into 6 pieces continuously from the basal part to the tip (Lendvai et al., 2007).

It seems that the levels of the dicot *RBR* mRNAs do not correlate with actual division events. Several experiments support this assumption. In alfalfa, similar mRNA levels of the *MsRBR1* gene were detected in leaf sections from the basal to the tip, and in rapidly dividing cells in suspension cultures (Lendvai et al., 2007). The *RBR* genes in the other two dicots, *Arabidopsis* (*AtRBR*) and

tobacco (*NtRBR1*) (Nakagami et al., 1999), are expressed constitutively in different organs, and act in the same way as the *MsRBR1* gene.

In summary, the single dicot gene appears to be ubiquitously expressed, while in the cereal monocot species a clear specialization can be observed between the members of the two RBR subfamilies suggesting that in monocot plants the members of the two RBR subfamilies have distinct functions (Sabelli et al., 2005; Lendvai et al., 2007; Miskolczi et al., 2007).

### **1.3. The role of phosphorylation/dephosphorylation in the regulation of the retinoblastoma protein**

The antiproliferative activity of pRB is mediated by its ability to inhibit the transcription of genes that are required for cell cycle progression. This transcriptional regulatory function of pRB is fulfilled through several distinct mechanisms. The best illustrated mechanism is the inhibition of E2F-regulated gene expression via the association between pRB and members of the E2F family.

The ability of the pRB protein to inhibit cellular proliferation is counterbalanced by the action of CDKs (Taya, 1997; Sherr and Roberts, 1999). In quiescent and early G1 cells, the pRB protein is non-phosphorylated; in this state it is able to bind to and repress E2F activity with the highest efficiency, preventing E2F-dependent transcription. As cells progress toward the S phase, pRB phosphorylation sites become sequentially phosphorylated and the protein is thereby inactivated. The initial phosphorylation of pRB is most likely catalyzed by Cyclin D-CDK4 or Cyclin D-CDK6 complexes. This event leads to the partial inactivation of pRB, which allows the transcription of E2F-controlled genes such as cyclin E1, which in turn activates the downstream CDK2 kinase. Subsequently, Cyclin E-CDK2 complexes phosphorylate the pRB protein further, increasing its phosphorylation level (Ortega et al., 2002; Inoue et al., 2007). Phosphorylation of pRB inactivates the pRB-E2F complex and releases E2F, allowing the expression of E2F-regulated genes required for cell cycle progression and S phase entry. During mitosis, pRB is rapidly dephosphorylated by a specific mitotic phosphatase that removes the phosphate group from the pRB protein, thus restoring the dephosphorylated or hypophosphorylated form of the pRB protein at the start of the G1 phase (Ludlow et al., 1993). DeCaprio et al. (1988) reported, based on differential mobility in SDS gels and phosphopeptide patterns, that a number of differentially phosphorylated forms of pRB appeared to

exist in cells and the state of phosphorylation appears to change even after the initial hyperphosphorylation of pRB in late G1.

Taken together, the *RB* gene product pRB undergoes phosphorylation-dephosphorylation changes in a cell cycle dependent manner.

### **1.3.1. The phosphorylation of pRB by CDKs**

Detailed analyses of phosphorylation sites both *in vivo* and *in vitro* indicated that 16 serine or threonine residues of pRB can become phosphorylated. Tyrosine residues, on the other hand, remain unphosphorylated. All of the phosphorylation sites were shown to contain a Ser/Thr-Pro motif (Zarkowska and Mitnacht, 1997; Knudsen and Knudsen, 2006).

At least three different cyclin-CDK complexes have been suggested to phosphorylate pRB during the cell cycle. It is proposed that cyclin D-CDK4/6 phosphorylates pRB during early G1, cyclin E-CDK2 phosphorylates the protein near the end of G1, and cyclin A-CDK2 may maintain phosphorylation of pRB during the S phase (Sherr and Roberts, 1999; Harbour and Dean, 2000). Cyclin B-CDC2 may contribute to the phosphorylation of pRB in G2 and the M phase, but the only evidence supporting this suggestion is the fact that pRB is a good substrate for this kinase complex *in vitro* (Taya, 1997).

Kitagawa et al. (1996) showed that cyclin D1/CDK4 preferentially phosphorylates S780, Zarkowska et al. (1997) and Connell-Crowley et al. (1997) identified T826 and S795 as cyclin D1/CDK4 specific sites respectively. The data seem to suggest that different kinases can phosphorylate different sites on pRB.

Several studies have found that phosphorylation of pRB on different sites inactivates different molecular functions (Adams, 2001). Knudsen et al. (1996) showed that phosphorylation of pRB on T821 and T826 is required for inactivation of binding to the LXCXE motif. Brown et al. (1999) suggested that accumulation of phosphates on a number of sites is sufficient for E2F/pRB complex disruption and that there is no requirement for specific sites. It has been shown that the phosphorylation of either the C-terminus or spacer region of pRB can release E2F (Knudsen et al.

1997). Interestingly Harbour et al. (1999) showed, the phosphorylation of just one serine in the A domain, S567, can disrupt the interaction of the A and B domains and release of E2F.

In summary, pRB is phosphorylated by different CDKs during different phases of the cell cycle on specific phospho-acceptor sites, modifying in each case the function of pRB specifically.

In addition to CDKs, there are still other kinds of serine/threonine-specific kinases, like the mitogen-activated protein kinases (MAPKs) and aurora kinases that are involved in the modulation of RB phosphorylation. P38, one MAPK, phosphorylates RB on ser567 in a cell cycle-independent manner, this phosphorylation leading to an interaction between RB and the human homolog of murine double minute 2 (MDM2), following by the degradation of RB, releasing of E2F1 and cell death (Delston et al., 2011). Aurora B kinase directly phosphorylates RB at serine 780 both *in vitro* and *in vivo*. The phosphorylation inhibits the endoreplication after an aberrant mitosis via the regulation of the postmitotic checkpoint (Nair et al., 2009).

### **1.3.2. Dephosphorylation of pRB by protein phosphatase 1 (PP1) in mammalian cells**

As mentioned above, pRB phosphorylation has been very well documented. In contrast, little is known about the way in which phosphates are removed from pocket proteins during the cell cycle. In 1993, Durfee et al. found that the catalytic subunit of a novel type 1 protein phosphatase (PP-1 $\alpha$ 2) interacts with pRB according to the yeast two-hybrid system. Cell-cycle synchrony experiments revealed that this interaction occurs from mitosis to early G1, raising the intriguing possibility that this phosphatase dephosphorylates RB during the mid-M or early G1 phase of the cell cycle. In the same year, Alberts et al. (1993) examined the effects of the elevation of protein phosphatase levels on cellular events that control the transition from the G1 to S phase of the cell cycle via the microinjection of protein phosphatase type1(PP1) and 2A (PP2A). Microinjection of either PP1 or PP2A into cells before the S phase increased the affinity of pRB for the nucleus (e.g. it inhibits cell cycle progression into the S phase) while injection of either of the two phosphatases into cells that had entered the S phase did not block DNA synthesis, suggesting that the effect of the two phosphatases on cell cycle arrest was specific. In the experiment carried out by Ludlow et al. (1993), dephosphorylation of pRB by mitotic cell extracts was sensitive to inhibitors of PP1. Moreover, a

high molecular weight form of PP1 found in mitotic cell extracts was shown to be able to dephosphorylate pRB *in vitro* (Nelson et al., 1997).

Berndt et al. (1997) showed that unless a threonine at position 320 is phosphorylated, the PP1- $\alpha$  (one of the isoforms of PP1) has the potential to arrest cell growth in G1. This result is in agreement with the observation of Nelson et al. (1997) that PP1 can regulate the cell cycle indirectly by modulating the activity of pRB. Tamrakar and Ludlow (2000) studied the co-precipitation of truncated pRB proteins isolated from a series of pRB deletion mutants with PP1 to map the pRB domain involved in binding to PP1. The results showed that the carboxyl-terminal region of pRB is both necessary and sufficient for physical interaction with PP1. The interaction between pRB and PP1 does not depend upon PP1 being catalytically active.

Yan et al. (1999) proposed that PP1 is involved in the direct dephosphorylation of pRB while protein phosphatase 2A (PP2A) is involved in the dephosphorylation components of pathways that play a role in the regulation of G1 cyclin-dependent kinase activity. PP2A may also have an important cell division regulatory role, since its activity contributes to the control of mitotic kinases and microtubule organization in alfalfa (Ayaydin et al., 2000).

### **1.3.3. PP2A phosphatases can dephosphorylate the mammalian pocket proteins under stress conditions**

PP1 has been implicated in the dephosphorylation of pRB from mitosis to a point in G1, where pRB becomes hyperphosphorylated by G1 cyclin-CDKs (Grana, 2008). In contrast to PP1, the role of PP2A in modulating the phosphorylation of pocket proteins is not restricted to mitosis and early G1, but can be shown throughout the cell cycle (Garriga et al., 2004). The catalytic subunit of PP2A was found to interact with both p130 and p107 throughout the cell cycle and it was shown that dephosphorylation occurs throughout the cell cycle (Garriga et al., 2004).

PP2A has been implicated in dephosphorylation of pocket proteins in response to certain stress. Following a treatment with  $\gamma$  irradiation, PP2A is required for postdamage dephosphorylation of phospho-RB and recruitment of RB to replication initiation sites (Avni et al., 2003). Similarly, oxidative stress elicited a rapid PP2A-dependent dephosphorylation of pRB, p107 and p130 (Cicchillitti et al., 2003). In addition, PP2A was shown to dephosphorylate p107 rapidly in cells

which are exposed to UV irradiation (Voorhoeve et al., 1999a). Vuocolo et al. (2003) found that the mechanism of growth inhibition by the differentiation agent all-trans-retinoic acid (ATRA) is involved in the induction of PP2A, which in turn dephosphorylates p130, making it stable. Kolupaeva et al. (2008) reported that in chondrocytes fibroblast growth factor (FGF) activates the PP2A phosphatase to promote p107 dephosphorylation, which is a key event in FGF-induced cell cycle arrest. A specific PP2A B'' subunit, PR70, was identified by Magenta et al. (2008). It is associated with pRB both *in vitro* and *in vivo*. PR70 overexpression caused pRB dephosphorylation; conversely, PR70 knockdown prevented both pRB dephosphorylation and oxidative stress induced inhibition of DNA synthesis. Moreover,  $\text{Ca}^{2+}$  induced the PP2A phosphatase activity of PR70. Low concentration of okadaic acid (OA) is ideally an inhibitor of PP2A. An immunocytochemical experiment revealed higher quantity of phosphorylated Rb-like protein, the main substrate of PP2A phosphatase, in 1  $\mu\text{M}$  OA treated cells (Polit JT & Kazmierczak A, 2007).

#### **1.3.4. Phosphorylation of plant RBR proteins by the cyclin D/cyclin-dependent kinase complex**

In plants, the RBR protein functions are controlled by phosphorylation and protein-protein interactions (Abraham et al., 2011). Phosphorylation of RBR also occurs in a cell-cycle-dependent manner. The highest phosphorylation state occurs during the G1-S transition and is maintained till mid/late S phase. Same as in animals, this phosphorylation results in an inactivation of RBR and the subsequent release of E2F-DP transcription factors in their active form (summarized in Gutzat et al., 2012).

The NtRBR1 protein of tobacco was phosphorylated by the cyclin D3;3-CDKA complex. This kinase activity was detected in extracts from G1 and S phase cells of synchronized tobacco BY-2 culture (Nakagami et al., 2002). The recombinant C-terminal domain [glutathione S-transferase (GST)-ZmRBR-C] of the maize RBR protein could serve as a substrate for p13SUC1-bound kinase complex from synchronized wheat cells. This kinase activity remained high for several hours after release from the hydroxyurea (HU) block (Boniotto and Gutierrez, 2001). Kawamura et al. (2006) generated antibodies against the C-terminal region of the NtRBR1 protein and different phosphoserine peptides containing sequences from NtRBR1. The NtRBR1 protein was

phosphorylated by immunoprecipitated CDKA and CDKB from actively growing cells. Antibodies recognizing specific phosphoserines cross-reacted differentially with the NtRBR1 protein in various phases of the cell cycle. The PsRBR1 protein from pea was found to form a complex with D-type cyclin (Pissa; cyclin D3;1) containing the canonical pRB-binding LXCXE motif in the N-terminal region (Shimizu-Sato et al. 2008). Ábrahám et al. (2011) reported that both the MedsaCDKA1;1/1;2 and the mitotic MedsaCDKB2;1 complexes could phosphorylate the C-terminal fragment of the MsRBR1 from alfalfa.

### **1.3.5. Dephosphorylation of plant phospho-RBRs**

Up till now not a single report has been published on plant phosphatases that are responsible for the dephosphorylation of phosphorylated RBR. In a previous yeast two-hybrid screen in our lab, a gene product from *Medicago truncatula* was identified as an interactor of the full-length MsRBR11 protein. This protein shares 39% identity and 57% homology with the murine PR59 PP2A regulatory subunit, and strongly associates with OsRBR1 but not with OsRBR2 (Lendvai et al., 2007). It is interesting to note that a similar selectivity occurred among murine RBs. Only the p107 protein strongly interacts the PR59 PP2A B'' regulatory subunit while pRB did not show association (Voorhoeve et al., 1999b). This analogous behavior also supports the assumption that plant RBRs belonging to different subfamilies (see 1.2.1) have different roles in the regulation of plant cell division and differentiation (Dudits et al., 2011).

The rice homologue of the *MsPP2A B''* gene, *OsPP2A B''*, has been isolated and the pairwise interaction studies confirmed that the OsPP2A B'' regulatory subunit interacts specifically with the OsRBR1 protein (Yu et al., 2014).

### **1.4. The PP2A protein phosphatase**

The PP2A protein phosphatase is ubiquitously expressed and comprises 0.2-1% of all cellular protein in various organs. It is responsible for 30% to 50% of cellular serine/threonine dephosphorylation activity depending on cell type. It is known that PP2A regulates diverse cellular processes, including processes related to development, metabolism, proliferation, transformation,



transcription, translation, apoptosis and stress response (reviewed by Davis, 2005). Ayaydin et al. (2000) treated alfalfa suspension cells with 1  $\mu$  M endothal and then studied the cells via cytological analysis and other molecular methods. The results showed that PP2A involved in mitotic kinase activities and affected the microtubule organization. Similarly, 1  $\mu$  M OA treated synchronized *Vicia faba* root meristems exhibited earlier DNA replication and mitosis entrance than in the control cells (Polit JT & Kazmierczak A 2007). Greatwall kinase (Gwl), a protein active only in the M phase, once activated by MPF via phosphorylation, it promotes the inhibition of PP2A-B55  $\delta$  to keep the MPF-driven phosphorylations. Extracts with depleted Gwl do not enter M phase but this defect can be compensated by depletion of B55  $\delta$  (Castilho PV et al. 2009). In contrast, extracts with silenced B55 enter into mitosis roughly twice faster than in the control, besides, they can never return back to interphase (Hunt T, 2013) .

#### 1.4.1. The structure of PP2A

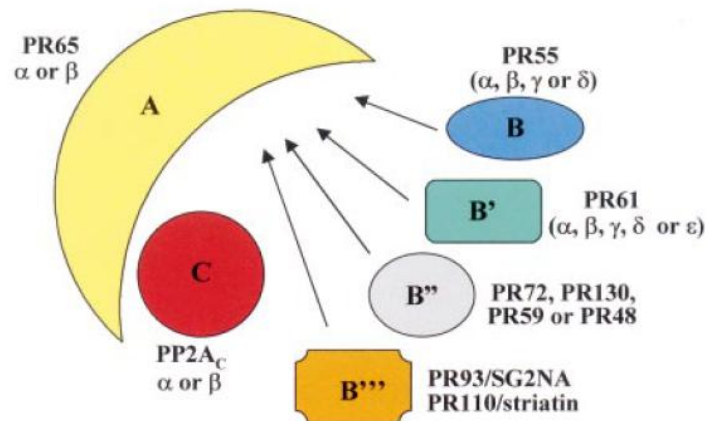
PP2A consists of three distinct functional components: a structural core subunit (PP2A<sub>A</sub>/PR65), a catalytic subunit (PP2A<sub>C</sub>) and a B regulatory subunit (**Fig. 5**). PP2A can exist *in vivo* in a dimeric (PP2A<sub>D</sub>) or a trimeric (PP2A<sub>T</sub>) form. The core enzyme (PP2A<sub>D</sub>) is composed of a 36-kDa catalytic subunit tightly bound to the 65 kDa scaffold subunit (PR65/A). There exists a wide variety of B regulatory subunits that can associate with the core enzyme; so far more than 90 heterotrimeric holoenzymes of PP2A have been identified (Lechward et al., 2001; Kolupaeva and Janssens, 2013).

There are two distinct PR65 isoforms,  $\alpha$  and  $\beta$  in mammals, which share 86% sequence identity; both are ubiquitously expressed. The structure of PR65/A is unusual, since it is entirely composed of 15 tandem repeats of a 39-amino-acid sequence, called a HEAT (Huntington-elongation-A subunit-TOR) motif. The catalytic subunit also contains  $\alpha$  and  $\beta$  isoforms, which share 97% identity. Catalytic subunit has a highly conserved C-terminal tail, which affords important residues for the interaction to A and B subunits (Mumby, 2007). In *Drosophila*, depletion of PP2A-A subunit gene will decrease the protein level of catalytic subunit. Shut off PP2A C or A subunits will issue in bipolar monoastral spindles (Chen et al, 2007). The number of genes encoding the subunit A

and C is quite limited and the gene products show remarkable sequence conservation among eukaryotes, whereas B subunit is multivariate. 15 genes have been identified in the human genome that encode at least 26 different alternative transcripts and variant splice forms representing each a B subunit of the PP2A holoenzyme. B subunits can be classified into four families of unrelated proteins: B (PR55), B' (PR61), B'' (PR72/PR130/PR59/PR48), and B''' (PR93/PR110).

The binding of different B subunits to the AC core enzyme generates a broad variety of holoenzymes thereby regulating phosphatase activity and substrate specificity (Janssens and Goris, 2001; Lechward et al., 2001; Eichhorn et al., 2009).

Database searches have revealed the presence of five PP2A catalytic subunits in the rice genome (*Os02g0217600*, *Os03g0167700*, *Os3g0805300*, *Os06g0574500* and *Os10g0410600*) and a single PP2A A regulatory subunit (*Os09g0249700*) (Dudits et al., 2011).



**Figure 5. Structure of PP2A.**

C is the catalytic subunit, A is the scaffolding subunit, and B/B'/B''/B''' represent variable subunits, which are structurally unrelated. (Janssens and Goris, 2001)

#### 1.4.2. The role of the PP2A regulatory subunits

Two striking features of the B subunits are their diversity, stemming from the existence of entire subunit families, and the lack of sequence similarity between these gene families, even though they recognize similar segments of the A subunit (Janssens and Goris, 2001).

As mentioned above, B subunits are classified into four families. Members of the four unrelated protein families have been identified in different holoenzyme complexes. It has been speculated that each PP2A holoenzyme serves a distinct function (Lechward et al., 2001). In plants, three distinct B-type protein families, named B, B', and B'', have been confirmed as components of PP2A holoenzymes so far (Matre et al., 2009).

Cegielska et al. (1994) demonstrated that the regulatory B subunits of PP2A play a role in the regulation of SV40 DNA replication in infected cells, each holoenzyme in its own way. The PR72/B'' containing holoenzyme activates the T antigen by dephosphorylating the serines 120 and 123, whereas PR55/B containing PP2A inhibits this process by dephosphorylating the p34cdc2 target site. Experiments from Sablina et al. (2010) implicated that not just one specific B regulatory subunit containing PP2A complex involved in the cell transformation induced by SV40 small t. A loss-of-function screen confirmed that four PP2A regulatory subunits (B56  $\alpha$ , B56  $\gamma$ , PR72/PR130 and PTPA) participated that process. If suppressed, the first three regulatory subunits containing PP2A complexes failed to dephosphorylate c-Myc, Wnt and PI3K/Akt adequately and hence activated the pathways regulated by them, cell transformation events up-regulated. But the suppression of PTPA made PP2A heterotrimeric complex unable to assemble, which inhibited PP2A phosphatase activity stronger. PP2A-B55 $\alpha$  plays a major role in restricting the phosphorylation state of p107 and inducing its activation in human cells (Jayadeva et al., 2010). Another member of this family, PR59B'', was found to bind to and dephosphorylate p107 after UV-irradiation (Voorhoeve et al., 1999a). Studies revealed that the PR59-containing PP2A heterotrimer holoenzyme could dephosphorylate p107 *in vivo*. Over-expression of the PR59 regulatory subunit in human osteosarcoma cells demonstrated the inhibition of cell-cycle progression and accumulation of the cells in G1 phase (Voorhoeve et al., 1999b). The loss of the G5PR subunit was shown to lead to B cell receptor-induced apoptosis, demonstrating the importance of G5PR for B cell survival (Xing et al., 2005). PR70, which also belongs to the B'' family, has been demonstrated to bind to pRB and mediates its dephosphorylation (Magenta et al., 2008). PR48 localized in the nucleus and was found to interact with CDC6, a human replication initiator protein (Yan et al., 2000). Based on the experimental data, it may be presumed that the PR48/59/72/130/G5PR/B'' family may be involved in the regulation of the G1/S transition (Lechward et al., 2001). In *Drosophila*, PP2A participate several processes of the cell cycle, including

spindle bipolarity, kinetochore function and anaphase entrance, but the specific B subunit in heterotrimeric complexes is different (Chen et al, 2007) Remarkably, all the known B'' family members contain a  $\text{Ca}^{2+}$  binding EF-hand domain which regulates the binding of these proteins to the core enzyme and determines the subsequent phosphatase activity.

The cDNA sequences of five *Arabidopsis* B'' genes (B'' $\alpha$ , B'' $\beta$ , B'' $\gamma$ , B'' $\delta$ , and B'' $\epsilon$ ) can be found in GenBank database now. Two of them, B'' $\alpha$  and B'' $\beta$ , have been identified as the EF-hand type  $\text{Ca}^{2+}$  binding proteins (Leivar et al., 2011). Sequence analysis of the OsPP2A B'' regulatory protein (encoded by the *Os10g0476600* gene) revealed that it contains EF-hand domains that can potentially regulate the function of this protein via  $\text{Ca}^{2+}$ -binding (Yu et al., 2014).

### **1.4.3. Post-translational regulation of B regulatory subunit**

The ability of PP2A to dephosphorylate specific intracellular proteins depends on its activity and appropriate intracellular localization, which in turn is largely determined by a diversity of regulatory subunits and several levels of post-translational modification. Up till now little is known about post-translational modification of the regulatory subunits of PP2A. The regulatory subunits B56 $\alpha$ , B56 $\beta$ , B56 $\gamma$ 3, B56 $\delta$ , and B56 $\epsilon$  are phosphoproteins in intact cells (McCright et al., 1996). A 74 kDa B'' regulatory subunit is phosphorylated by Protein Kinase A (PKA) *in vitro*. Phosphorylation of this B'' subunit does not affect interaction with the PP2A core dimer, but does stimulate activity toward two general phosphatase substrates (Usui et al., 1998). B56 $\alpha$  is phosphorylated by double-stranded RNA-dependent protein kinase (PKR) (Xu and Williams, 2000). Similar to 74 kDa B'' subunit, phosphorylation of B56 $\alpha$  does not affect interaction with the PP2A dimer. Other reports indicate that phosphorylation of Ser566 in the B56 $\delta$  subunit by PKC is solely responsible for PP2A activity (Ahn et al., 2007a; Ahn et al., 2011).

Even less is known about B regulatory subunits in plants. Terol et al. (2002) identified five B' isoforms, which showed a high degree of conservation in sequence and structure with respect to the *Arabidopsis* B' subunit genes and proteins. Matre et al. (2009) reported the differential subcellular targeting within the *Arabidopsis* B' $\eta$  subfamily.

## 2. AIMS OF STUDY

The retinoblastoma tumor suppressor gene encodes a nuclear phosphoprotein (pRB) that regulates the G1/S transition of the cell cycle. The activity of pRB is modulated by phosphorylation/dephosphorylation cycles on serine and threonine residues. The phosphorylation of pRB is well documented, whereas, in contrast, little is known about the way in which phosphates are removed from the pocket proteins during the cell cycle. Two phosphatases, PP1 and PP2A, are involved in the dephosphorylation of the pocket proteins in mammalian cells. But up till now not a single report has been published on plant phosphatases responsible for the dephosphorylation of phospho-RBR.

The OsPP2A B'' regulatory subunit, which we identified earlier on the basis of yeast two-hybrid screens, showed a strong association with OsRBR1 but did not interact with OsRBR2. The PP2A protein phosphatase complex containing the identified B'' regulatory subunit may play an important role in the dephosphorylation of the rice RBR proteins during cell cycle progression and/or in response to extracellular stimuli. The main aims of our work were the following:

- 1) Measure the cell cycle phase-dependent variation in the amounts of the phospho-MsRBR1 and the phospho-OsRBR proteins, which helps to understand the differences in the phosphorylation of RBR proteins in monocots and dicots.
- 2) To analyse the interaction between OsRBR1 and the OsPP2A B'' regulatory proteins *in vivo* and *in vitro*.
- 3) To understand which domains in both OsRBR1 and the OsPP2A B'' regulatory proteins are important for their interaction.
- 4) Based on the results of the computer-assisted phosphorylation site analysis, to study the effect of phosphorylation on the function of the OsPP2A B'' regulatory protein:
  - identify the phosphorylated amino acid residues;
  - study the effect of phosphorylation on OsRBR1 binding and phosphatase
  - phosphatase activity using non-phosphorylatable and phosphorylation-mimicking mutants.

### 3. RESOURCES AND METHODS

#### 3.1. The yeast two-hybrid system

##### 3.1.1. Construction of *Oryza sativa* var. *japonica* cv. UNGGI-9 cDNA libraries for yeast two hybrid screening

The cDNAs for cDNA library construction were obtained from the total RNA of rice cell suspensions and rice leaves by using the TRI reagent method (Chomczynski and Sacchi, 1987) optimized for plant RNA extraction. The rice cell suspensions were grown in the culture medium according to Li and Murai (1990). The leaves were collected from greenhouse-grown whole rice plants. The tissues were frozen in liquid nitrogen and stored at -80°C before RNA extraction.

RNA was quantified by means of spectrophotometric OD<sub>260</sub> measurements, and the quality was assessed by calculating the ratio of OD<sub>260</sub>/OD<sub>280</sub> and by electrophoresis on 1% formaldehyde agarose gels followed by ethidium bromide staining. RNA samples were stored at -80°C.

The cDNA Synthesis Kit provided the reagents required to convert mRNA into cDNA inserts prior to unidirectional insertion into the HybriZAP-2.1 vector. The protocols for preparing cDNA inserts were found in the section *cDNA Insert Preparation* of the Instruction manual (Stratagene Company, La Jolla, California, USA).

The cDNA inserts were ligated into the HybriZAP-2.1 expression vector between the Xho I and EcoR I restriction sites. For titering the packaging reaction and amplifying the HybriZAP-2.1 Library, XL1-Blue MRF' cells were used. For the amplification of the cDNA libraries following *in vivo* mass excision of the pADGal4-2.1 phagemid vector from the HybriZAP-2.1 vector, the non-suppressive *E. coli* strain XL0LR was used instead of XL1-Blue MRF'. At the same time, the average length of cDNA fragments contained in the libraries was checked. A pair of AD primers was used (Table 1).

For a final quality control test we selected eighteen to twenty colonies from each library (leaves and suspension) to make colony PCR. The results showed that in the case of the suspension culture library, zero out of twenty colonies was empty and for the leaf cDNA library there were two empty clones out of eighteen. Digestion experiments told us that the average insert size in these two cDNA libraries was around 1000 bps.

### **3.1.2. Plasmid construction for yeast two-hybrid experiments and for recombinant protein production**

In all experiments, the full length coding regions of the three genes (OsRBR1, OsRBR2, OsPP2A B'' regulatory subunit) were used as template to generate different types of mutations. OsRBR1 cloned into pGBT9, using the BamHI and SalI restriction sites, was used to generate deletion mutants (N-terminal region; N plus A domain; N plus A domain and link region; N plus whole pocket domain), except for OsRBR1 A+L+B domain which was used as a pGBT9 (+2) construct. The mutated fragments were also ligated into pET-28a and pGEX-4T1 protein expression vectors. OsRBR2 was used to generate the N-terminal and C-terminal fragments which were inserted into pBD-GAL4, pET-28a and pGEX-4T1 using the EcoRI and SalI restriction sites. There were deletion mutations (full length minus one C terminal EF hand; a fragment containing the first two CDK kinase phosphorylation sites; a fragment that did not contain any CDK kinase phosphorylation sites) and site-directed mutations (three single mutants, three double mutants and one triple mutant containing alanine instead of serine or threonine and one triple mutant containing glutamic acid instead of serine or threonine) were generated from full length of the OsPP2A B'' regulatory subunit. The mutated fragments were introduced into pAD-Gal4, pET-28a and pGEX-4T1 using the EcoRI and SalI restriction sites. In some experiments we use the pGAD424 or pGADT7 instead of pAD-Gal4 2.1. Plasmid construction for yeast two-hybrid experiments and for recombinant protein production is described detailly in table1 and 2 in Appendix. All the nucleotide sequences of the inserts were verified by sequencing (by the ABI 3100 Genetic Analyzer from Applied Biosystem, Foster City, California, USA). Plasmid DNA was purified according to a standard alkali lysis protocol described by Birnboim and Doly. (1979).

### **3.1.3. Yeast two-hybrid screens and pairwise interaction analysis.**

The *Saccharomyces cerevisiae* yeast strain PJ69-4a (James et al., 1996), which can eliminate nearly all false positives using simple plate assays, was used for all the yeast experiments. In yeast two-hybrid screens, after the transformation of the bait plasmids, the self activation properties of pGBT9 or pBD-Gal4/baits were assayed first. The ability of the colonies to grow on SD plates minus tryptophan, adenine and histidine was used to test these bait characteristics. Successful bait plasmids

do not induce the expression of the *lacZ* reporter, the *HIS3* and the *ADE2* selective marker genes; no colonies should grow on the selective plates. Once it has been shown that self activation does not exist, the screening procedure continues with the transformation of the produced cDNA libraries into a yeast strain containing the bait plasmids. The transformants were then plated onto SD plates (–Trp, –Leu, –His, –Ade) monitoring the activation of the *HIS3* and *ADE2* selective marker genes. The plasmids were isolated from the positive yeast clones and then transformed into the XL1-Blue MRF' strain. The following steps consisted of colony PCR (a pair of AD primers were used, Table 1), plasmid isolation, sequencing of the plasmids and a BLAST analysis using the NCBI nucleotide database to identify the possible interactors.

In the case of pairwise interaction analysis, the binding domain containing bait (without self-activation property) and the activation domain containing prey were co-transformed into yeast. The transformants were grown on –Trp, –Leu and –Trp, –Leu, –His, –Ade medium, allowing the monitoring of the transformation efficiency and the activation of the *HIS3* and *ADE2* reporter genes that demonstrate the interaction of the fusion proteins.

#### **3.1.4. Quantitation of $\beta$ -galactosidase activity in yeast extracts**

The protein-protein interaction strength was quantified by the  $\beta$ -galactosidase activity assay; the ONPG (o-nitrophenyl- $\beta$ -D-galactopyranoside) substrate was used (Bartel et al., 1993). Enzyme activity was determined according to Horváth et al. (1998) with minor modifications. Five ml cultures were grown overnight in non-selective medium (SD –Trp, –Leu). 1 ml of the culture was used for the optical density measurement; the other 4 ml of the culture were collected into 2 ml Eppendorf tubes, centrifuged (1 min, 12 000 rpm) and lysed by vortexing with 0.5 mm diameter glass beads. The samples were vortexed more than ten times, each time just 1min, because the samples had to be kept at a low temperature. After centrifugation (3 min, 12 000 rpm), the supernatants (lysates) were transferred into 1.5 ml Eppendorf tubes and 2  $\mu$ l of the lysate was used for a protein concentration measurement. The protein concentrations were determined by the method of Bradford (Bradford, 1976). The remaining lysate was divided into two aliquots; the  $\beta$ -galactosidase activity of the first aliquot was measured using ONPG substrate at 420 nm, whereas the second aliquot served as



a blank control for the OD measurement at 420 nm. The blank control was treated in the same way as measured samples, except that the substrate buffer (Z buffer plus  $\beta$ -mercaptoethanol, pH 7.0) lacked ONPG. The  $\beta$ -galactosidase activities, based on three independent measurements, were calculated from the OD values as described by Bartel et al. (1993).

### 3.2. Plant suspension culture synchronization (alfalfa and rice)

Alfalfa (*Medicago sativa* ssp.  *varia* genotype A2) cell suspension cultures were maintained by weekly subculturing in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). Synchronization of the cell cycle was started by a 1:4 dilution of a 7-day-old alfalfa suspension culture. After 3 days cells were treated with 10 mM hydroxyurea (HU) (Sigma, St Louis, MO, USA) for 36 h. The cells were then washed three times with pre-conditioned MS medium (taken from an A2 suspension culture of the same age after subculturing). The cells were then cultured further for synchronous growth in the original volume of pre-conditioned MS medium (Ayaydin et al., 2011). 2 h after washing the HU from the cells, samples were collected at the indicated time points for protein extraction, cytology, and flow cytometric analysis.

The rice cell suspensions (*Oryza sativa* var. *japonica* cv. UNGGI-9) were cultured and synchronized in the same way as *Medicago* cells.

### 3.3. Flow cytometry

For flow cytometric analysis, 1 ml of cell culture was filtered through Miracloth (EMD Millipore, Darmstadt, Germany), then the cells were chopped with a sharp razor blade in Galbraith's buffer (45 mM  $\text{MgCl}_2$ , 20 mM MOPS, 30 mM sodium citrate, 0.1% Triton X-100, pH 7.0) in 6 cm plastic Petri dishes on ice (Galbraith et al., 1993). Nuclei (in 1 ml of buffer) were filtered through 20  $\mu\text{m}$  sieves into 1.5 ml Eppendorf tubes. Nuclei were stained with propidium iodide (4  $\mu\text{g ml}^{-1}$ ). Nuclei ( $1 \times 10^4$ ) were used for flow cytometric determination of the relative DNA content with a FACSCalibur flow cytometer from Becton Dickinson (Franklin Lakes, NJ, USA). Cell cycle phase analysis was carried out by the ModFit-software, immunolocalization and confocal laser scanning microscopy.

### **3.4. Recombinant protein purification**

#### **3.4.1. GST- tagged fusion protein expression and purification**

The BL21 (DE3) strain was used for the glutathione-S-transferase (GST) fusion protein expression and purification. *E. coli* was grown at 22 °C (the temperature depended on the construct used: for plasmids containing full length *OsRBR1* and *OsRBR2* the *E. coli* cells were grown and induced at 16 °C, whereas for short fragment containing plasmids, such as ZmCKS, OsRBR1 and OsRBR2 C terminal region, were grown and induced at 37 °C). When the OD<sub>600</sub>-value attained 0.3-0.4, 0.2 mM IPTG was added. After 4h of induction, bacterial cultures were centrifuged (10 min, 4000 g) and the bacterial cells were washed with ice cold 1x PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4). Following centrifugation the bacterial pellet was resuspended in 1x PBS containing 2 mM PMSF, 1 mM DTT and lysosyme at final concentration of 100 µg/ml. After this, Triton X-100 was added to the solution to a final concentration of 0.25% (v/v), and cells were sonicated. The lysate was placed on a shaker for 20 min and kept at RT. Following a 15 min centrifugation (10.000 g) the clear supernatant was used for affinity purification. All steps were carried out at 4 °C, unless otherwise stated. The affinity purification of the GST-fusion proteins was carried out using the Glutathione Sepharose purification protocol according to the manufacturer's instructions (Amersham-Pharmacia Biotech AB). The protein concentrations were determined by the method of Bradford (Bradford, 1976). Purity of the GST-fusion proteins was checked by polyacrylamide gel electrophoresis (PAGE) on 10 % SDS-polyacrylamide gels (Laemmli 1970)

#### **3.4.2. His<sub>6</sub>-tagged protein expression and purification**

The BL21 (DE3) strain was also used for His-tagged fusion protein expression and purification. For bacterial growth, induction and purification the same methods were used as in GST-tagged protein purification, although with different buffers: native wash buffer (50mM Na<sub>3</sub>PO<sub>4</sub> , 300mM NaCl, 20mM imidazole, 10% glycerol, pH 8.0) and elution buffer (50mM Na<sub>3</sub>PO<sub>4</sub>, 300mM NaCl, 250mM imidazole, 10% glycerol, pH 8.0). NiNTA agarose (80-90µl 50% slurry/200ml bacterial culture) was used instead of Glutathione Sepharose. For proteins used in the

phosphorylation experiment, the elutions were dialyzed against MgCl<sub>2</sub>-free kinase buffer (50mM Tris pH7.5, 5mM EGTA, 0.1% NP40, 1mM DTT). The protein concentrations were again determined by the method of Bradford (Bradford, 1976). Purity of the His-tagged fusion proteins was checked by PAGE on 10 % SDS-polyacrylamide gels too.

### **3.5. Pull down assays**

Total protein extracts were made from *Oryza sativa ssp. Japonica* cv. ‘Unggi 9’ cultured cells 5 days after subculturing using IP buffer. In a pull down assay 2mg of total protein was used.

For GST-pull down assays, recombinant GST-OsPP2A B” and GST (used as negative control) were produced in *E. coli* BL21 (DE3) strain and subjected to affinity purification on glutathione-sepharose (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Glutathione-Sepharose-bound OsPP2A B” served as affinity matrix to isolate putative interacting proteins. Protein extracts were added to beads and incubated for 90 min at 4°C with constant agitation. Beads were washed 3 times with extraction buffer. Bound proteins were eluted with buffer containing 50mM Tris-HCl pH8, 5% glycerol and 10mM reduced glutathione. Protein samples were separated on 8% SDS-polyacrylamide gel, blotted onto PVDF membrane (Immobilon-P, Merck Millipore, Billerica, MA, USA) and visualized with anti-OsRBR1 monoclonal antibody.

For His<sub>6</sub>-pull down assays, His<sub>6</sub>-tagged proteins were affinity purified on Ni-NTA-agarose (Qiagen Inc., Valencia, CA, USA). Rice cell extracts (negative control) or Ni-NTA-bound wild type- or mutant OsPP2A B” affinity matrices, incubated and eluted as before. Immunoblotting was performed with monoclonal anti-OsRBR1 or anti-PP2Ac antibody, respectively.

### **3.6. Antibodies and Western blotting**

40µg of total protein from synchronized suspension cultures was loaded on a denaturing polyacrylamide gel, and then blotted onto Immobilon-P PVDF membranes (Merck Millipore, Billerica, MA, USA). The chicken polyclonal anti-AtRBR1 antibody was used for the detection of the MsRBR11 protein. A mouse monoclonal antibody raised against the N-terminal 419 amino acids

of OsRBR1 was used for the detection of the OsRBR1 protein. For the production of this antibody affinity purified recombinant protein was used as antigen to immunize Balb/c.

Polyclonal antibodies (Phospho-RB (Ser807/811) Antibody #9308, Cell Signaling Technology, Beverly, UK) produced against a phosphopeptide corresponding to residues around Ser807/811 of human RB protein(based on SwissPro database, accession # P06400) were used according to the manufacturer's instructions to monitor the phospho-MsRBR1 and phospho-OsRBR1 proteins level. Commercially available  $\alpha$ - PP2A mAb (clone 1D6, Merck Millipore, Billerica, MA, USA) was used for the detection of the C subunit of OsPP2A and  $\alpha$  - PSTAIRE mouse mAb (clone PSTAIR ascites fluid, P7962, Sigma-Aldrich, St. Louis, MO, USA) to detect PSTAIRE motif containing kinases.

To generate polyclonal antibody against OsPP2A B'' subunit, the coding region of *Os10g047660 cDNA* was amplified from the AK067811 plasmid (Rice Genome Project of the National Institute of Agrobiological Sciences, Japan) with primers OsPP2A FL-EcoFw and OsPP2A FL-SalIRev (table 1 in Appendix). The amplicon was digested with EcoRI and SalI and ligated into pET28a vector (EMD Millipore, Billerica, MA, USA). The recombinant protein with an N-terminal hexahistidine tag was expressed in *E. coli* BLR strain (EMD Millipore, Billerica, MA, USA). Purification of His-tagged OsPP2A B'' on a Ni-NTA agarose column (Qiagen Inc., Valencia, CA, USA) resulted in a homogenous protein band of expected size in SDS-PAGE. The immunization of rabbits was carried out as described earlier (Kokai et al., 2006). Anti-OsPP2A B'' sera were prepared 14 days after the last injection and were purified on an NHS-activated Sepharose (GE Healthcare Bio-Sciences, Pittsburg, PA, USA) coupled polypeptide affinity column.

### **3.7. Protein phosphorylation**

Rice suspension cells were harvested 4 days after subculturing and ground in liquid N<sub>2</sub>. The powder was transferred to 2ml Eppendorf tubes and stored at -80 °C. Whole cell protein was extracted with buffer prepared according to Magyar et al. (1997). This buffer contains 25 mM TRIS-HCl pH 7.6, 15 mM MgCl<sub>2</sub>, 15 mM EGTA, 75 mM NaCl, 60 mM  $\beta$ -glycerophosphate, 1 mM DTT, 0.1% NP-40, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 1 mM PMSF, protease inhibitors (Complete, Roche,

Mannheim, Germany), and the PhosSTOP phosphatase inhibitor cocktail (Roche, Mannheim, Germany). The homogenate was centrifuged at 15 000g for 15 min at 4 °C. In the meantime, freshly prepared ZmCKS1 agarose (ZmCKS1-coated Glutathione Sepharose) was equilibrated with CKS1 wash buffer (50 mM TRIS-HCl pH 7.5, 250 mM NaCl, 5 mM EGTA, 5 mM EDTA, 0.1% NP-40, 2.5 mM NaF) and kinase buffer (50mM Tris pH7.5, 15mM MgCl<sub>2</sub>, 5mM EGTA, 0.1% NP40, 1mM DTT). The clear lysates were transferred into ZmCKS1 agarose, and the sample was incubated at 4 °C for 2h while shaken at a constant rotation rate. Subsequently, the samples were centrifuged, the supernatant was removed and the kinase reaction mixture was added. The mixture consisted of the recombinant protein (1-5µg), 0.5µl 10mM ATP, 0.5µl fresh <sup>32</sup>P-γ-ATP, and kinase buffer was added to a total of 50µl. During the incubation of the ZmCKS1 agarose at 30 °C for 2h, the beads were mixed carefully in every half hour. Subsequently, the mix was centrifuged and the supernatant was pipetted into 1.5ml Eppendorf tubes. The liquid was heated to 90 °C for 15 min. The subsequent steps were the following: SDS-polyacrylamide gel electrophoresis, drying of the gel for 2h at 80 °C, the placement of the dried gel into the PhosphorImager cassette, and the scanning of the gel using a Phosphor-Imager SI system (Molecular Dynamics, Sunnyvale, USA).

### **3.8. Immunoprecipitation**

Immunoprecipitations (IPs) were carried out using magnetic beads (Dynabeads<sup>R</sup>, Thermo Fisher Scientific Inc., Waltham, MA, USA) coated with Protein G. Polyclonal antibody raised against OsPP2A B'' was used to immunoprecipitate a functional PP2A phosphatase complex. Rabbit anti-mouse IgG (Sigma-Aldrich, MO, St. Louis, USA) served as negative control. 20 µg of antibody was diluted in 500 µl of PBS (phosphate buffered saline), mixed with prewashed magnetic beads and incubated while gently mixing for 40 min at room temperature. Unbound proteins were removed by five washing with PBS. Dynabeads<sup>R</sup> Protein G-immunoglobulin complex was mixed with total protein extract prepared from a rice cell suspension culture (that was in the exponential growth phase) in IP buffer (20 mM Tris-HCl (pH 7.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% 2-mercapto-ethanol and protease inhibitor cocktail (Complete, Roche, Basel, Switzerland). Cleared cell lysate was added to Dynabeads<sup>R</sup>-Ig and incubated for 1 h at 4°C while mixing constantly.

Unbound proteins were removed by washing with IP buffer 4 times. 50 mM glycine (pH 2.5) was used to elute the bound proteins (eluted samples were immediately neutralized).

### 3.9. Protein phosphatase activity

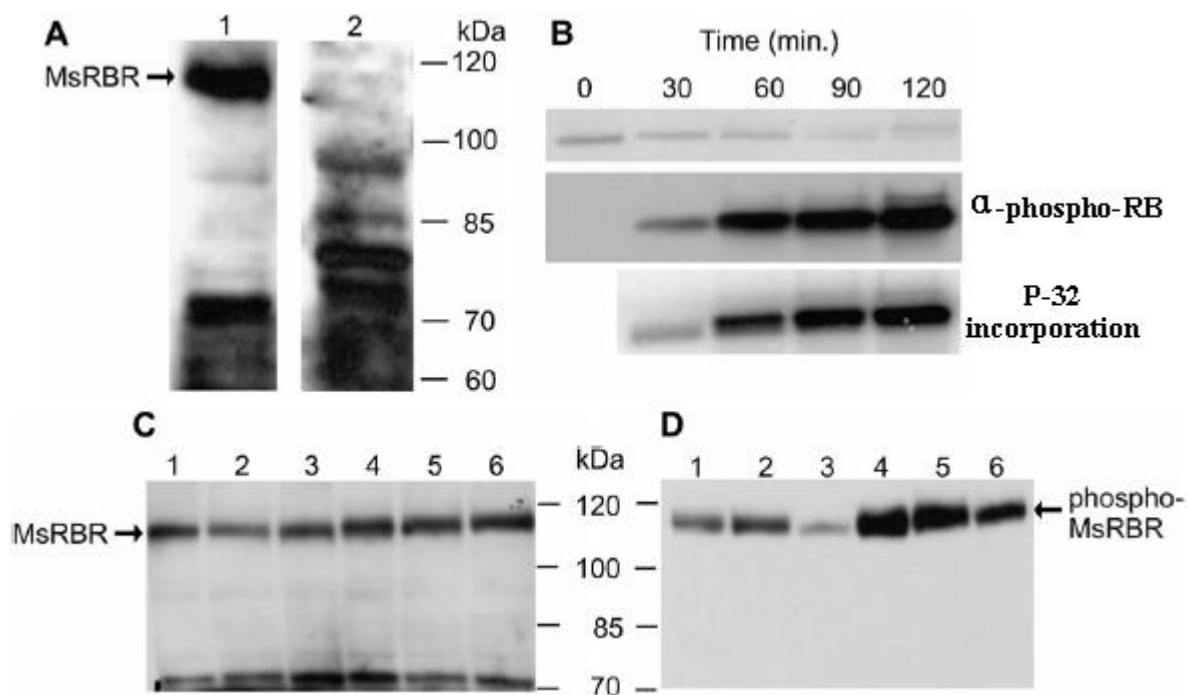
The functional PP2A phosphatase complex was isolated by a pull down technique. Total protein extract was prepared from an exponential growth phase rice cell suspension culture using IP buffer containing protease inhibitor cocktail (Complete, Roche, Basel, Switzerland). Cleared cell lysate was added to Ni-NTA agarose-bound recombinant. Wild type or mutated His<sub>6</sub>-OsPP2A B'' proteins incubated for 1 h at 4°C while mixing continuously. Ni-NTA agarose was also incubated with protein extract only (negative control). Beads were washed 4 times with extraction buffer and once with phosphatase buffer (50 mM Tris-HCl pH 7.5, 0.1% β-mercaptoethanol, 2mM MnCl<sub>2</sub>, complete protease inhibitor cocktail) and were used immediately for the phosphatase assay. Recombinant purified full length GST-OsRBR1 protein was *in vitro* phosphorylated using P13<sup>SUC1</sup>-bound CDK complex and used as phosphatase substrate. An equal amount of phosphatase buffer containing 2 μg phospho-OsRBR1 and 5μg of BSA was added to the beads and incubated at 37 °C while mixing constantly. Aliquots were taken at the indicated time points and used for SDS-PAGE (8 % gel). Proteins were blotted onto PVDF membrane (Immobilon-P, Merck Millipore, Billerica, MA, USA) and the phosphorylation status of OsRBR1 was followed by using anti-Phospho-Rb antibody (Cell Signaling Technology, Beverly, UK).

## 4. RESULTS

### 4.1. Immunodetection of MsRBR1 and phospho-MsRBR1 proteins in cultured alfalfa cells

A polyclonal antibody was produced against the recombinant C-terminal fragment of the Arabidopsis AtRBR1 protein consisting of 236 amino acids (Horváth et al., 2006). The cross-reactivity of this anti-AtRBR1 antibody with the His-tagged C-terminal fragment of the MsRBR1 protein was tested. This antibody recognized even a low amount (5 ng) of recombinant protein and a 115 kDa protein was detected as a major band in Western blots of total protein extract (**Fig. 6A**, lane 1). The 115 kDa protein band corresponds to the predicted molecular mass (114.2 kDa) of the alfalfa RBR protein. The specificity of this cross-reaction was tested using an antigen competition assay. Pre-incubation of this antibody with the purified His<sub>6</sub>-tagged C-terminal part of the MsRBR1 protein removed the cross-reacting antibody and resulted in the disappearance of the 115 kDa band (**Fig. 6A**, lane 2). This finding supports the use of this antibody in the alfalfa experimental system. Since the phosphorylation status of the MsRBR1 protein has functional significance, the level of this phosphoprotein was monitored using an antibody produced against the phosphorylated pRB peptide containing Ser807/811 (Cell Signaling Technology). The NVYVSPLRS motif in the C-terminus of the alfalfa MsRBR1 protein may correspond to the NIYISPLKS motif of the human pRB protein which has a conserved 807/811serine (S) phosphorylation site. In order to test the specificity of this peptide antibody, the recombinant His<sub>6</sub>-tagged C-terminal fragment of the MsRBR1 protein was phosphorylated using alfalfa CDK complex bound to GST-p13<sup>SUC1</sup> beads (**Fig. 6B**, middle panel). The phosphorylation reaction was also monitored by detection of incorporated <sup>32</sup>P (**Fig. 6B**, lower panel). The detected level of <sup>32</sup>P incorporation and the signal given by the anti-phospho-RB antibody correlate and this allowed us to use the antibody to monitor the changes in the level of phospho-MsRBR1 protein.

Using whole-cell extracts, both antibodies produced against either the AtRBR1 protein or phospho-pRB peptide recognized the same 115 kDa size protein (**Fig. 6C, D**). The sensitivity of the band corresponding to the phospho-MsRBR1 protein to a calf intestinal alkaline phosphatase (CIAP) treatment is also demonstrated. Dephosphorylation reduced the amount of detected protein cross-reacting with the anti-phospho-pRB antibody (**Fig. 6D**, lanes 3 and 6).



**Figure 6. Specificity test of antibodies used for detection of the Medicago retinoblastoma-related protein (MsRBR1) and its phosphorylated form (phospho-MsRBR1) in cells at stationary or exponential growing phase.**

(A): Antigen competition assay for anti-AtRBR1 antibody: 1, Western blot of total protein extract with anti-AtRBR1 antibody detected the MsRBR1 protein (115kDa); 2, immunoblot of total protein extract with anti-AtRBR1 antibody pre-incubated with the purified His6-tagged C-terminal part of the MsRBR1 (115kDa). (B): Functional test of anti-human pRB phosphopeptide antibody by Western blot of the in vitro phosphorylated recombinant C-terminal fragment of MsRBR1 protein after incubation with p13SUC1-bound kinase complex. Upper panel, Ponceau S-stained filter used for immunoblot assay, loading control; middle panel, immunoblot with antibody produced against the phosphopeptide corresponding to residues around Ser807/811 of human pRB; lower panel, detection of incorporated [ $^{32}$ P] inorganic phosphate by the Phosphor Imager SI (Molecular Dynamics). (C): Alfalfa cells at exponential phase have an increased amount of the MsRBR1 protein in comparison with cells at stationary phase. (D): Western blots with the anti-human pRB phosphopeptide antibody detected reduced amounts of phospho-MsRBR1 protein after phosphatase treatment and showed significantly higher amount of phospho-MsRBR1 protein in cells at exponential growing phase. In both C and D, Lanes 1-3, protein extracts from 7-day-old cultures (stationary phase); lanes 4-6, protein extracts from 4-day-old cultures (Exponential phase); 1, 4, control cultures; 2, 5, protein extracts treated with phosphatase buffer; 3, 6, protein extracts with calf intestinal alkaline phosphatase (CIAP).

The results of these Western blots provide additional information on the accumulation of MsRBR1 and phospho-MsRBR1 proteins in cells in different growth phases of the A2 suspension culture. The frequency of dividing cells harvested 4 d after subculturing is high, because the suspension culture is in the exponential growth phase.

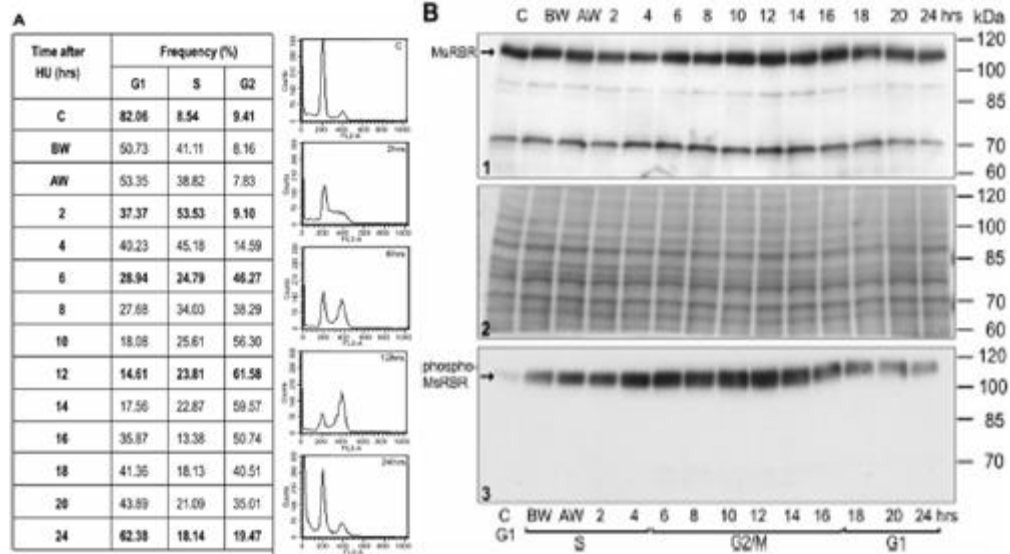


As shown in **Fig. 6C, D**, lanes 4–6 these cells contain higher amounts of both forms of the MsRBR1 protein than do cells harvested 7 d after subculturing representing the stationary growth phase (**Fig. 6C, D**, lanes 1–3). In particular, the elevated level of phospho-MsRBR1 protein in dividing cells is demonstrated by this experiment.

Careful tests using the antibody set discussed above have opened up a way to monitor the level of MsRBR1 and phospho-MsRBR1 proteins in synchronized alfalfa cells.

#### **4.2. Cell cycle phase-dependent variations in MsRBR1 and phospho-MsRBR1 protein levels**

Rapidly growing suspension cultures are a suitable system to follow cell cycle-associated changes in most biochemical or cytological parameters; because large numbers of cells are grown in an aqueous environment under well-defined conditions, which can be exposed uniformly to synchronizing treatments. Ayaydin et al. (2011) described an efficient *Medicago sativa* cell suspension culture synchronization procedure. Hydroxyurea (HU) was used for the synchronization treatment and the ethynyl deoxyuridine (EdU)-based fluorescent staining method was used for the detection of cells in S phase. In our experiment, 10mM HU was used for synchronization. As shown by representative profiles and frequency data from flow cytometry in **Fig. 7A** the control culture represents mostly G1 cells (82%). Alfalfa cells were exposed to HU for 36 h and then the inhibitor was removed; subsequently, the cells were grown under standard conditions. A sharp increase of fraction of S phase cells following HU removal clearly demonstrated that the chemical block is reversible and that the cells re-start their DNA synthesis at the same time following the removal of the inhibitor. The S phase cells increased from 38% to 53% in two hours (**Fig. 7A**). After 4 hours, most of the cells went into the G2/M phase. The maximum number of G2/M cells could be detected in samples from cultures collected after 10–16 h (56, 61, 59, and 50%). The frequency of G1 cells increased after 16 h, indicating the initiation of a new cycle. A Western blot of total protein extracts detected the 115 kDa full size MsRBR1 protein in all samples (**Fig. 7B**, lane 1).

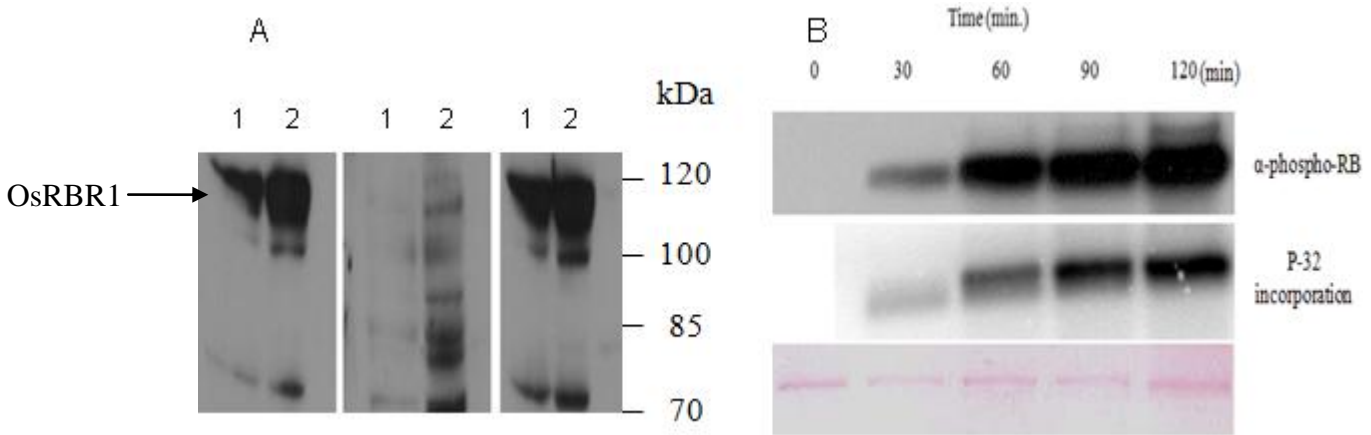


**Figure 7. Variation in the amounts of the MsRBR1 and the phospho- MsRBR1 proteins in alfalfa cells during cell cycle progression after synchronization with 10mM hydroxyurea (HU) treatment.**

(A) Frequency data of G1, S, and G2 cells at various time points after removal of the inhibitor (HU) with characteristic DNA histograms from flow cytometry. (B) Western blot of protein extracts by anti-AtrRBR1 antibody (1) and anti-human phospho-pRb peptide antibody (3). Ponceau S-stained filter with 50μg protein samples (2). C, control, non-synchronized, actively dividing culture; BW, before wash out HU; AW, after wash out HU.

The amounts of cross-reacting MsRBR1 protein showed limited variation during cell cycle progression. Interestingly, the G2/M cells had slightly elevated levels of this protein. After 16 h the blot showed a gradual decrease of the MsRBR1 protein level, this is in good agreement with the increasing frequency of G1 cells. Western blotting of the same protein samples with anti-phospho-pRb antibody indicated a low protein phosphorylation level in the control culture with 82% G1 cells (**Fig. 7B**, lower panel). The phosphoprotein level was elevated in samples containing S phase cells (53% and 45%). The protein cross-reacting with antibodies against the phospho-pRB peptide was found to be abundant in protein extracts isolated from cell populations representing a significant frequency (56, 61, and 59%) of G2/M phase cells. Subsequently the phospho-MsRBR1 protein level was reduced in samples containing more G1 cells. In this experiment both antibodies cross-reacted with the full size MsRBR1 (or phospho-MsRBR1) protein of 115 kDa. Both forms of the MsRBR1 protein are present in all cell cycle phases, but the phospho-MsRBR1 protein exhibits higher variation between cell cycle phases.

### 4.3. Cell cycle phase-dependent phosphorylation and dephosphorylation of OsRBR1



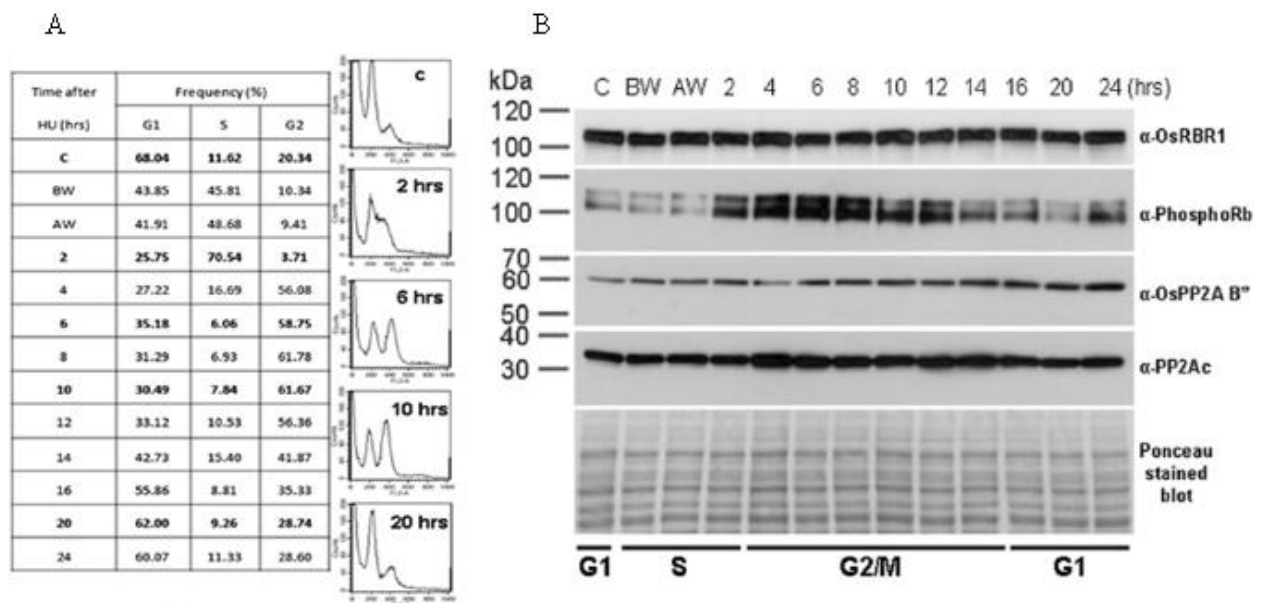
**Figure 8. Specificity test of antibodies used for detection of the rice retinoblastoma-related protein 1 (OsRBR1) and its phosphorylated form (phospho-OsRBR1).**

(A) Antigen competition assay for the anti-OsRBR1 antibody. Left panel, no competitor; middle panel, competitor: OsRBR1 antigen; right panel, competitor: OsRBR2 antigen. 1, 20µg total protein extracts from cell suspension; 2, 40 µg total protein extract from cell suspension. An immunoblot of total protein extract with anti-OsRBR1 antibody pre-incubated with the purified His6-tagged N-terminal part of the OsRBR1 protein failed to detect the OsRBR1 protein (middle panel). (B) Test of anti-human pRB phosphopeptide antibody by Western blot of the in vitro phosphorylated recombinant full size OsRBR1 protein after incubation with the p13SUC1-bound kinase complex. upper panel, immunoblot with antibody produced against a phosphopeptide corresponding to residues around Ser807/811 of human pRB; middle panel, detection of incorporated [32P] phosphate by Phosphor Imager SI (Molecular Dynamics); lower panel, Ponceau stained filter used for immunoblot assay, loading control.

A monoclonal antibody was produced against the recombinant N-terminal 419 amino acids of the OsRBR1 protein, an approximately 112 kDa protein was detected as a major band in Western blots of total protein extract (**Fig. 8A**, left panel), which agrees with the calculated 111.5 kDa molecular weight of the OsRBR1 protein. The specificity of this antibody was tested using an antigen competition assay. Pre-incubation of this antibody with the purified His<sub>6</sub>-tagged N-terminal part of the OsRBR1 protein removed the cross-reacting antibody and resulted in the out-titration of the band (**Fig.8A**, middle panel). This antibody recognized recombinant OsRBR1 but not the OsRBR2 protein (**Fig. 8A**, right panel). The phosphoprotein level was monitored as was done in the previous section by immunoblotting with an antibody produced against the phosphorylated pRB peptide containing Ser807/811. The conserved 807/811 serine (S) phosphorylation site in the NIYISPLKS motif of the human pRB protein may equate the NVYVSPLRTS motif of OsRBR1, which is located within the

C-terminal region of this protein. In order to test the specificity of this peptide antibody, the recombinant His<sub>6</sub>-tagged OsRBR1 protein was phosphorylated using GST-p13<sup>SUC1</sup> beads bound CDK complex (**Fig. 8B**, upper panel). The phosphorylation reaction was also monitored by <sup>32</sup>P incorporation (**Fig. 8B**, middle panel). The two approaches gave very similar results, thus the phospho-Rb antibody can be used in our experiments.

During the cell division cycle the OsRBR1 protein level was monitored in a synchronized rice cells (**Fig. 9A**). The suspension of actively dividing rice cells was subjected to 36 h hydroxyurea (HU) treatment. After removing the inhibitor, cells progressed through S phase with the highest frequency at 2 h after the release of the HU block (71%, **Fig. 9A**). Cultures collected after 4-14 h contained an elevated percentage of G2/M phase cells. The frequency of G1 cells increased after 14 h, indicating the initiation of a new cycle.



**Figure 9. Changes in the OsRBR1, phospho- OsRBR1, OsPP2A B''regulatory subunit, and OsPP2A catalytic subunit (PP2Ac) protein levels in rice suspension cells during cell cycle progression after synchronization with 10 mM hydroxyurea (HU) treatment.**

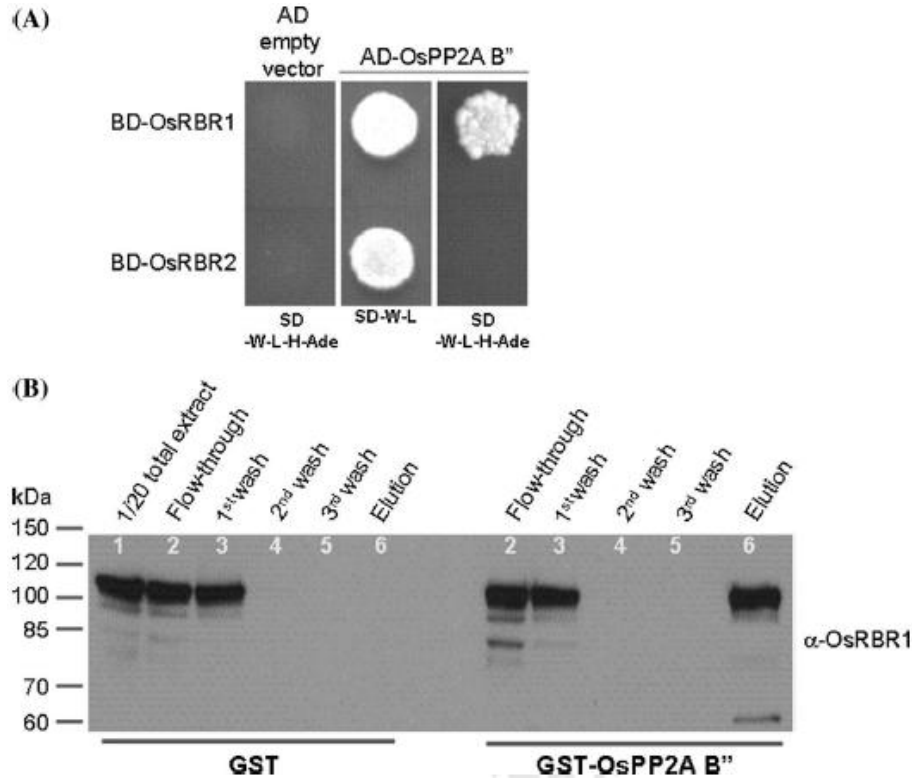
A: Distribution of G1, S, and G2 cells in the synchronized cell culture at various time points after removal of HU with characteristic DNA histograms obtained by flow cytometry. B: Western blot of rice suspension cell culture total protein extract probed with the monoclonal OsRBR1 antibody, phospho-RB peptide antibody, polyclonal OsPP2A B'' antibody and PP2A catalytic subunit monoclonal antibody. Aliquots of extracts containing 40 μg of total protein were loaded. C, control, asynchronous culture; BW, before washing out HU; AW, after washing out HU.

The Western blot of protein extracts (**Fig. 9B**) revealed that the amounts of OsRBR1 did not show significant variation during cell cycle progression while the amounts of phospho-OsRBR1 exhibited a quite different pattern. The phospho-OsRBR1 antibody detected two protein bands with a lower (at approximately 112-115kDa, the size of the OsRBR1) and a higher ( $\sim$ 118-120kDa) molecular weight (**Fig. 9B**). These may represent OsRBR1 proteins differing in their phosphorylation-status: hyperphosphorylated and hypophosphorylated. The Western blotting analysis (**Fig. 9B**) indicated a low protein phosphorylation level in the control culture containing mainly G1 cells. The ratio of phospho-OsRBR1 was elevated in samples containing a large population of S phase cells at 2 h, in these cells the level of the hyper- and hypophosphorylated forms was almost equal. The phosphorylation of OsRBR1 protein peaked at 6 h (G2 phase) and it remained high in samples of cells in the middle G2 phase (8 h). Subsequently, the level of phosphorylated OsRBR1 was reduced in samples containing more late G2/M-phase cells (12-14 h). When the majority of the cells re-entered into the G1 phase again (16-24 h), the phospho-OsRBR1 protein decreased into a very low level. These findings show that the OsRBR1 protein underwent dynamic phosphorylation and dephosphorylation during the cell cycle. Under the same synchronization conditions the levels of the PP2A regulatory B'' and catalytic subunits were also investigated. The results showed that the levels of the OsPP2A catalytic and B'' regulatory subunits changed only slightly throughout the whole cell cycle (**Fig. 9B**). However, it is still possible that the phosphatase activity was regulated by posttranslational modifications, e.g. by phosphorylation.

#### **4.4. Retinoblastoma-related protein OsRBR1 interacts with the rice phosphatase OsPP2A B'' regulatory subunit**

A *Medicago truncatula* interactor of full-length alfalfa MsRBR11 protein obtained from yeast two-hybrid screening – a homologue of the murine PR59 PP2A regulatory subunit (39% identity and 57% homology) – was demonstrated to interact strongly with OsRBR1 but did not associate with OsRBR2 (Lendvai et al., 2007). During our work we have isolated the rice homologue of the *MsPP2A B''* gene, *OsPP2A B''* (*Os10g0476600*) (74% identity and 80% similarity with the *Medicago B''* regulatory subunit). After cloning the OsPP2A B'' coding sequence into the pAD-Gal4 2.1 (yeast

two-hybrid active domain containing vector) we carried out pairwise interaction studies to verify the specific interaction between the OsPP2A B'' regulatory subunit and the OsRBR1 protein. As shown in **Fig. 10A** the OsPP2A B'' regulatory subunit interacted only with the OsRBR1 protein. (**Fig.10A**, right panel). Since the OsPP2A B'' subunit showed strong autoactivation when it was cloned in a DNA binding domain-containing vector, we could not perform the experiments in a reverse setup.



**Figure 10. OsPP2A B'' regulatory subunit associates with OsRBR1 specifically.**

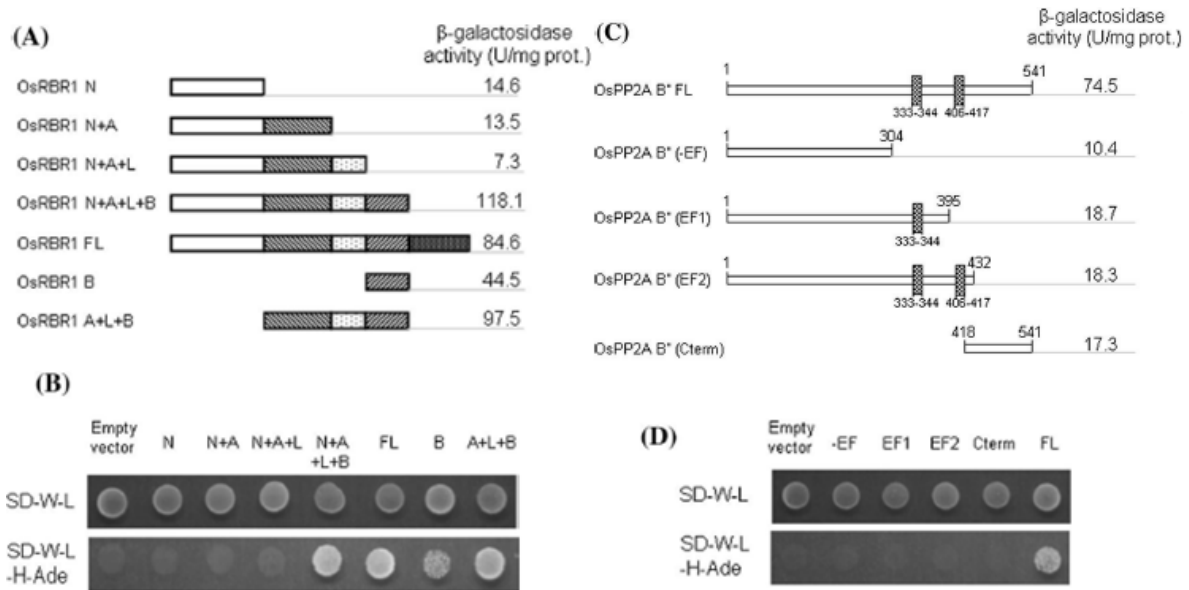
A: Pairwise yeast two hybrid assays. The empty vector (AD) or pAD-GAL4 2.1- OsPP2A B'' vector (AD-OsPP2A B'') were cotransformed with pBD-GAL4 2.1-OsRBR1 (BD-OsRBR1) or pBD-GAL4 2.1-OsRBR2 (BD-OsRBR2) to yeast strain PJ69-4A. Yeast cells were grown on synthetic dropout (SD) medium lacking tryptophan and leucine (SD-W-L) for testing transformation efficiency or SD lacking tryptophan, leucine, adanine, and histidine (SD-W-L-H-Ade) for testing protein-protein interactions. B: Pull down assays. GST-tagged OsPP2A B'' (right side) or GST (left side), each bound to glutathione-sepharose, were incubated with an extract of rice suspension cells. Lane1, loading control from the total protein extract (1/20 of the total amount). Lanes 2-5 show the elimination of weakly bound proteins and lanes 6 contain the eluted fractions. A monoclonal antibody against OsRBR1 was used to detect the OsRBR1 protein in the samples.

The interaction between the OsPP2A B'' and OsRBR1 proteins was also confirmed by pull down assays using a monoclonal antibody against OsRBR1 (**Fig. 10B**). Total protein extract of rice

cultured cells were incubated with GST coated (negative control) and GST-OsPP2A B'' coated glutathione-sepharose beads. After washing the beads three times with wash buffer; OsRBR1 was detected in the flow through and in the first wash fraction (**Fig. 10B**, lanes 2, 3) in both experiments. After elution, however, OsRBR1 was found only in the sample obtained from the GST-OsPP2A B'' coupled resin (lane 6) indicating the specific interaction between these two proteins. The results show that OsPP2A B'' can bind to OsRBR1 in vitro and suggest the B'' regulatory subunit may play a role in the dephosphorylation of retinoblastoma-related protein(s) in rice.

#### **4.5. The B pocket domain in OsRBR1 is sufficient, and the C-terminal region in OsPP2A B'' is indispensable for the interaction between those two proteins**

Similarly to the animal pocket proteins, plant RBRs contain an N-terminal region, A and B pocket domains in the so called small pocket, and a C-terminal region (**Fig. 11A**). To map the structural elements in that are required for OsPP2A B'' binding, stepwise truncations of OsRBR1 as well as deletion mutants containing the small pocket or the B domain were generated by in vitro mutagenesis (**Fig. 11A**, Table 1 and 2 in Appendix). Full and truncated OsRBR1 coding sequences were cloned into the pGBT9 vector while the full OsPP2A B'' coding sequence was inserted in the pGADT7 vector of the yeast two hybrid system. According to yeast colony growth assays (**Fig. 11B**) and  $\beta$ -galactosidase activity data (**Fig. 11A**), the A and B pocket domains of OsRBR1 are both required for a strong binding to OsPP2A B''. However, the B domain alone is sufficient for a moderate interaction, suggesting a predominant role of the second binding pocket in the docking to the regulatory subunit of PP2A phosphatase. The contribution of the N-terminal, spacer, and C-terminal regions of OsRBR1 is negligible in this context. In comparison, Jayadeva et al. (2010) observed that the animal PP2A-B55  $\alpha$  subunit binding site of p107 is located with the spacer region in between the two pocket domains, and the C-terminal region of p107 is not required for the association. On the other hand, the interaction between pRB and the catalytic subunit of PP1 occurs through the C-terminal region of pRB (Tamrakar and Ludlow, 2000; Bianchi and Villa-Moruzzi, 2001). The results suggest that the phosphatase binding to plant RB depends on different structural elements than in the case of animal pRB or p107 pocket proteins.



**Figure 11. Yeast two hybrid assays mapping the role of selected protein domains in the interaction between OsRBR1 and OsPP2A B''.**

A: Schematic representation of the OsRBR1 deletion mutants and their interaction strength with OsPP2A B'' measured as β-galactosidase activity. The structural elements of OsRBR1 are labeled as: N, N-terminal region (white); A, A pocket domain (left stripped); L, linker region between A and B domains (dotted); B, B pocket domain (right stripped), and the C-terminal region (squared). FL indicates full length OsRBR1. B: In the yeast two hybrid tests the wild type or truncated pGBT9-OsRBR1 coding vectors were combined with pGAD-Gal4-OsPP2A B'' and were tested as in **Fig. 10A**. The empty pGBT9 vector was used as background control. C: Schematic representation of full length (FL) OsPP2A B'' and its deletion mutants (-EF, EF1, EF2, and Cterm) cloned in pGADT7 vector, and their interaction strength with OsRBR1 measured as β-galactosidase activity. D: Yeast two hybrid interaction assays between wild type OsRBR1 and different OsPP2A B'' variants using vector combinations pBD-Gal4-OsRBR1 with pGADT7-OsPP2A B''. The media used for the selection of transformants in B and D are abbreviated as in **Fig.10A**.

Sequence comparisons of OsPP2A B'' with its human orthologs (Dovega et al. 2014) revealed the presence of two functional  $\text{Ca}^{2+}$ -binding EF-hand domains in the plant protein (**Fig. 11C**). The mutation of the second EF-hand close to the C-terminal region in animal PR72/B'' abolished heterotrimer formation, suggesting an important role for this EF-hand motif in the phosphatase holoenzyme (Janssens et al., 2003). Davis et al. (2008) reported that the C-terminal region of the B'' regulatory subunit is important in substrate binding. To test the role of the EF domains and the C-terminal region (residues 418-541) in the RBR binding we produced a series of deletion mutants of OsPP2A B'' in the PGADT7 prey vector of the yeast two hybrid system (**Fig.11C**). According to the yeast two hybrid tests, none of the truncated versions of OsPP2A B'' could interact with OsRBR1 (**Fig.11C, D**). Interestingly, while the inclusion of the C-terminal region to OsPP2A B'' restored the



interaction, the coiled C-terminal part of the protein alone was not able to associate with OsRBR1. Another experiment has verified that all prey protein species were produced in the yeast cell, the lack of interactions with the mutated proteins was not caused by the insufficient prey protein production or stability (data not show). The reverse vector combination was not informative because OsPP2A B'' fused on the DNA binding domain of Gal4 (expressed from pGBT9-OsPP2A B'') showed a remarkably strong self-activation. Thus the deletion of the C-terminal tail of the B'' regulatory subunit abolishes the RBR binding in all of the combinations tested, however alone this segment is not sufficient for the interaction. The data collectively support the notion that the C-terminal substrate binding region of OsPP2A B'' has an important function in recruiting OsRBR1 to the vicinity of the phosphatase catalytic subunit.

#### **4.6. OsPP2A B'' interacts with and is phosphorylated by cyclin-dependent kinase**

Up till now, we have very limited knowledge about the post-translational modifications of B regulatory subunit: these subunits are phosphoproteins; the phosphorylation of B'' do not affect the association with PP2A<sub>D</sub> heterodimer; the phosphorylation of B'' stimulates the activity of PP2A heterotrimeric holoenzyme (McCright et al., 1996; Usui et al., 1998; Xu and Williams, 2000).

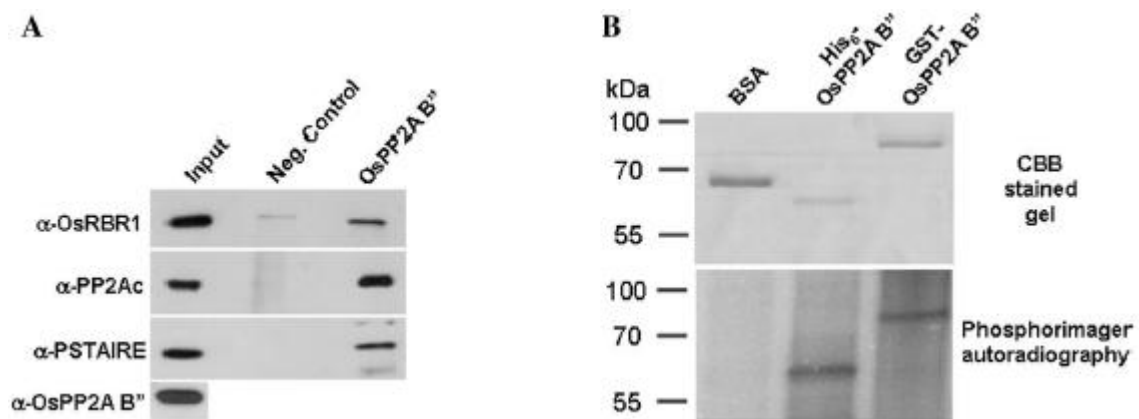
We were wondering if the homologue of the MsPP2A B'' regulatory subunit, OsPP2A B'', can be phosphorylated; and if it can be phosphorylated, which residues are phosphorylated, and whether phosphorylation affects interaction with the PP2A core dimer and OsRBR1. Furthermore, we posed the question if the absence of phospho-acceptors will influence the phosphatase activity of the PP2A holoenzyme. I will first answer if OsPP2A B'' can be phosphorylated or not.

Sequence analysis of OsPP2A B'' by the Scansite algorithm (<http://scansite.mit.edu/>) revealed the presence of three putative CDK phosphorylation sites in the N-terminal region of the protein. These are Ser95 in APPLSPRST, Ser102 in STSGSPRVM and Ser119 in SSLGSPLKV; all three in a sequence context similar to the canonical (S/T) PX(R/K) recognition site for CDKs.

The interaction of OsPP2A B'' with a CDK was experimentally substantiated as the polyclonal antibody against OsPP2A B'' immunoprecipitated a protein complex that harbors OsRBR1, the PP2A catalytic subunit (PP2Ac) and a PSTAIRE motif containing CDK (**Fig. 12A**),

Thus, the PP2A phosphatase holoenzyme and the PSTAIRE type protein kinase are in the same protein complex with their potential substrate OsRBR1. Another proof comes from a pull down assay which demonstrated that OsPP2A B'' and OsRBR1 are in association with the CDK (data not show). The functional significance of this interaction was revealed by the fact that CDK-cyclin complexes isolated from actively dividing rice cells in suspension culture by ZmCKS-agarose phosphorylate both GST- and His-tagged OsPP2A B'' (**Fig. 12B**).

Data from our lab demonstrated that B'' regulatory subunit associated with members of the subfamily II of OsPP2Ac catalytic subunit preferentially, while the OsPP2Ac isoforms here (**Fig. 12A**) could not be distinguished because of the narrow range of molecular weight (35.1-35.6) (Yu et al., 2014).

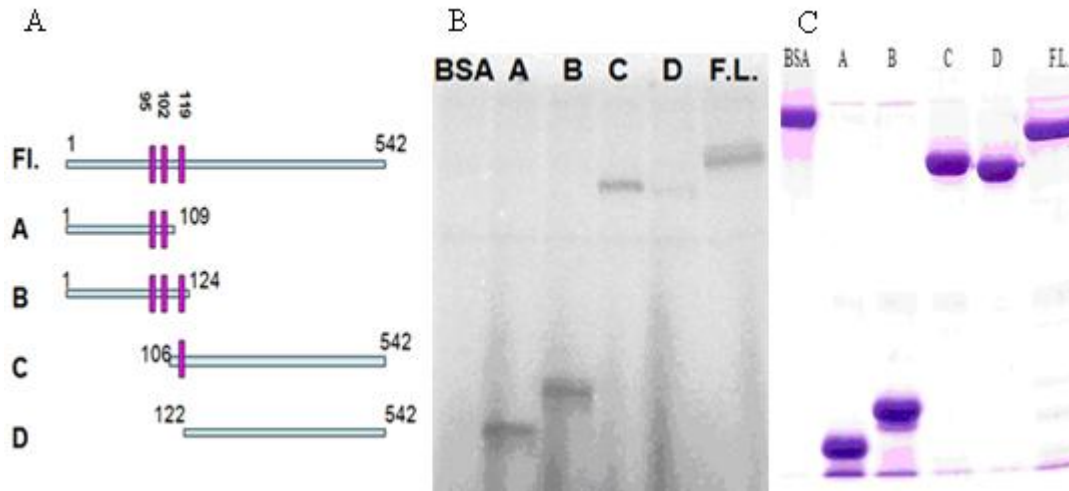


**Figure 12. OsPP2A B'' interacts with and is phosphorylated by cyclin-dependent kinase.**

(A) Co-immunoprecipitation of OsRBR1, PP2A catalytic subunit and PSTAIRE cyclin-dependent kinases by a polyclonal antibody raised against of OsPP2A B''. 1 mg of total protein extract prepared from a rice cell suspension culture (exponential growth phase) was used in the experiment and 40 $\mu$ g was loaded as input control. Anti-mouse IgG produced in rabbit served as negative control. Western blot was performed using monoclonal antibodies raised against OsRBR1, the catalytic subunit of PP2A and the PSTAIRE sequence. A Western blot that shows the level of OsPP2A B'' in 40 $\mu$ g total protein extract is also included. (B) Phosphorylation of OsPP2A B'' regulatory subunit with PSTAIRE-motif contained kinases in vitro. His<sub>6</sub>- and GST- tagged OsPP2A B'' was phosphorylated by a PSTAIRE CDK complex isolated from actively dividing rice suspension cells by p13SUC1 -agarose with  $\gamma$  [32P]-ATP. The upper panel shows the loading control stained for proteins with Coomassie brilliant blue R250 (CBB). In bottom panel, the phosphate incorporation was detected by autoradiography using phosphorImager SI system (Molecular Dynamics, Sunnyvale, USA).

#### 4.7. Confirmation of the putative phosphorylation sites of OsPP2A B'' regulatory subunit via site-directed mutagenesis and proteomic analysis

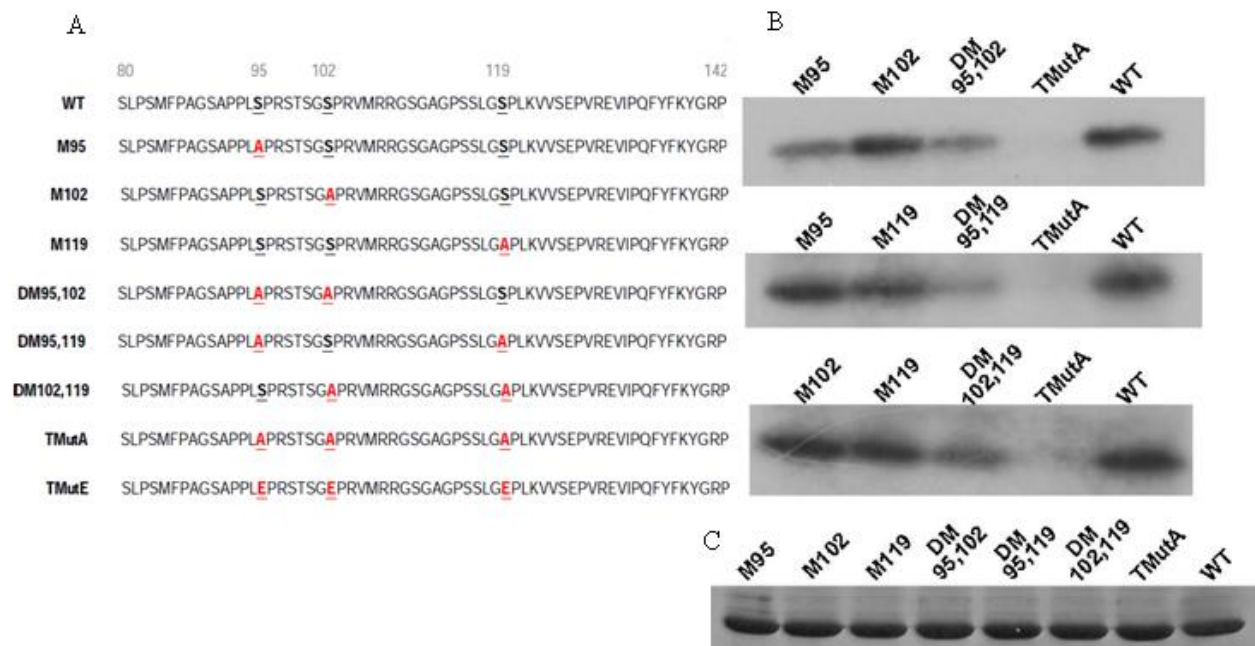
After that it has been clearly shown that the B'' regulatory subunit can be phosphorylated *in vitro* we can address the question if those putative CDK phosphorylation sites can really be phosphorylated. Data from collabotory verified the phosphorylation of Ser95 and Ser119 by LC-MS/MS analysis (**Fig. 18** in Appendix). Unfortunately, Ser102 was not represented by any unmodified or phosphorylated peptides in their experiment either.



**Figure 13. Confirmation of the putative OsPP2A B'' phosphorylation sites by deletion mutagenesis.** (A) Schematic representation of the predicted CDK phosphorylation sites Ser95, Ser102, and Ser119 in full length (F.L.). Fragment A contained Ser95 and Ser102, fragment B contained all three phosphorylation sites, Fragment C had only Ser119 and fragment D lacked all three putative phosphorylation sites. (B) The OsPP2A B'' regulatory subunit variants described in panel A were phosphorylated by rice PSTAIRE CDK-cyclin complex isolated by p13SUC1/CKS beads. After SDS-polyacrylamide gel electrophoresis, <sup>32</sup>P incorporation was detected by phosphorImager SI system (Molecular Dynamics, Sunnyvale, USA). BSA was used as the negative control in experiments. (C) Loading control of (B) panel, stained with CBB.

To check whether all of the putative sites, including Ser102, can be phosphorylated by CDKs and to be sure there are no more phosphorylation sites in the full size of OsPP2A B'' protein apart from those three predicted ones, we generated a set of truncations as well as phosphorylation-site mutations in His<sub>6</sub>-tagged OsPP2A B'' and used them as substrates for rice CDK. All mutants of the B'' regulatory subunit were produced by PCR (**Fig. 13A** and **Fig.14**, primers used in this experiment were listed in table 1 and table 2). Fragment A contained Ser95 and Ser102, fragment B contained all

three sites, fragment C contained only the third site, Ser119, while fragment D had no predicted phosphorylation site (**Fig. 13A**). The phosphorylation reaction was monitored by detection of  $^{32}\text{P}$  incorporation into the proteins after in vitro phosphorylation with radioactive ATP (**Fig. 13B**). Except for fragment D, all the truncated mutants were phosphorylated. These results mapped all phosphorylation sites to the N-terminal segment of the protein and confirmed the phosphorylation of Ser119 in fragment C. They also excluded the possibility that additional non-predictable phosphorylation sites were present in the C-terminal half of the protein.



**Figure 14. Confirmation of the putative OsPP2A B'' phosphorylation sites by site-directed mutagenesis.**

(A) Site-directed mutants of the OsPP2A B'' regulatory subunit. Three single mutants (M95, M102, M119), three double mutants (DM95,102; DM95,119; DM102,119) and two triple mutants (TMutA and TMutE). Amino acid sequences are shown in the region containing the putative CDK phosphorylation sites. Phosphorylation target serines are bold and underlined, alanines or glutamates replacing serines are highlighted in red. (B) Phosphorylation of wild type (WT), and serine-alanine single (M95, M102, M119), double (DM95,102; DM95,119; DM102,119), as well as triple (TMutA) mutants of OsPP2A B'' by affinity purified rice PSTAIRE kinase complex in vitro. Phosphorylated proteins were separated by SDS-PAGE and  $^{32}\text{P}$  incorporation was detected by autoradiography using X-ray film. (C) The loading control for (A) was stained with CBB.

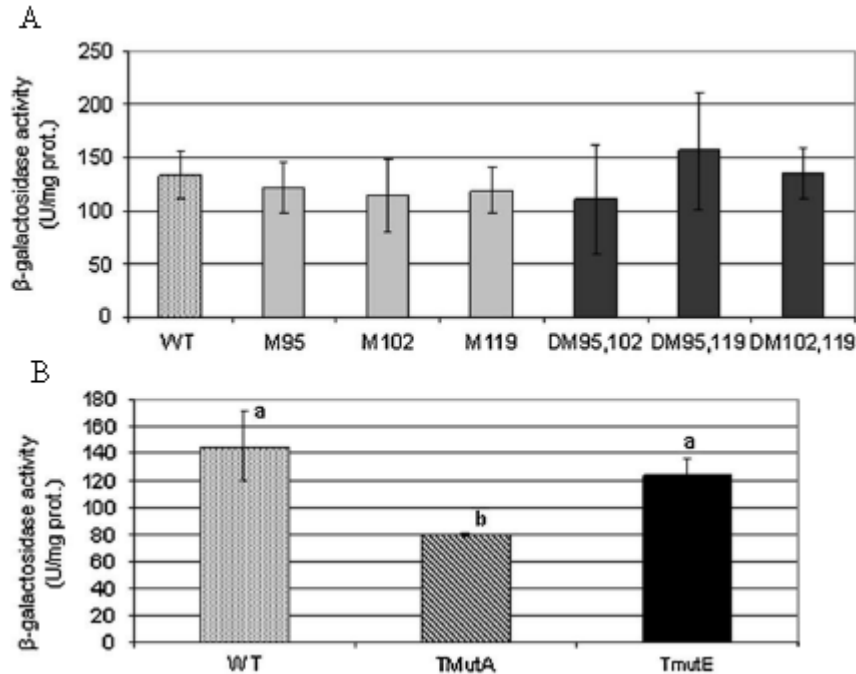
In order to investigate the phosphorylation sites further and to examine the role of these modifications in the function of the OsPP2A B'' regulatory subunit, a series of point mutants was generated by serine-to-alanine exchanges eliminating the predicted CDK phosphorylation sites (**Fig. 14A**). Three recombinant single site mutants (M95, M102, and M119), three double mutants (DM95, 102; DM95, 119; DM102, 119) and one triple mutant (TM95, 102, 119, named TMutA) were constructed with His<sub>6</sub>-tag (**table 1** and **2** in Appendix). The wild type recombinant His<sub>6</sub>-tagged OsPP2A B'' and all of its mutants were expressed in *E. coli*, were affinity purified, and phosphorylated in vitro using a GST-p13<sup>SUC1</sup> bound CDK complex (**Fig.14B**). In the triple mutant no <sup>32</sup>P incorporation was detected, the double mutants exhibited a quite strong reduction of <sup>32</sup>P incorporation, whereas the single mutants showed phosphate incorporation close to that of the wild type OsPP2A B'' (**Fig, 14B**). The lack of phosphorylation in the triple mutant indicates that only the predicted residues can act as phosphate acceptors. The phosphorylation of the double mutants DM95, 102 and DM102, 119 confirmed the phosphorylation of Ser119 and Ser95 respectively, while the phosphorylation of the double mutant DM95, 119 revealed that Ser102 is also a valid target of the kinase. These results collectively indicate that all of the putative sites including Ser102 can be phosphorylated by CDK.

#### **4.8. Phosphorylation of OsPP2A B'' may promote its association with OsRBR1**

Above we demonstrated that the OsPP2A B'' regulatory subunit can be phosphorylated and the sites of phosphorylation (Ser95, Ser102, and Ser119) were determined as well. Based on these results the following question emerged: will the phosphorylation of the B'' regulatory subunit affect the interaction with OsRBR1 and/or with the PP2A core dimer?

The pairwise yeast two hybrid assays with the pBD-GAL4 vector encompassing the OsRBR1 coding region and a series of pGAD424 vectors each containing the coding sequence of OsPP2A B'' phosphorylation site mutants. In this experimental setup the OsPP2A B'' variants may be phosphorylated by the yeast CDKs. Protein-protein interaction affinity was quantified by  $\beta$ -galactosidase activity assay (**Fig. 15A**). Although there was a difference in the growth rates among the transfected yeast cells, we were not able to find a significant difference in the binding affinity of

any single or double mutants relative to the wild type OsPP2A B'' due to the large experimental error. Furthermore, we constructed a phosphorylation mimicking mutant (TMutE, serine-to-glutamic acid in three sites, **Fig. 14A**). A more dramatic effect was observed when the interactions of OsRBR1 with wild type and triple alanine mutant TMutA and triple glutamate mutant TMutE OsPP2A B'' proteins were tested (**Fig. 15B**). In this in vivo system, a significant difference was found between the non-phosphorylated TMutA and the presumably phosphorylated wild type TMutE (or the phosphorylation mimicking). Thus, the dephosphorylation of OsPP2A B'' appears to decrease its interaction with OsRBR1.

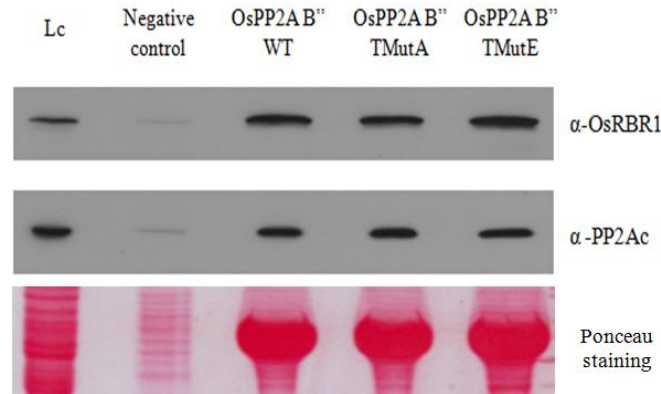


**Figure 15. Pairwise assay to check the effect of phosphorylation on the interaction between B'' regulatory subunit and OsRBR1.**

(A) Yeast two hybrid interaction strengths measured as  $\beta$ -galactosidase activities between OsRBR1 and wild type (WT) or site-directed mutants of OsPP2A B''. OsRBR1 inserted into pBD-GAL4 vector was combined with pGAD424 vectors containing wild type or mutant OsPP2A B''. The empty pBD-GAL4 vector was used as background control. (B) Strength of interaction was measured as in (A) between OsRBR1 and OsPP2A B'' wild type (WT), triple phosphorylation site alanine (TMutA) or glutamate (TMutE) mutant in the yeast two hybrid system. The results shown in (A) and (B) are the averages of three independent transformations and  $\beta$ -galactosidase measurements. In (B) letters a and b indicate significant divergence at the level  $p < 0.05$ .

The pull down assay supported this conclusion. This experiment checked the association of OsRBR1/PP2A catalytic subunit to OsPP2A B<sup>''</sup> WT/ TMutA/TMutE. His<sub>6</sub>-tagged OsPP2A B<sup>''</sup> wild type (WT), and two mutants, TMutA and TMutE incubated in total protein extract prepared from a rice cell suspension culture (exponential growth phase) and immunoblotting was performed with monoclonal anti-OsRBR1 and anti-PP2Ac antibody, respectively. Similar to the results from the yeast two hybrid system, the non-phosphorylated TMutA showed somewhat lower affinity to OsRBR1 compare to wild type and phosphorylation mimicking TMutE (**Fig. 16**, upper panel). While for PP2Ac, Three OsPP2A B<sup>''</sup> variants showed same interaction intensity to OsPP2A catalytic subunit (**Fig. 16**, middle panel).

Similar results were reported by Davis (2005), on B<sup>''</sup>/PR70. Mutation of the phosphorylation sites, by either ablating the phosphorylation site or producing a phosphorylation mimic, had no apparent effect on binding of PR70 to the PP2A core dimer (A and C subunits). All these data suggest that the phosphorylation sites have no effect on the properties of the binding domains.



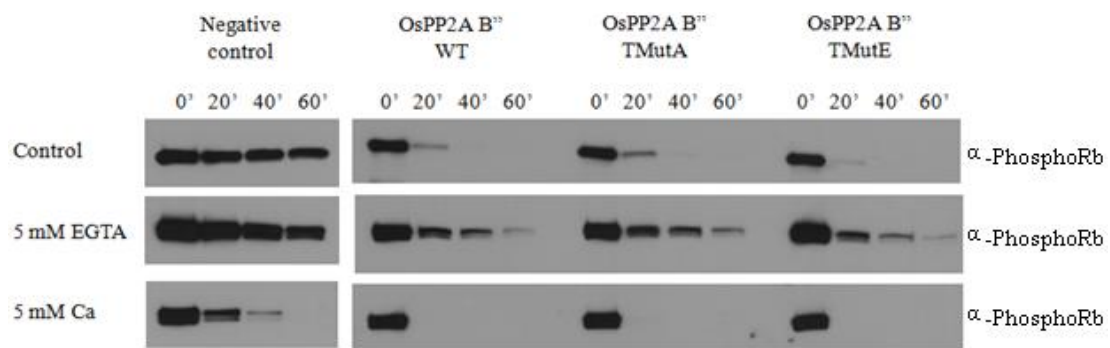
**Figure 16. Pull down assay for the association of OsPP2A B<sup>''</sup> variants and OsRBR1.**

His<sub>6</sub>-tagged OsPP2A B<sup>''</sup> the wild type (WT), triple A mutant (TMutA) and triple E mutant (TMutE) incubated in total protein extract prepared from a rice cell suspension culture (exponential growth phase). Western blot probed with monoclonal antibodies raised against OsRBR1, the catalytic subunit of PP2A. Lc, loading control, 1/20 of the total amount (total protein extract of rice cultured cells).

#### 4.9. Phosphorylation of OsPP2A B<sup>''</sup> regulatory subunit Effects on the phosphatase activity of holoenzyme

To test whether the phosphorylation of OsPP2A B<sup>''</sup> also modulated by the PP2A catalytic activity, phosphatase holoenzymes were isolated by pull down from the extract of rice suspension

cells using His<sub>6</sub>-tagged wild type OsPP2A B'' (WT) or the triple mutants TMutA or TMutE. The GST-tagged full length OsRBR1 protein was first *in vitro* phosphorylated by incubation with p13<sup>SUC1</sup>-bound CDK kinase complexes and then used as substrate for the dephosphorylation experiment to compare the holoenzyme activities. The dephosphorylation was monitored by phospho-RB (Ser807/811) polyclonal antibody (**Fig. 17**). In this assay the phosphatase complexes containing triple mutant TMutE had somewhat higher, and the TMutA mutant complexes has somewhat lower PP2A activity than the wild type (**Fig. 17**), demonstrating that the activity of OsPP2A B''-containing phosphatase complex can be regulated by CDK mediated phosphorylation of the regulatory subunit.



**Figure 17. Protein phosphatase activity assays with OsPP2A holoenzymes containing wild type (WT) and phosphorylation site mutated alanine (TMutA) and triple glutamate mutant (TMutE) OsPP2A B''.**

The holoenzymes were isolated from rice suspension cell extracts by pull down with the His<sub>6</sub>-tagged wild type or mutant B'' subunits. The activity of the holoenzymes was compared using phospho-OsRBR1 substrate prepared by the phosphorylation of GST-tagged full length OsRBR1 protein with GST-p13SUC1 bound CDK complex. The amount of phospho-OsRBR1 was monitored before the reaction (0') and 20, 40, and 60 min after adding the phosphatase with a phospho-Rb specific antibody. Control, 5mM EGTA, and 5mM Ca refer to different extraction buffer and binding conditions. Control, standard condition; 5mM EGTA, buffer containing 5mM EGTA to chelate Ca<sup>2+</sup>; 5mM Ca, buffer containing 5mM Ca<sup>2+</sup>.

There are two functional Ca<sup>2+</sup>-binding EF-hand domains presented in OsPP2A B'' (**Fig. 11C**). Will the presence of Ca<sup>2+</sup> effect on the phosphatase activity of PP2A heterotrimers? This is the reason why we carried out this experiment under three different conditions (standard, lacking Ca<sup>2+</sup> and abundant Ca<sup>2+</sup>). Under free Ca<sup>2+</sup> absence condition (EGTA chelating, **Fig. 17**, middle panel), all PP2A holoenzymes showed a lower phosphatase activity compare to the enzymes under standard



condition (upper panel). While all PP2A holoenzymes showed high phosphatase activity and can dephosphorylated all substrates in 20 minutes (**Fig. 17**, lower panel) when with abundant  $\text{Ca}^{2+}$  (5mM).

## 5. DISCUSSION

Proper regulation of the cell cycle is vital for normal cell division, growth and differentiation. The family of retinoblastoma proteins (pRB) or pocket proteins is one of the main players in this well-orchestrated process. The RB protein was originally identified as product of a tumor suppressor gene; deleted or mutated in retinoblastoma as well as in a wide-variety of other cancers (Friends et al., 1986; Weinberg, 1995). Later two other proteins were isolated, p107 and p130, which share structural and functional similarity with pRB (Ewen et al., 1991; Baldi et al., 1996). In mammalian cells, pRB is a negative regulator of genes required for cell cycle progression through its ability to recruit members from the E2F family of transcriptional factors, which act together as heterodimers of E2F and DP subunits. Mitogenic signaling induces cyclin D kinases which phosphorylate pRB and sequentially render it inactive, resulting in the dissociation of E2F, allowing the expression of E2F-regulated genes. The expression of this group of genes is central to the progression of cells into, and through, the S phase (reviewed by de Jager et al., 2001).

pRB regulation is a conserved feature of both the mammalian and plant cell cycle. Plant retinoblastoma-related proteins (RBRs) have the same structure as RB family members and also interact with various cellular proteins, such as E2F family proteins, D-type cyclins, mammalian viral oncoproteins, and plant virus proteins (Miskolczi et al., 2007). The RBR proteins contain several phosphorylation sites and Ábrahám et al. (2011) reported that both the MedsaCDKA1;1/1;2 and the mitotic MedsaCDKB2;1 complexes could phosphorylate the C-terminal fragment of MsRBR1 from alfalfa. Phosphorylation occurs in a cell-cycle-dependent manner. The maximum phosphorylation state is reached during the G1-S transition, and remains high until the mid/late S phase (Bonioti and Gutierrez, 2001; Nakagami et al. 2002). Moreover, as reviewed by de Jager et al. (2009), activation of E2F transcription factors at the G1-to-S phase boundary, with the resultant expression of genes needed for DNA synthesis and S-phase, is due to phosphorylation of the RBR protein by cyclin D-dependent kinase, particularly CYCD3-CDKA.

### 5.1. Specific antibodies used in the detection of MsRBR1/OsRBR1 and phospho-MsRBR1/OsRBRs

Studies on plant RBR proteins have uncovered a wide variety of additional roles such as cell fate determination and the influencing chromatin dynamics or genomic imprinting (Reviewed by Dudits et al., 2011). For a deeper understanding of the molecular basis of these divergent functions of plant RBR proteins it is essential to know the cell cycle phase-dependent changes in levels and post-translational modifications of these proteins. In this study, immunodetection techniques were used to monitor the MsRBR1 and the phospho-MsRBR1 proteins in samples from synchronized alfalfa cells. In other experiments, similarly to this, the levels of OsRBR and phospho-OsRBR proteins were monitored with the same methods as well.

Antibodies against the C-terminal fragment of AtRBR1 protein specifically recognized the 115 kDa MsRBR1 proteins, as shown by the antigen competition assay (**Fig. 6A**). In total protein extracts from cultured alfalfa cells this polyclonal antibody detected smaller cross-reacting bands, indicating a wider spectrum of unspecific interactions for the antibody tested. The higher signal intensity in lane 2 of **Fig. 6A** may originate from a higher concentration of antibodies during the pre-incubation procedure. Our results suggest that antibodies generated either against the C-terminal fragment of the Arabidopsis AtRBR1 protein or the phosphopeptide of the human RB protein cross-reacted with the same 115 kDa alfalfa protein having the predicted molecular size; this provides a sufficient basis to use these antibodies in alfalfa cells (**Fig. 6C, 6D**). According to the sequence data, the C-terminal regions of the Arabidopsis and alfalfa RBR proteins share a significant degree of amino acid sequence identity (74%). The homologous regions cover a substantial portion of these fragments and include the NVYVSPLRGS motif that may correspond to the NIYISPLKS motif of the human RB protein which harbors the conserved 807/811serine (S) phosphorylation site. When interpreting the results of the experiments aimed at the detection of the phosphorylated form of the MsRBR1 protein by using anti-phosphopeptide antibody, it has to be emphasized that this methodology detects the phosphorylation only in this relatively short region, whereas post-translational modifications can occur at additional residues with different functional consequences. Therefore, the methodology used here is not sufficient for determining the ratio between the MsRBR1 and the phospho-MsRBR1 proteins. The functionality and specificity of this anti-

phosphopeptide antibody was demonstrated by detection of the *in vitro* phosphorylated form of the *Medicago* RBR protein C-terminal fragment by Western blot (**Fig. 6B**). The RB proteins from mammalian cells show phosphorylation-dependent migration patterns, and the hyperphosphorylated RB protein is usually detected as a more slowly migrating band (Farkas et al., 2007). As a result of the protein separation protocol employed, the MsRBR1 protein band was diffuse and a clear shift between the two protein forms was not observed. Similarly, no band shift was reported in the case of the phosphorylation of the NtRBR1 proteins (Kawamura et al., 2006). After inorganic phosphate incorporation into pea axillary buds, the anti-PsRBR antibody immunoprecipitated higher and lower molecular mass forms of the phosphorylated PsRBR1 that were sensitive to  $\lambda$ -phosphatase treatment (Shimizu-Sato et al., 2008). In the present study the intensity of the band corresponding to the phospho-MsRBR1 (115 kDa) protein was reduced by phosphatase treatment, which indicates that the phosphorylated form is correctly recognized by the anti-phosphopeptide antibody used (**Fig. 6D**, right panel, lanes 3 and 6).

A monoclonal antibody raised against the N-terminus of OsRBR1 can recognize a 112kDa protein from the total protein extract of rice cultured cells, which correspond to the 111.5kDa molecular weight of the OsRBR1 (**Fig. 8A**, left panel). The antigen competition assay shows the specificity of this antibody for OsRBR1 protein (**Fig. 8A**, middle and right panels). The phosphorylation of recombinant His<sub>6</sub>-tagged OsRBR1 protein was probed by the human pRB anti-phosphopeptide antibody and in parallel monitored by <sup>32</sup>P incorporation. These two approaches agree quite well with each other (**Fig. 8B**, upper and middle panel), which means that the anti-phosphopeptide antibody can be used in our experiments for phospho-OsRBR1 detection. This result is also relevant for all the following experiments on testing of phospho-OsRBR1.

## **5.2. Variation in cell-cycle dependent RBR and phospho-RBR protein levels in alfalfa and rice cell cultures**

Cultured plant cells at present provide an ideal experimental system for the efficient synchronization of cells in certain phases of the cell division cycle, which allows the analysis of phase-specific events (Ayaydin et al., 2011). In our study the A2 cell line of *Medicago sativa* that was long-term adapted to *in vitro* conditions, was used. Its short cycle time allowed the enrichment of

cell populations in various phases by using specific cell cycle inhibitors such as HU, roscovitine, or colchicine. Western blotting of total protein extracts from alfalfa cells with either an antibody against the C-terminal fragment of the AtRBR1 protein or an anti-human phospho-pRb peptide antibody allows the detection of the RBR protein (115 kDa) with the expected size and additional cross-reacting bands of various sizes and quantities (**Fig. 7B**). These signals are considered to be the results of non-specific interactions or degradation. Therefore, the present analysis is restricted to the full-size MsRBR1 protein and its phosphorylated form. Western blot analysis of protein samples detected the MsRBR1 protein during the whole division cycle and only moderate differences between phases were found (**Fig. 7B**, upper panel). The observed increase in MsRBR1 protein levels in samples with a higher frequency of G2/M phase cells was shown more clearly by the colchicine-blocked cells, although a cell cycle phase independent action of this inhibitor on RBR protein accumulation cannot be excluded (Ábrahám et al., 2011). Cell cycle phase dependence could be more clearly recognized in changes in phospho-MsRBR1 protein levels (**Fig. 7B**, lower panel). A reduced level of the phosphorylated form in G1 cells can be safely concluded from the presented data. Comparing amounts of cross reacting MsRBR1 and phospho-MsRBR1 proteins in **Fig. 7B** reveals that G1 cells primarily have the hypophosphorylated form.

Hirano et al. (2008) demonstrated, using a pull-down assay that the non-phosphorylated AtRBR1 protein could bind to the E2Fa protein. These findings are in agreement with the general model of G1/S phase transition control, where the hypophosphorylated RB proteins inhibit E2F activation of S phase specific genes (Poznic, 2009). The release of this inhibition is expected to occur when the RBR proteins are phosphorylated by CDK complexes. The role of CDKs in phosphorylation of RBR protein was shown by different studies on plant systems. *In vitro* studies suggest that in alfalfa cells both the MedsaCDKA1;1 and the mitosis-specific MedsaCDKB2;1 can be responsible for this modification of the MsRBR1 protein (Ábrahám et al., 2011).

The specific antibody against the N-terminal fragment of OsRBR1 and the human pRB anti-phosphopeptide antibody were used to monitor the phase specific level of OsRBR1 and phospho-OsRBR proteins. The variation in phospho-OsRBR levels during phase progression shows the same trend as the change in phospho-MsRBR1 (**Fig. 9B**). The total amount of OsRBR1 barely changed throughout the cell cycle, while the phospho-forms of OsRBR proteins showed a clear difference

between the G1 phase and S phase. In contrast to phospho-MsRBR1, phospho-OsRBR presented two clear bands which can be explained as two different states of the phosphoprotein: hyper- and hypo-phosphorylated OsRBR1. The NVYVSPLRTS motif of OsRBR1, which is located within the C-terminal region of this protein, may equate to the conserved 807/811 serine (S) phosphorylation site containing NIYISPLKS motif of the human RB protein. Similarly, in the C-terminal region of OsRBR2 protein the NVYVSPLRQTK motif may also correspond to the NIYISPLKS motif. It indicates that both phospho-OsRBR proteins can be detected by the human pRB anti-phosphopeptide antibody. However, those two bands may be explained by the presence of two different OsRBR proteins as well. We already know from the study of Lendvai et al. (2007) that in actively dividing cultured cells, *OsRBR2* gene was expressed around 10 times less than the *OsRBR1* gene, whereas in the mature leaves the *OsRBR2* gene transcripts were present in quantities several hundred times higher than the mRNA levels of *OsRBR1*, but interestingly, no matter how abundant it is, the antibody can not detect phospho-OsRBR in different segments of mature leaves (Yu et al., 2014). What's more, partial depletion of OsRBR1 in cell suspension protein extract by specific anti-OsRBR1 antibody resulted in the decrease of the phospho-OsRBR band intensities (Yu et al., 2014). In addition, the size of the OsRBR1 protein is 111.5 kDa, whereas the OsRBR2 protein is 107.9 kDa (Lendvai et al., 2007). Considering the differences between the two proteins in expression level as well as the differences in the molecular size, we may conclude that two bands in the second panel of **Fig. 9B** are not due to two different OsRBR proteins but represent the hyper- and hypo-phosphorylated OsRBR1. The results also suggest that the human pRB anti-phosphopeptide antibody recognizes only the phosphorylated form of the OsRBR1 protein.

### **5.3. PP2A phosphatase B" regulatory subunit interacts with OsRBR1 specifically and preferentially associates with members of the subfamily II of OsPP2A catalytic subunits**

Reversible protein phosphorylation is crucial in many cellular processes. It is a key feature in signal transduction, gene expression and in the regulation of metabolic enzymes. While hundreds of articles have been published to explain the details of the phosphorylation of RB proteins, our knowledge about dephosphorylation is still limited. Two laboratories almost simultaneously published the first evidence that PP1 and/or PP2A were directly responsible for this

dephosphorylation (Alberts et al., 1993; Ludlow et al., 1993). Whereas PP1-mediated RB dephosphorylation is mainly restricted to mitotic exit, it was proposed that PP2A is important for maintaining the phosphorylation status of three pocket proteins (pRb, p107, and p130) during cell cycle progression by counteracting CDKs (Kolupaeva and Janssens, 2013). The PP2A-depleted extracts have only around 20-30% of the anti-CDK phosphatase activity that is present in interphase cycling extracts (Castilho et al., 2009). Low concentration of OA and endothal are ideally inhibitors of PP2A. Studies on 1  $\mu$  M OA and endothal treated synchronized cells showed shorter G1 and G2 phases than in the control cells (Polit and Kazmierczak, 2007); PP2A also took part in the regulation of mitotic kinase activities and the microtubule organization (Ayaydin et al., 2000).

PP1 has already been identified as the major cell cycle-related pRB phosphatase, while PP2A is expected to be a major phosphatase that is responsible for p107 and p130 dephosphorylation during the cell cycle or when the cells are suffering from stress, such as oxidative stress, DNA damage, UV radiation, etc. (Kolupaeva and Janssens, 2013). First, the PP2A catalytic subunit was found to be associated with dephosphorylation of both p130 and p107 throughout the cell cycle and in quiescent cells, with a noticeable increase as cells progress through the S phase (Garriga et al., 2004). Second, the PR59/B'' $\gamma$  subunit of PP2A showed a strong yeast-two hybrid based interaction with p107 but not with pRB. In U2OS cells, overexpression of PR59/B'' $\gamma$  did not affect RB phosphorylation, but ectopic co-expression of p107 and PR59/B'' $\gamma$  resulted in the accumulation of dephosphorylated p107. A further proof for the specific association between p107 and PR59/B'' $\gamma$  is that PR59/B'' $\gamma$  and p107 are co-localized in nuclear dots, while no co-localization was observed for pRB (Voorhoeve et al., 1999b). Third, B55 $\alpha$  containing PP2A holoenzymes play a major role in restricting the phosphorylation state of p107 and in inducing its activation in human cells (Jayadeva et al., 2010). Further evidence comes from 1  $\mu$  M OA treated suspension cells, in which the phosphorylation of Rb-like proteins increased obviously (Polit and Kazmierczak, 2007).

In the study of Lendvai et al. (2007), a protein from *Medicago truncatula* was identified as an interactor of full-length MsRBR11 protein, which shares 39% identity and 57% homology with the murine PR59/B'' PP2A regulatory subunit. This so called MtPP2A B'' regulatory subunit displayed a strong association with MsRBR1 and OsRBR1, but could not interact with OsRBR2 (**Fig. 10A**). This selective interaction is similar to the discrimination of PR59/B'' $\gamma$  between pRB and p107. During our

work we have isolated the rice homologue of the *MtPP2A B''* gene. Cloning this homologue into the yeast two-hybrid vector allowed us to carry out pair wise interaction studies that verified the specific interaction between the OsPP2A B'' regulatory subunit and the OsRBR1 protein (**Fig. 10A**). The interaction was proven *in vitro* as well, using a pull-down assay (**Fig. 10B**). We already know that there are two RBR proteins in rice, OsRBR1 and OsRBR2. They belong to distinct subfamilies (**Fig. 3**). OsRBR1 and MsRBR1 share 61% identity, whereas, in the case of OsRBR2 the level of identity is 51%. The identity between OsRBR1 and OsRBR2 is 54%. These data may explain the differences in interaction strength between the OsPP2A B'' regulatory subunit and the two rice RBR proteins. The levels of OsPP2A B'' regulatory subunit and catalytic subunit changed only slightly during the whole cell cycle (**Fig. 9B**). Considering the expression pattern of the two genes: *OsRBR2* is more abundant in differentiated cells while the expression of *OsRBR1* is much higher in actively dividing cells, we suggest that plant RBRs belonging to the RBR1 and RBR2 subfamilies have different roles in the regulation of plant cell division and differentiation. The identified B'' regulatory subunit containing PP2A heterotrimeric holoenzyme may have an important role in the dephosphorylation of rice retinoblastoma-related protein(s) during cell cycle progression and/or in the response to extracellular (environmental) stimuli. The results also offer an important starting point for further research, since the regulatory functions of pocket domain containing RB-like proteins (pRB, p107 and p130) in cellular processes depend on their phosphorylation status.

In *Oryza sativa*, there are five isoforms of the PP2A catalytic subunits OsPP2Ac-1, -2, -3, -4, and -5 that are encoded by the *Os06g0574500*, *Os03g0805300*, *Os02g0217600*, *Os10g0410600*, and *Os03g0167700* genes, respectively. They can be grouped into two subfamilies: subfamily I (containing isoenzymes 1 and 3) and II (including isoenzymes 2, 4, and 5) (Yu et al., 2005). According to the data from our lab, the protein sequences of these catalytic subunits are highly similar, except for the N-terminal segment. LC-MS analysis of OsPP2Ac tryptic fragments was used to identify the association between B'' regulatory subunit and catalytic subunit isoforms. The results showed that B'' interact with subfamily II prior to subgroup I. Considering the expression patterns of OsPP2A catalytic subunit family members the proposed selective binding of B'' regulatory subunit to the subfamily II OsPP2Ac members may underline the functional differences between different heterotrimeric PP2As (Yu et al., 2014).



#### **5.4. The B pocket domain in OsRBR1 is sufficient, and the C-terminal region in OsPP2A B'' is obbligator for the interaction between these two proteins**

As reviewed by Knudsen and Wang (1996), pRB contains at least three distinct protein binding domains. The small A/B pocket binds proteins containing the LXCXE motif, the large A/B pocket binds the transcription factor E2F, and the C pocket binds the nuclear c-Abl tyrosine kinase. The C pocket is restricted to the C-terminal region and does not overlap with the large A/B pocket. The small and large A/B pocket domains are affected in all known naturally occurring mutants of RB proteins in mammals, suggesting an important role of this region of the protein in tumor suppression. The N-terminus of pRB may function in part to facilitate the binding of growth promoting factors in subnuclear regions actively involved in RNA metabolism (Durfee et al. 1994).

Durfee et al. (1993) reported a direct association between pRB and the catalytic subunit of PP1 (PP-1 $\alpha$ 2). The sequence of pRB required for binding to PP-1 $\alpha$ 2 is similar but not identical to the region that binds the SV40 large T antigen. Tamrakar and Ludlow (2000) demonstrated that the C-terminal region of pRB is both necessary and sufficient for physical interaction with PP1. The author showed that pRB binds to PP1 in a non-competitive manner as well.

Since OsRBR1 associates with the OsPP2A B'' regulatory subunit both *in vivo* and *in vitro* (**Fig. 10A, 10B**), we wondered about the segment of the binding domain in OsRBR1 necessary for the formation of the complex. Yeast two-hybrid experiments between a set of deletion mutants of OsRBR1 and the OsPP2A B'' regulatory subunit demonstrated that B domain (B pocket) is essential and sufficient for the interaction. The C terminus of OsRBR1 seemed to be unnecessary for association (**Fig. 11A, 11B**). As Voorhoeve et al. (1999b) reported, interaction between PR59 and p107 - which does not need an intact "B pocket" - differs from the stable LXCXE-motif-mediated interactions with the 'A+B pocket' of p107. According to the literature, the p107 PP2A-binding domain is located within the linker region in between the pocket domains (reviewed by Kolupaeva and Janssens, 2013).

In the B'' subfamily of regulatory subunits, PR72 contains two well-conserved canonical EF-hand domains which bind Ca<sup>2+</sup>, resulting in a conformational change of the protein structure. Similar to PR72, G5PR/PR130/PR48/PR70, all the other known B'' family members also harbour EF-hand

domain which regulate the binding to the PP2A core enzyme and subsequent phosphatase activity, which is regulated by the  $\text{Ca}^{2+}$  level (Eichhorn et al., 2009).

Several examples in the literature have already demonstrated such a regulation. The mutation of the EF-hand 2 (close to the C-terminal region of PR72/B'') in PR72/B'' abolishes heterotrimer formation (Janssens et al., 2003), which hints at the involvement of EF-hand subunits in the formation of the holoenzyme. Another report suggested that  $\text{Ca}^{2+}$  binding to EF-hand 1 of the B''/PR72 subunit increased PP2A affinity for certain substrates like Thr-75-DARPP32 protein further, or altered the orientation of phospho-serine or -threonine in the active site of the catalytic subunit. This also confirmed essential role of EF-hand 2 in heterotrimeric assembly (Ahn et al., 2007b).

In plants, *Arabidopsis thaliana* contains five genes encoding PP2A B'' subunits that carry putative calcium-binding EF-hand motifs (reviewed by Farkas et al., 2007). Leivar et al. (2011) identified the B'' $\alpha$  and B'' $\beta$ , which contain two EF-hand domains. The  $^{45}\text{Ca}^{2+}$  binding experiment showed that the GST-B'' $\alpha$  chimera bound  $^{45}\text{Ca}^{2+}$ . And the presence of  $\text{Ca}^{2+}$  increased the interaction between B'' $\alpha$ /B'' $\beta$  and PR65 (A subunit), compared to the  $\text{Ca}^{2+}$  absence condition. Database searches in the rice genome revealed the presence of three PP2A B regulatory subunits (*Os02g0224200*, *Os02g0191700* and *Os06g0563300*), seven B' subunits (*Os03g0681800*, *Os03g0844500*, *Os05g0555100*, *Os07g0274800*, *Os08g0122000*, *Os09g0542700* and *AK108850*) and two B'' subunits (*Os10g476600* and *AK067811*). A sequence analysis of the identified OsPP2A B'' (*Os10g476600*) revealed that it contained two EF-hand domains which potentially regulate its function by  $\text{Ca}^{2+}$ -binding (Dovega et al., 2014). To determine if the EF-hand domain is necessary for the formation of the OsRBR1/OsPP2A B'' complex, yeast two-hybrid experiments were carried out with the wild type OsRBR1 and a set of deletion mutants of OsPP2A B'' (shown in **Fig. 11C**, fragment minus C-terminal; minus EF2; minus EF1 and EF2). It was clearly shown that none of the truncated version of OsPP2A B'' could associate with OsRBR1 (**Fig. 11D**). We can safely conclude that the C-terminal substrate binding region of OsPP2A B'' has an important function in recruiting OsRBR1 to the vicinity of the phosphatase catalytic subunit. However, no matter how important it is, the C-terminal alone is not sufficient for the interaction.

The C-terminus of the B'' protein shows acidity character, it is the most intensity region of the negative charged amino acids. Especially in the very end of C-terminus, in 25 amino acids sequence, 9 negatively charged amino acids are there and without positively charged amino acids. This structure character may related to its role in the association between OsPP2A B'' and OsRBR1.

### **5.5. Phosphorylation of the OsPP2A B'' regulatory subunit and identification of its putative phosphorylation sites**

The ability of the PP2A holoenzyme to dephosphorylate specific intracellular proteins depends on its activity and intracellular localization, which in turn is largely determined by the nature of its regulatory subunit. McCright et al. (1996) found that the human B56 gene family encodes retinoic acid-inducible phosphoproteins which can differentially target PP2A to the cytoplasm and nucleus. Moreover, most of the B56 isoforms (B56 $\alpha$ , B56 $\beta$ , B56 $\gamma$ 3, B56 $\delta$ , and B56 $\epsilon$ ) are phosphoproteins, which are phosphorylated on serine. All information suggests that the regulation of PP2A activity may be mediated by post-translational modification of B56, by targeting of PP2A heterotrimers to specific intracellular locations mediated by association with B56, and by tissue and developmental stage specific expression of B56 genes.

Three sites (Ser60, Ser75, and Ser573) of human 74 kDa B'' (which is different from human B56 $\delta$  only in a 32-amino acid insertion in B56 $\delta$  near the N terminus) have been shown to be phosphorylated by Protein Kinase A (PKA) under *in vitro* conditions. On the basis of this observation it can be proposed that the reversible phosphorylation of the regulatory 74 kDa B'' subunit serves as a control mechanism for PP2A function in the cell. In the first place, phosphorylation of B'' changed the phosphatase activity of the PP2A heterotrimeric holoenzyme CAB'' and its spectrum of substrate specificity. Secondly, this phosphorylation may also regulate the targeting of CAB'' to a certain compartment in the cell by interaction with a specific protein (Usui et al., 1998). B56 $\alpha$  was reported as an interactor of double-stranded RNA-dependent protein kinase (PKR) by yeast two-hybrid screening (Xu and Williams, 2000). Later experiments proved that B56 $\alpha$  is phosphorylated by PKR *in vivo*. Similar to human 74 kDa B'', phosphorylation of B56 $\alpha$  does not affect the interaction with the PP2A core dimer, but does stimulate the activity of the PP2A holoenzyme. In the case of the B'' regulatory subunit used in our experiments, the immunolocalization experiments indicated that

during the G1 and S phases this protein can be detected in the nucleus and its localization partially overlapped with OsRBR1 (Ábrahám et al., manuscript in preparation). The study of Ábrahám et al. (2011) suggests that the phosphorylated form of MsRBR1 has different localization than the unphosphorylated form. We therefore want to postulate that the phosphorylated form of B'' may localize differently than the nonphosphorylated form. Further experiments will be needed to test this idea.

Compared with the B regulatory subunit in mammalian cells, much less is known about the B regulatory subunit in plants. Terol et al. (2002) identified five B' isoforms, which showed a high degree of conservation in sequence and structure with respect to the Arabidopsis B' subunit genes and proteins. Similar to the products of B56 gene family, these proteins exhibit high levels of similarity in their central regions but a considerable divergence in their amino- and carboxy-terminal regions. We may presume that the highly variable amino- and carboxy-terminal regions of these proteins play an important role in defining properties such as the substrate specificity and/or the cellular localization of the Ser/Thr PP<sub>2A</sub> given their similarity to the primary structure of the human B56 gene family products. Actually, in some mammal isoforms of the B and B' regulatory subunits, several regions in their carboxy termini have been identified as being responsible for PP<sub>2A</sub> subcellular targeting, and also a target for phosphorylation (McCright et al. 1996; Usui et al., 1998; Ahn et al., 2007a). Those results support the opinion that variable regions of the B' regulatory subunits control PP<sub>2A</sub> function. Matre et al. (2009) described the differential subcellular targeting within the Arabidopsis B'η subfamily. B'η and B'γ targeted to the cytosol and nucleus. B'ξ localized to the cytoplasm and partly co-localized with mitochondrial markers when its N-terminus was free. The B'θ subunit targeted to the peroxisomes if its C-terminus was free. A B''-like subunit in *Arabidopsis*, TON2 (TONNEAU2), shows similarity to the human PR72/B'' subunit and was proven to associate with the PP<sub>2A</sub> catalytic subunit in two-hybrid assays. The *ton2* mutation affects seedling body organization and causes abnormalities in microtubule assembly and in the formation of the pre-prophase band before mitosis (Camilleri et al., 2002).

In our work, co-immunoprecipitation and recombinant protein phosphorylation experiments demonstrated that the OsPP<sub>2A</sub> B'' regulatory subunit could be phosphorylated by PSTAIRE motif containing CDKs (**Fig. 12A, 12B**). Computer-assisted analysis revealed the presence of three putative

CDK phosphorylation sites in the N-terminal region of the protein. These three sites are Ser95 in APPLSPRST, Ser102 in STSGSPRVM and Ser119 in SSLGSPLKV, which are all three SPR/L motif. Phosphorylation studies on a series of deletion mutants indicated that the three potential phosphorylation sites can be indeed phosphorylated (**Fig. 13**). Phosphorylation of OsPP2A B'' by p13<sup>SUC1</sup> bind A and B-type CDKs was analyzed as well by a proteomic method. The results verified the phosphorylation of Ser95 and Ser119 clearly (**Fig. 18** in Appendix). Unfortunately, Ser102 can not be confirmed with this method, maybe because of technical limitation.

Elimination of protein phosphorylation by serine to alanine exchange is a quite popular method and this is used to verify putative phosphorylation sites. With this method, a series of site-directed mutants of the OsPP2A B'' regulatory subunit was created (**Fig. 14A**) to check whether all of the putative sites, including Ser102, can be phosphorylated by CDKs. The single mutants, double mutants, and triple mutant showed significant difference in phosphorylation level (**Fig. 14B**) which confirmed the predicted three phosphorylation sites. The results also make it clear that no more potential CDK phosphorylation sites within B'' regulatory subunit.

### **5.6. Non-phosphorylation mutant of OsPP2A B'' regulatory subunit influences PP2A phosphatase activity, but has no affect on the formation of the PP2A heterotrimer**

Besides phosphorylation elimination of OsPP2A B'' (TMutA), we made the phosphorylated state mimicking by site directed serine-to-glutamic acid exchanges on three CDK phosphorylation sites (TMutE). Two triple mutants (TMutA and TMutE) were used to check the PP2A holoenzyme complex assembly and the interaction with OsRBR1. The results agreed with those of Davis (2005): mutations of the phosphorylation sites, by either ablating the phosphorylation site or producing a phosphorylation mimic, had no apparent affect on binding of PR70 to the PP2A core dimer (A and C subunits). Data from pull down assays (**Fig. 16**) and yeast two-hybrid interaction assays between pBD-Gal4-OsPP2A B'' mutants and pAD-Gal4-OsRBR1 (**Fig. 15A, 15B**) also supported the observation that these site-directed mutations did not affect the association between B'' and PP2A catalytic subunit, this may show that all these changes did not influence the interaction interface between these two subunits. As concerned to OsRBR1, non-phosphorylated version (TMutA) and phosphorylation mimicking version (TMutE) exhibited significant different association strength with

it. We do not know the exact mechanism responsible for this phenomenon. However *vica verse*, Vietri et al. (2006) reported that mutant pRB with 14 out of 16 Ser/Thr substitutions to Ala was still able to bind PP1 with similar affinity as wild-type pRB and, therefore, we can conclude that phosphorylation does not play a role in the association between B'' and OsRBR1.

When B56 $\alpha$  was phosphorylated by PKR, phosphatase activity towards phospho-MBP was enhanced. Similar results were obtained when recombinant eIF2 $\alpha$  protein phosphorylated by PKR was used as a substrate for PP2A (Xu and William, 2000). In addition, if the B56 $\delta$  subunit is phosphorylated by PKA, this increases the overall activity of PP2A *in vitro* and *in vivo* (Ahn et al., 2007a). But what will happen if the phosphorylation of the B regulatory subunit is eliminated? Moreover, considered two Ca<sup>2+</sup> binding EF-hand motif in B'' regulatory subunit, what kind of role will the free Ca<sup>2+</sup> perform in phosphatase activity? Compared to the wild type B'' regulatory subunit, the triple mutant TMutA containing PP2A holoenzyme showed lower phosphatase activity under standard condition and lower Ca<sup>2+</sup> concentration (**Fig. 17**). It is prove the importance of post-transcriptional regulation of the B regulatory subunit from another aspect. On the other hand, PP2A<sub>TmE</sub> showed higher phosphatase activity than the PP2A<sub>Twt</sub> under standard and Ca<sup>2+</sup> absence condition, which means that the phosphorylation of B'' indeed elevates the activity of the PP2A heterotrimeric holoenzyme (**Fig. 17**). The results also indicated an effect of Ca<sup>2+</sup> on the activity of PP2A. Lowering of free Ca<sup>2+</sup> concentration decreased PP2A activity significantly.

Just like the study from Dudits et al. (2011), such finding supports the hypothesis that the dephosphorylation of plant RBR proteins is influenced by the increase in intracellular Ca<sup>2+</sup>; responding in this way to extracellular stimuli, like e.g. environmental stress factors.

## 6. ACKNOWLEDGEMENT

I am deeply grateful to my supervisor, Dr. Gábor V. Horváth. First for his kindness to give me a chance to be an ITC student and lately became a PhD student in his group. I appreciate his excellent assistance, scientific guidance, teachings, and encouragement. I thank him for supporting me always and for his great patience during my studies. His tolerance for my mistakes, calmness and truthfully scientist character with immeasurable knowledge impressed me a lot. The experience I obtained in his group under his guidance will always help me in the future. I would also like to thank Prof. Dénes Dudits for the possibility to join his lab and for his help during my studies.

I sincerely thank to our diligent and creative colleague Dr. Edit Abraham for her immense help in learning the molecular biology techniques and for her continuous presence and assistance in my work. She is serious and strict in the sciences but very kind in all other aspects. That is a great model for my future career. I also thank Aladár Pettkó-Szandtner who teaches me the techniques at the beginning of my lab work for his patience.

I would like to thank to all the past and present members, my colleagues and friends from the Plant Cell Division Cycle and Stress Adaptation Group for their help. I think I will never forget Maria Szecsenyi's calmness, Zoltan Zombori and Bettina Zomborine Nagy couple's kindness and patient, Petra Majer's sweatness, Zoltan Turoczy's energetic, helpful and high qualified cook.

I am very gratefully to all my friends through these years, ITC and PhD students and researchers from Hungary and abroad, for so many great memories and hopefully everlasting friendships. My stay in Szeged wouldn't have been the same without these wonderfully times. I will always cherish the memories. Special thank to Emine Dinc, Ozge Sozor, Burcin gungor, Ildiko Stier, Hajnalka Heverne Laczko-Dobos and Himabindu Gali for their constant spiritual support.

I appreciate the great help form Dr. Gert Schansker for checking the grammar and sentence structure of this thesis.

I thank my parents and two sisters, my constant source of inspiration, for their support. The life I spent in abroad making me realize the importance of home.

## 7. REFERENCES

- Ábrahám E, Miskolczi P, Ayaydin F, Yu P, Kotogány E, Bakó L, Ötvös K, Horváth GV, and Dudits D. (2011) Immunodetection of retinoblastoma-related protein and its phosphorylated form in interphase and mitotic alfalfa cells. *Journal of Experimental Botany*, 62: 2155-2168
- Ach RA, Durfee T, Miller AB, Taranto P, Hanley-Bowdoin L, Zambryski P, and Gruissem W. (1997) RBR1 and RBR2 encode maize retinoblastoma-related proteins that interact with a plant D-type cyclin and geminivirus replication protein. *Molecular Cell Biology*, 17: 5077-5086
- Adams PD. (2001) Regulation of the retinoblastoma tumor suppressor protein by cyclin/cdks. *Biochimica et Biophysica Acta*, 1471: M123-M133
- Ahn JH, Kim Y, Kim AS, Greengard P, and Nairn AC. (2011) Protein Kinase C-dependent dephosphorylation of tyrosine hydroxylase requires the B56 $\delta$  heterotrimeric form of protein phosphatase 2A. *PLoS One*, 6: e26292
- Ahn JH, McAvoy T, Rakhilin SV, Nishi A, Greengard P, and nairn AC. (2007a) Protein kinase A activates protein phosphatase 2A by phosphorylation of the B56{delta} subunit. *Proceedings of the National Academy of Sciences of the USA*, 104: 2979–2984
- Ahn JH, Sung JY, McAvoy T, Nishi A, Janssens V, Goris J, Greengard P, and Narin AC. (2007b) The B $\gamma$ /PR72 subunit mediates Ca<sup>2+</sup>-dependent dephosphorylation of DARPP-32 by protein phosphatase 2A. *Proceedings of the National Academy of Sciences of the USA*, 104: 9876-9881
- Alberts AS, Thorburn AM, Shenolikar S, Mumby MC, and Feramisco JR. (1993) Regulation of cell cycle progression and nuclear affinity of the retinoblastoma protein by protein phosphatase. *Proceedings of the National Academy of Sciences of the USA*, 90: 388-392
- Avni D, Yang H, Martelli F, Hofmann F, ElShamy WM, Ganesan S, Scully R, and Livingston DM. (2003) Active localization of the retinoblastoma protein in chromatin and its response to S phase DNA damage. *Molecular Cell*, 12: 735–746
- Ayaydin F, Kotogány E, Ábrahám E, and Horváth VG. (2011) Synchronization of *Medicago sativa* cell suspension culture. *Methods in Molecular Biology*, 761: 227-238
- Ayaydin F, Vissi E, Meszáros T, Miskolczi P, Kovács I, Fehér A, Dombrádi V, Erdödi F, Gergely P, and Dudits D. (2000) Inhibition of serine/threonine-specific protein phosphatases causes premature activation of cdc2Msf kinase at G2/M transition and early mitotic microtubule organization in alfalfa. *The Plant Journal*, 23: 85-96



- Baldi A, Boccia V, Claudio PP, De Luca A, and Giordano A. (1996)** Genomic structure of the human retinoblastoma-related Rb2/p130 gene. *Proceedings of the National Academy of Sciences of the USA*, 93: 4629-4632
- Baldi A, De Luca A, Clausio PP, Baldi F, Giordano GG, Tommasino M, Paggi MG, and Giordano A. (1995)** The Rb2/p130 gene product is a nuclear protein whose phosphorylation is cell cycle regulated. *Journal of Cell Biochemistry*, 59: 402-408
- Bartel PL, Chien CT, Sternglanz R, and Fields S. (1993)** Using the two-hybrid system to detect protein-protein interactions in development: a practical approach. Oxford University Press, Oxford, pp. 153-179
- Beijersbergen RL, Carlee L, Kerkhoven RM, and Bernards RE. (1995)** Regulation of the retinoblastoma protein-related p107 by G1 cyclin complexes. *Genes & Development*, 9: 1340–1353
- Berndt N, Dohadwala M, and Liu CWY. (1997)** Constitutively active protein phosphatase 1 alpha causes Rb dependent G1 arrest in human cancer cells. *Current Biology*, 7: 375–386
- Bianchi M, and Villa-Moruzzi E. (2001)** Binding of phosphatase-1 delta to the retinoblastoma protein pRb involves domains that include substrate recognition residues and a pRB binding motif. *Biochemical and Biophysical Research Communications*, 280: 1-3
- Birnboim HC, and Doly J. (1979)** A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research*, 7: 1513
- Boniotti MB, and Gutierrez C. (2001)** A cell-cycle-regulated kinase activity phosphorylates plant retinoblastoma protein and contains, in Arabidopsis, a CDKA/cyclin D complex. *The Plant Journal*, 28: 341-350
- Bradford MM. (1976)** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72: 248-254
- Borghi L, Gutzat R, Futterer J, Laizet Y, Hennig L, and Gruissem W. (2010)** Arabidopsis retinoblastoma-related is required for stem cell maintenance, cell differentiation, and lateral organ production. *Plant Cell*, 22: 1792-1811
- Brown VD, Phillips RA, and Gallie BL. (1999)** Cumulative effect of phosphorylation of pRB on regulation of E2F activity. *Molecular and Cellular Biology*, 19: 3246-3256
- Burkhardt LD, and Sage J. (2008)** Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nature reviews*, 8: 671-682
- Camilleri C, Azimzadeh J, Pastuglia M, Bellini C, Grandjean O, and Bouchez D. (2002)** The Arabidopsis TONNEAU2 gene encodes a putative novel protein phosphatase 2A regulatory subunit essential for the control of the cortical cytoskeleton. *Plant Cell*, 14: 833–845

- Castilho PV, Williams BC, Mochida S, Zhao Y, and Goldberg ML. (2009)** The M phase kinase Greatwall (Gwl) promotes inactivation of PP2A/B55  $\delta$ , a phosphatase directed against CDK phosphosites. *Molecular Biology of the Cell*, 20: 4777-4789
- Cegielska A, Shaffer S, Derua R, Goris J, and Virshup DM. (1994)** Different oligomeric forms of protein phosphatase type 2A activate and inhibit simian virus 40 DNA replication. *Molecular and Cellular Biology*, 14: 4616-4623
- Chen F, Archambault V, Kar A, Lio' P, D'Avino PP, Sinka R, Lilley K, Laue DE, Deak P, Capalbo L, and Glover MD. (2007)** Multiple protein phosphatases are required for mitosis in *Drosophila*. *Current Biology*, 17: 293-303
- Chen Z, Hafidh S, Poh SH, Twell D, and Berger F. (2009)** Proliferation and cell fate establishment during *Arabidopsis* male gametogenesis depends on the retinoblastoma protein. *Proceedings of the National Academy of Sciences of the USA*, 106: 7257-7262
- Chen Z, Higgins JD, Tan JLH, Li J, Franklin FCH, and Berger F. (2011)** Retinoblastoma protein is essential for early meiotic events in *Arabidopsis*. *The EMBO Journal*, DOI: 10.1038/emboj.2010.344
- Chomczynski P, and Sacchi N. (1987)** Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*, 162: 156-159
- Cicchillitti L, Fasanaro P, Biglioli P, Capogrossi MC, and Martelli F. (2003)** Oxidative stress induces protein phosphatase 2A-dependent dephosphorylation of the pocket proteins pRb, p107, and p130. *The Journal of Biology Chemistry*, 278: 19509-19517
- Classon M, and Dyson N. (2001)** p107 and p130: versatile proteins with interesting pockets. *Experimental Cell Research*, 264: 135-147
- Claudio PP, Howard CM, Baldi A, De Luca A, Fu Y, Condorelli G, Sun Y, Colburn N, Calabretta B, and Giordano A. (1994)** p130/pRb2 has growth suppressive properties similar to yet distinctive from those of retinoblastoma family members pRb and p107. *Cancer Research*, 54: 5556-5560
- Claudio PP, Tonini T, and Giordano A. (2002)** The retinoblastoma family: twins or distant cousins? *Genome Biology*, 3: 1-9
- Cobrinik, D. (2005)** Pocket proteins and cell cycle control. *Oncogene*, 24: 2796-2809
- Connell-Crowley L, Harper JW, and Goodrich DW. (1997)** Cyclin D1/Cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation. *Molecular Biology of the Cell*, 8: 287-301

- Dannenbergh JH, van Rossum A, Schuijff L, and te Riele H. (2000)** Ablation of the retinoblastoma gene family deregulates G (1) control causing immortalization and increased cell turnover under growth-restricting conditions. *Genes & Development*, 14: 3051–3064
- Davis AJ, Yan Z, Martinez B, and Mumby MC. (2008)** Protein phosphatase 2A is targeted to cell division control protein 6 by a calcium-binding regulatory subunit. *Journal of Biological Chemistry*, 283: 16104-16114
- Davis AJ. (2005)** Characterization of the protein phosphatase 2A regulatory subunit PR70. The University of Texas Southwestern Medical Center, Dallas, USA.
- de Jager SM, Menges M, Bauer UM, and Murray JA. (2001)** *Arabidopsis* E2F1 binds a sequence present in the promoter of S-phase-regulated gene AtCDC6 and is a member of a multigene family with differential activities. *Plant Molecular Biology*, 47: 555-568
- de Jager SM, Scofield S, Huntley RP, Robinson AS, den Boer BG, and Murray JA. (2009)** Dissecting regulatory pathways of G1/S control in *Arabidopsis*: common and distinct targets of CYCD3;1, E2Fa AND E2Fc. *Plant Molecular Biology*, 71: 345-365
- DeCaprio JA, Ludlow JW, Figge J, Shew JY, Huang CM, Lee WH, Marsillo E, Paucha E, and Livingston DM. (1988)** SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell*, 54: 275-283
- Delston RB, Matatall KA, Sun Y, Onken MD, and Harbour JW. (2011)** P38 phosphorylates Rb on ser567 by a novel, cell cycle-independent mechanism that triggers Rb-Hdm2 interaction and apoptosis. *Oncogene*, 30: 588-599
- Dimova DK, and Dyson NJ. (2005)** The E2F transcriptional network: old acquaintances with new faces. *Oncogene*, 24: 2810-2826
- Dovega R, Tsutakawa S, Quistgaard EM, Anandapadamanaban M, Low C, and Nordlund P. (2014)** Structural and biochemical characterization of human PR70 in isolation and in complex, with the scaffolding subunit of protein phosphatase 2A. *PLoS One*, 9:e101846
- Dudits D, Ábrahám E, Miskolczi P, Ayaydin F, Bilgin M, and Horváth VG. (2011)** Cell-cycle control as a target for calcium, hormonal and developmental signals: the role of phosphorylation in the retinoblastoma-centred pathway. *Annals of Botany*, 107: 1193-1202
- Durfee T, Becherer K, Chen PL, Yeh SH, Yang Y, Kilburn AE, Lee WH, and Elledge SJ. (1993)** The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes & Development*, 7: 555-569
- Durfee T, Feiler HS, and Grissem W. (2000)** Retinoblastoma-related protein in plants: homologues or orthologues of their metazoan counterparts? *Plant Molecular Biology*, 43: 635-642

- Durfee T, Mancini MA, Jones D, Elledge SJ, and Lee WH. (1994)** The amino-terminal region of the retinoblastoma gene product binds a novel nuclear matrix protein that co-localizes to centers for RNA processing. *The Journal of Cell Biology*, 127: 609-622
- Ebel C, Mariconti L, and Gruissem W. (2004)** Plant retinoblastoma homologues control nuclear proliferation in the female gametophyte. *Nature*, 429: 776-780
- Eichhorn PJA, Creighton MP, and Bernards R. (2009)** Protein phosphatase 2A regulatory subunits and cancer. *Biochimica et Biophysica Acta*, 1795: 1-15
- Ewen EM, Xing YG, Lawrence JB, and Livingston MD. (1991)** Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein. *Cell*, 66: 1155-1164
- Farkas I, Dombrádi V, Miskei M, Szabados L, and Koncz Cs. (2007)** Arabidopsis PPP family of serine/threonine phosphatase. *Trends in Plant Science*, 12: 169-176
- Friend SH, Bernards R, Rogelj S, Weinberg RA, Rapaport JM, Albert DM, and Dryja TP. (1986)** A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature*, 323: 643-646
- Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, and Firoozabady E. (1993)** Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science*, 220: 1049-1051
- Galderisi U, Cipollaro M, and Giordano A. (2006)** The retinoblastoma gene is involved in multiple aspects of stem cell biology. *Oncogene*, 25:5250–5256
- Garriga J, Jayaraman AL, Limon A, Jayadeva G, Sotillo E, Truongcao M, Patsialou A, Wadzinski BE, and Grana X. (2004)** A dynamic equilibrium between CDKs and PP2A modulates phosphorylation of pRB, p107 and p130. *Cell Cycle*, 3: 1320-1330
- Goodrich DW, Wang NP, Qian YW, Lee EY HP, and Lee WH. (1991)** The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. *Cell*, 67:293–302
- Grafi G, Burnett RJ, Helentjaris T, Larkins BA, DeCaprio JA, Sellers WR, and Kaelin WG. (1996)** A maize cDNA encoding a member of the retinoblastoma protein family: involvement in endoreduplication. *Proceedings of the National Academy of Sciences of the USA*, 93: 8962-8967
- Grana X, Garriga J and Mayol X. (1998)** Role of the retinoblastoma protein family, pRB, p107 and p130 in the negative control of cell growth. *Oncogene*, 17: 3365-3383
- Grana X. (2008)** Downregulation of the phosphatase nuclear targeting subunit (PNUTS) triggers pRB dephosphorylation and apoptosis in pRB positive tumor cell lines. *Cancer Biology & Therapy*, 7: 842-844

- Gutzat R, Borghi L, and Gruissem W. (2012)** Emerging roles of RETINABLASTOMA-RELATED proteins in evolution and plant development. *Trends in Plant Science*, 17: 139-148
- Gutzat R, Borghi L, Futterer J, Bischof S, Laizet Y, Hennig L, Feil R, Lunn J, and Gruissem W. (2011)** Retinoblastoma-related protein controls the transition to autotrophic plant development. *Development (Cambridge, England)*, 138, 2977-2986
- Hannon JG, Demetrick D, and Beach D. (1993)** Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins. *Genes & Development*, 7: 2378-2391
- Harbour JW, and Dean DC. (2000)** The Rb/E2F pathway: expanding roles and emerging paradigms. *Genes & Development*, 14: 2393-2409
- Hirano H, Harashima H, Shinmyo A, and Sekine M. (2008)** Arabidopsis retinoblastoma-related protein 1 is involved in G1 phase cell cycle arrest caused by sucrose starvation. *Plant Molecular Biology*, 66: 259-275
- Horváth BM, Magyar Z, Zhang Y, Hamburge AW, Bakó L, Visser RG, Bachem CW, and Bögre L. (2006)** EBP1 regulates organ size through cell growth and proliferation in plants. *EMBO Journal*, 25: 4904-4920
- Horváth VG, Pettkó-Szandtner A, Nikovics K, Bilgin M, Boulton M, Davies JW, Gutierrez C, and Dudits D. (1998)** Prediction of functional regions of the maize streak virus replication-associated proteins by protein-protein interaction analysis. *Plant Molecular Biology*, 38: 699-712
- Hunt T. (2013)** On the regulation of protein phosphatase 2A and its role in controlling entry into and exit from mitosis. *Advances in Biological Regulation*, 53: 173-178
- Huntley R, Healy S, Freeman D, Lavender P, de Jager S, Greenwood J, Makker J, Walker E, Jackman M, Xie Q, Bannister AJ, Kouzarides T, Gutierrez C, Doonan JH, and Murray JAH. (1998)** The maize retinoblastoma protein homologue ZmRb-1 is regulated during leaf development and displays conserved interactions with G1/S regulators and plant cyclin D (CycD) proteins. *Plant Molecular Biology*, 37: 155–169
- Ichimura K, Hanafusa H, Takimoto H, Ohgama Y, Akagi T, and Shimizu K. (2000)** Structure of the human retinoblastoma-related p107 gene and its intragenic deletion in a B-cell lymphoma cell line. *Gene*, 251: 37-43
- Inoue Y, Kitagawa M, and Taya Y. (2007)** Phosphorylation of pRB at Ser612 by Chk1/2 leads to a complex between pRB and E2F-1 after DNA damage. *The EMBO Journal*, 26: 208-2093
- James P, Halladay J, and Graig EA. (1996)** Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics*, 144: 1425-1436

- Janssens V, and Goris J. (2001)** Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochemical Journal*, 353: 417-439
- Janssens V, Jordens J, Stevens I, Van Hoof C, Martens E, De Smedt H, Engelborghs Y, Waelkens E, and Goris J. (2003)** Identification and functional analysis of two  $\text{Ca}^{2+}$ -binding EF-hand motifs in the B $\alpha$ /PR72 subunit of protein phosphatase 2A. *The Journal of Biological Chemistry*, 278: 10697-10706
- Jayadeva G, Kurimchak A, Garriga J, Sotillo E, Davis AJ, Haines DS, Mumby M, and Grana X. (2010)** B55 $\alpha$  PP2A holoenzymes modulate the phosphorylation status of the retinoblastoma-related protein p107 and its activation. *The Journal of Biological Chemistry*, 285: 29863-29873
- Johnston AJ, Kirioukhova O, Barrell PJ, Rutten T, Moore JM, Baskar R, Grossniklaus U, and Gruissem W. (2010)** Dosage-sensitive function of retinoblastoma related and convergent epigenetic control are required during the Arabidopsis life cell. *PLoS Genetics*, 6: e1000988
- Kawamura K, Kato K, Shinmyo A, and Sekine M. (2006)** Tobacco retinoblastoma-related protein is phosphorylated by different types of cyclin-dependent kinases during the cell cycle. *Plant Biotechnology*, 23: 467-473
- Kirchhefer U, Heinick A, Konig S, Kristensen T, Muller FU, Seidl MD, and Boknik P. (2014)** Protein phosphatase 2A is regulated by PKA  $\alpha$ -dependent phosphorylation of its targeting subunit B56  $\alpha$  at Ser41. *The Journal of Biological Chemistry*, 289: 163-176
- Kitagawa M, Higashi H, Jung HK, Suzuki-Takahashi I, Ikeda M, Tamai K, Kato J, Segawa K, Yoshida E, Nishimura S, and Taya Y. (1996)** The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2. *The EMBO Journal*, 15: 7060-7069
- Knudsen ES, and Knudsen KE. (2006)** Retinoblastoma tumor suppressor: where cancer meets the cell cycle. *Experimental Biology and Medicine*, 231: 1271-1281
- Knudsen ES, and Wang JY. (1996)** Differential regulation of retinoblastoma protein function by specific cdk phosphorylation sites. *The Journal of Biological Chemistry*, 271: 8313-8320
- Knudsen ES, and Wang JY. (1997)** Dual mechanisms for the inhibition of E2F binding to RB by cyclin-dependent kinase-mediated RB phosphorylation. *Molecular and Cellular Biology* 17: 5771-5783
- Kokai E, Szuperak M, Alphey L, Gausz J, Adam G, and Dombradi V. (2006)** Germ line specific expression of a protein phosphatase Y interacting protein (PPYR1) in *Drosophila*. *Gene Express Patterns*, 6: 724-729

- Kolupaeva V, and Janssens V. (2013)** PP1 and PP2A phosphatases—cooperating partners in modulating retinoblastoma protein activation. *The FEBS Journal*, 280: 627-643
- Kolupaeva V, Laplantine E, and Basilico C. (2008)** PP2A-mediated dephosphorylation of p107 plays a critical role in chondrocyte cell cycle arrest by FGF. *PLoS One*, 3: e3447
- Laemmli UK. (1970)** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227:680-685
- Lechward K, Awotunde OS, Swiatek W, and Muszynska G. (2001)** Protein phosphatase 2A: variety of forms and diversity of functions. *Acta Biochimica Polonica*, 48: 921-933
- Leivar P, Antolin-Llovera M, Ferrero S, Closa M, Arro M, Ferrer A, Boronat A, and Campos N. (2011)** Multilevel control of *Arabidopsis* 3-hydroxy-3-methylglutaryl coenzyme A reductase by protein phosphatase 2A. *The Plant Cell*, 23: 1494-1511
- Lendvai A, Pettkó-Szandtner A, Csordás-Tóth É, Miskolczi P, Horváth GV, Györgyey J, and Dudits D. (2007)** Dicot and monocot plants differ in retinoblastoma-related protein subfamilies. *Journal of Experimental Botany*, 58: 1663–1675
- Letourneux C, Rocher G, and Porteu F. (2006)** B56-containing PP2A dephosphorylate ERK and their activity is controlled by the early gene IEX-1 and ERK. *The EMBO Journal*, 25: 727-738
- Li Y, Graham C, Lacy S, Duncan MVA, and Whyte P. (1993)** The adenovirus E1A-associated 130-kD protein is encoded by a member of the retinoblastoma gene family and physically interacts with cyclins A and E. *Genes & Development*, 7: 2366-2377
- Li Z, and Murai N. (1990)** Efficient plant regeneration from rice protoplasts in general medium. *Plant Cell Report*, 9: 216-220
- Ludlow JW, Glendening CL, Livingston DM, and DeCaprio JA. (1993)** Specific enzymatic dephosphorylation of retinoblastoma protein. *Molecular and Cellular Biology*, 13: 367–372
- Ludlow JW, Shon J, Pipas JM, Livingston DM, and DeCaprio JA. (1990)** The retinoblastoma susceptibility gene product undergoes cell cycle-dependent dephosphorylation and binding to and release from SV40 large T. *Cell*, 60: 387-396.
- Magenta A, Fasanaro P, Romani S, Di Stefano V, Capogrossi MC, and Martelli F. (2008)** Protein phosphatase 2A subunits PR70 interacts with pRb and mediates its dephosphorylation. *Molecular and Cellular Biology*, 28: 873–882
- Magyar Z, Meszáros T, Miskolczi P, Deák M, Fehér A, Brown S, Kondorosi E, Athanasiadis A, Pongor S, Bilgin M, Bakó L, Koncz C, and Dudits D. (1997)** Cell cycle phase specificity of putative cyclin-dependent kinase variants in synchronized alfalfa cells. *The Plant Cell*, 9: 223-235

- Margolis SS, Perry JA, Forester CM, Nutt LK, Guo Y, Jardim MJ, Thomenius MJ, Freel CD, Darbandi R, Ahn JH, Arriti JD, Wang XF, Shenolikar S, Nairn AC, Dunphy WG, Hahn WC, Virshup DM, and Kornbluth S. (2006)** Role for the PP2A/B56delta phosphatase in regulating 14-3-3 release from Cdc25 to control mitosis. *Cell*, 127: 759-773
- Matre P, Meyer C, and Lillo C. (2009)** Diversity in subcellular targeting of the PP2A B'η subfamily members. *Planta*, 230: 935-945
- Mayol X, Garriga J, and Grana X. (1996)** G1 cyclin/CDK independent phosphorylation and accumulation of p130 during the transition from G1 to G0 lead to its association with E2F-4. *Oncogene*, 13: 237-246
- Mayol X, Grana X, Baldi A, Sang N, Hu Q, and Giordano A. (1993)** Cloning of a new member of the retinoblastoma gene family (pRb2) which binds to the E1A transforming domain. *Oncogene*, 8: 2561-2566
- McCright B, Rivers AM, Audlin S, and Virshup DM. (1996)** The B56 family of protein phosphatase 2A (PP2A) regulatory subunits encodes differentiation-induced phosphoproteins that target PP2A to both nucleus and cytoplasm. *The Journal of Biological Chemistry*, 271: 22081-22089
- Mihara K, Cao XR, Yen A, Chandler S, Driscoll B, Murphree AL, T'Ang A, and Fung YK. (1989)** Cell cycle-dependent regulation of phosphorylation of the human retinoblastoma gene product. *Science*, 246: 1300-1303
- Miskolczi P, Lendvai A, Horváth VG, Pettkó-Szandtner A, and Dudits D. (2007)** Conserved functions of retinoblastoma proteins: from purple retina to green plant cells. *Plant Science*, 172: 671-683
- Mumby M. (2007)** The 3D structure of protein phosphatase 2A: new insights into a ubiquitous regulator of cell signaling. *ASC Chemical Biology*, 2(2): 99-103
- Murashige T, and Skoog F. (1962)** A revised medium for rapid growth and bioassays with tobacco tissue culture. *Plant Physiology*, 15: 473-497
- Nair JS, Ho AL, Tse AN, Coward J, Cheema H, Ambrosine G, Keen N, and Schwartz GK. (2009)** Aurora B kinase regulates the postmitotic endoreduplication checkpoint via phosphorylation of the retinoblastoma protein at serine 780. *Molecular Biology of the Cell*, 20: 2218-2228
- Nakagami H, Kawamura K, Sugisaka K, Sekine M, and Shinmyo A. (2002)** Phosphorylation of retinoblastoma-related protein by the cyclin D/ cyclin-dependent kinase complex is activated at the G1/S-phase transition in tobacco. *The Plant Cell*, 14: 1847-1857



- Nakagami H, Sekine M, Murakami H, and Shinmyo A. (1999)** Tobacco retinoblastoma-related protein phosphorylated by a distinct cyclin-dependent kinase complex with Cdc2/cyclin D *in vitro*. *Plant Journal*, 18: 243-252
- Nelson DA, Krucher NA, and Ludlow JW. (1997)** High molecular weight protein phosphatase type 1 dephosphorylates the retinoblastoma protein. *Journal of Biological Chemistry*, 272: 4528–4535
- Nevins J. (1998)** Toward an understanding of the functional complexity of the E2F and retinoblastoma families. *Cell Growth & Differentiation*, 9: 585–593
- Ortega S, Malumbres M, and Barbacid M.(2002)** Cyclin D-dependent kinases, INK4 inhibitors and cancer. *Biochimica et Biophysica Acta*, 1602: 73-87
- Poznic M. (2009)** Retinoblastoma protein: a central processing unit. *Journal of Biosciences*, 43: 305-312
- Sabelli PA, Dante RA, Leiva-Neto JT, Jung R, Gordon-Kamm WJ, and Larkins BA. (2005)** RBR3, a member of the retinoblastoma-related family from maize, is regulated by the RBR1/E2F pathway. *Proceedings of the National Academy of Sciences of the USA*, 102: 13005–13012
- Sabelli PA, Hoerster G, Lizarraga LE, Brown SW, Gordon-Kamm WJ, and Larkins BA. (2009)** Positive regulation of minichromosome maintenance gene expression, DNA replication, and cell transformation by a plant retinoblastoma gene. *Proceedings of the National Academy of Sciences of the USA*, 106: 4042-4047
- Sablina AA, Hector M, Colpaert N, and Hahn WC. (2010)** Identification of PP2A complexes and pathways involved in cell transformation. *Cancer Research*, 70(24): 10474-10484
- Schmitz MH, Held M, Janssens V, Hutchins JR, Hudecz O, Ivanova E, Goris J, Trinkle-Mulcahy L, Lamond AI, Poser I, Hyman AA, Mechtler K, Peters JM, and Gerlich DW. (2010)** Live-cell imaging RNAi screen identifies PP2A-B55alpha and importinbeta as key mitotic exit regulators in human cells. *Nature Cell Biology*, 12: 886-893
- Sherr CJ, and Roberts JM. (1999)** CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes & Development*, 13: 1501-1512
- Shimizu-Sato S, Ike Y, and Mori H. (2008)** PsRBR1 encodes a pea retinoblastoma-related protein that is phosphorylated in axillary buds during dormancy-to-growth transition. *Plant Molecular Biology*, 66: 125-135
- Shirodkar S, Ewen M, DeCaprio JA, Morgan J, Livingston DM, and Chittenden T. (1992)** The transcription factor E2F interacts with the retinoblastoma product and p107 cyclin A complex in a cell cycle regulated manner. *Cell*, 68: 157-166

- Tamrakar S, and Ludlow JW. (2000)** The carboxyl-terminal region of the retinoblastoma protein binds non-competitively to protein phosphatase type 1 $\alpha$  and inhibits catalytic activity. *The Journal of Biological Chemistry*, 275: 27784-27789
- Taya Y. (1997)** RB kinases and Rb-binding proteins: new points of view. *Trends in Biochemical Sciences*, 22: 14-17
- Terol J, Bagues M, Carrasco P, Perez-Alonso M, and Paricio N. (2002)** Molecular characterization and evolution of the protein phosphatase 2A B' regulatory subunit family in plants. *Plant Physiology*, 129: 808-822
- Usui H, Inoue R, Tanabe O, Nishito Y, Shimizu M, Hayashi H, Kagamiyama H, and Takeda M. (1998)** Activation of protein phosphatase 2A by cAMP-dependent protein kinase-catalyzed phosphorylation of the 74-kDa B" (delta) regulatory subunit in vitro and identification of the phosphorylation sites. *FEBS Letters*, 430: 312-316
- Vietri M, Bianchi M, Ludlow JW, Mittnacht S, and Villa-Moruzzi E. (2006)** Direct interaction between the catalytic subunit of Protein Phosphatase 1 and pRb. *Cancer Cell International*, 6: 3
- Voorhoeve PM, Hijmans EM, and Bernards R. (1999b)** Functional interaction between a novel protein phosphatase 2A regulatory subunit, PR59, and the retinoblastoma-related p107 protein. *Oncogene*, 18: 515-524
- Voorhoeve PM, Watson RJ, Farlie PG, Bernards R, and Lam EW. (1999a)** Rapid dephosphorylation of p107 following UV irradiation. *Oncogene*, 18: 679-688
- Vuocolo S, Purev E, Zhang D, Bartek J, Hansen K, Soprano DR, and Soprano KJ. (2003)** Protein phosphatase 2A associates with Rb2/p130 and mediates retinoic acid-induced growth suppression of ovarian carcinoma cells. *The Journal of Biological Chemistry*, 278: 41881-41889
- Weinberg RA. (1995)** The retinoblastoma protein and cell cycle control. *Cell*, 81: 323-330
- Wildwater M, Campilho A, Perez-Perez JM, Heidstra R, Blilou I, Korthout H, Chatterjee J, Mariconti L, Grissem W, and Scheres B. (2005)** The retinoblastoma-related gene regulates stem cell maintenance in Arabidopsis roots. *Cell*, 123: 1337-1349
- Wlodarchak N, Guo F, Satyshur KA, Jiang L, Jeffrey PD, Sun T, Stanevich V, Mumby MC, and Xing Y. (2013)** Structure of the Ca<sup>2+</sup>-dependent PP2A heterotrimer and insights into Cdc6 dephosphorylation. *Cell Research*, 23: 931-946
- Xiao ZX, Ginsberg D, Ewen M, and Livingston DM. (1996)** Regulation of the retinoblastoma protein-related protein p107 by G1 cyclin-associated kinases. *Proceedings of the National Academy of Sciences of the USA*, 93: 4633-4637

- Xie Q, Sanz-Burgos AP, Hannon GJ, and Gutierrez C. (1996)** Plant cells contain a novel member of the retinoblastoma family of growth regulatory proteins. *The EMBO Journal*, 15: 4900-4908
- Xing Y, Igarashi H, Wang X, and Sakaguchi N. (2005)** Protein phosphatase subunit G5PR is needed for inhibition of B cell receptor-induced apoptosis. *Journal of Experimental Medicine*, 202: 707-719
- Xu Z, and Williams BR. (2000)** The B56alpha regulatory subunit of protein phosphatase 2A is a target for regulation by double-stranded RNA-dependent protein kinase PKR. *Molecular and Cellular Biology*, 20: 5285-5299
- Yan Y, and Mumby MC. (1999)** Distinct roles for PP1 and PP2A in phosphorylation of the retinoblastoma protein. *The Journal of Biological Chemistry*, 274: 31917-31924
- Yan Z, Fedorov SA, Mumby MC, and Williams RS. (2000)** PR48, a novel regulatory subunit of protein phosphatase 2A, interacts with Cdc6 and modulates DNA replication in human cells. *Molecular and Cellular Biology*, 20:1021–1029
- Yu P, Abraham E, Farkas I, Darula Z, Varga E, Lukacs N, Ayaydin F, Medzihradszky KF, Dombradi V, Dudits D, and Horvath GV. (2015)** The B'' regulatory subunit of protein phosphatase 2A mediates the dephosphorylation of rice retinoblastoma-related protein-1. *Plant Molecular Biology*, 87: 125-141
- Yu RM, Wong MM, Jack RW, and Kong RY. (2005)** Structure, evolution and expression of a second subfamily of protein phosphatase 2A catalytic subunit genes in the rice plant (*Oryza sativa* L.). *Planta*, 222: 757-768
- Zarkowska T, and Mitnacht S. (1997)** Differential phosphorylation of the retinoblastoma protein by G1/S cyclin dependent kinases. *The Journal of Biological Chemistry*, 272: 12738–12746
- Zhu L, van der Heuvel S, Helin K, Fattaey A, Ewen M, Livingston D, Dyson N, and Harlow E. (1993)** Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. *Genes & Development*, 7: 1111-1125

## 8. SUMMARY

### Introduction

The ability of the pRB (retinoblastoma protein) to inhibit cellular proliferation is counterbalanced by the action of CDKs. In quiescent and early G1 cells, the RB protein is in a non-phosphorylated and efficiently E2F binding state. The pRB-E2F complex down-regulates the activity of E2F and prevents E2F-dependent transcription. pRB becomes phosphorylated as the cell progresses towards the S phase. Phosphorylation of pRB inactivates the pRB-E2F complex and releases E2F, allowing the expression of E2F-regulated genes required for cell cycle progression and S phase entry. The initial phosphorylation of pRB is most likely catalyzed by Cyclin D-CDK4 or Cyclin D-CDK6 complexes. Subsequently, Cyclin E-CDK2 complexes phosphorylate the pRB protein further, increasing the phosphorylation level. During mitosis, pRB is rapidly dephosphorylated by a specific mitotic phosphatase that removes the phosphate group from the pRB protein, thus restoring the dephosphorylated or hypophosphorylated form of the pRB protein at the start of the G1 phase.

Phosphorylation of plants RBR (RB-related protein) also occurs in a cell-cycle-dependent manner. The highest phosphorylation state occurs during the G1-S transition and is maintained till mid/late S and G2 phases. Considering the phylogenetic conservation of several cell cycle regulation elements (CDKs; cyclins) between animals and plants, this phosphorylation also results in an inactivation of RBR and the subsequent release of E2F-DP transcription factors in their active form.

Protein phosphatases types 1 and 2A (PP1 and PP2A) have been implicated in the control of cell cycle events in yeast and mammalian cells and have been characterized as RB or RB-like protein phosphatase. Yan et al. (1999) proposed that PP1 is involved in the direct dephosphorylation of pRB while protein phosphatase 2A (PP2A) is involved in the dephosphorylation components of pathways that play a role in the regulation of G1 cyclin-dependent kinase activity. PP2A may also have an important cell division regulatory role, since its activity contributes to the control of mitotic kinases and microtubule organization in alfalfa (Ayaydin et al., 2000).

PP2A consists of three distinct functional components: a structural core subunit (PP2A<sub>α</sub>/PR65), a catalytic subunit (PP2A<sub>α</sub>) and a B regulatory subunit. A and C subunits usually bind each other to form the core enzyme PP2A<sub>D</sub>. The binding of different B subunits to the AC core

enzyme generates a broad variety of holoenzymes thereby regulating phosphatase activity and substrate specificity (Janssens and Goris, 2001). The ability of the PP2A holoenzyme to dephosphorylate specific intracellular proteins depends on its activity and appropriate intracellular localization, which in turn is largely determined by the nature of its regulatory subunit.

In a previous yeast two-hybrid screen in our lab, a gene product from *Medicago truncatula* was identified as interactor of the full-length MsRBR11 protein. This protein shares 39% identity and 57% homology with the murine PR59 PP2A regulatory subunit, and strongly associates with OsRBR1 but not with OsRBR2 (Lendvai et al., 2007). It is interesting to note that a similar selectivity occurred among Murine RBs. Only the p107 protein strongly interacts the PR59 PP2A B'' regulatory subunit while pRB did not show association (Voorhoeve et al., 1999b). This analogous behavior also supports the assumption that plant RBRs belonging to different subfamilies (see 1.2.1) have different roles in the regulation of plant cell division and differentiation (Dudits et al., 2011).

In our work, the rice homologue of the *MsPP2A B''* gene, *OsPP2A B''*, has been isolated. The pair wise interaction studies confirmed that the OsPP2A B'' regulatory subunit interacts specifically with the OsRBR1 protein.

### **Aims of study**

Initially we were looking for the specific antibodies for non-phosphorylated and phosphorylated forms of RBR proteins of *Medicago* and rice, which will help to monitor the protein during the cell cycle of cultured cells and detect the presence of the proteins in all other experiments. For a deeper understanding of the molecular basis of these divergent functions of plant RBR proteins it is essential to know the cell cycle phase-dependent changes in levels and post-translational modifications of these proteins.

The results of previous work indicated the specific interaction between OsPP2A B'' and OsRBR1. We were wondering which domain within the OsRBR1 essential for the formation of OsPP2A B''-OsRBR1 complex and if the EF-hand of OsPP2A B'' important for the binding. In addition, for the OsPP2A B'' regulatory subunit, we were interested in its post-transcriptional modification.

To sum up, we focused on the following points:

- 1) Measure the cell cycle phase-dependent variation in the amounts of the phospho-MsRBR11 and the phospho-OsRBR1 proteins, which helps to understand the differences in the phosphorylation of RBR proteins in monocots and dicots.
- 2) To analyse the interaction between OsRBR1 and the OsPP2A B'' regulatory proteins *in vivo* and *in vitro*.
- 3) To understand which domains in both OsRBR1 and the OsPP2A B'' regulatory proteins are important for their interaction.
- 4) Based on the results of the computer-assisted phosphorylation site analysis, to study the effect of phosphorylation on the function of the OsPP2A B'' regulatory protein:
  - identify the phosphorylated amino acid residues;
  - study the effect of phosphorylation on OsRBR1 binding and phosphatase
  - activity using non-phosphorylatable and phosphorylation-mimicking mutants.

## Resources and Methods

- Construction of *Oryza sativa* var. japonica cv. UNGGI-9 cDNA libraries for yeast two hybrid screening
- Yeast two-hybrid screens, pairwise interaction analysis and the interaction quantitation ( $\beta$ -galactosidase activity).
- Plant suspension culture synchronization (alfalfa and rice) and flow cytometry.
- GST- and His-tagged proteins purification
- Protein phosphorylation (deletion mutants and site-directed mutants of B'' regulatory subunit)
- Immunoprecipitation
- Protein phosphatase activity (OsPP2A heterotrimeric holoenzyme contains different B'' regulatory subunit: wild type, non-phosphorylatable and phosphorylation-mimicking mutants) under different conditions

## Results and short discussion

Our work started with the testing of the specific antibodies for MsRBR1/OsRBR1 and phospho-MsRBR1/OsRBR1 detection. The anti-AtRBR1 antibody produced against the recombinant C-terminal fragment of the Arabidopsis AtRBR1 protein consisting of 236 amino acids (Horváth et al., 2006). The antigen competition experiment supports the use of this antibody in the alfalfa experimental system. Since the NVYVSPLRS motif in the C-terminus of the alfalfa MsRBR1 protein may correspond to the NIYISPLKS motif of the human pRB protein which has a conserved 807/811serine (S) phosphorylation site. It has the possibility that the level of phospho-MsRBR1 can be monitored using an antibody produced against the phosphorylated pRB peptide containing Ser807/811. Experiments carried out to phosphorylate the recombinant His<sub>6</sub>-tagged C-terminal fragment of the MsRBR1 protein, and then monitored the phosphorylation using the antibody and the incorporation of <sup>32</sup>P in parallel. Two parameters agree each other quite well; this means the antibody can be used to detect the phosphorylated MsRBR1 protein. Similar experiments carried out on rice found that the monoclonal antibody produced against the recombinant N-terminal fragment of the OsRBR1 and the human anti-phospho-pRB antibody can be used in rice experimental system as well.

Probed by those antibodies, we checked the variation of RBR and phospho-RBR proteins in alfalfa and rice cultured cells with cell-cycle dependent manner. Total amount of MsRBR1/OsRBR1 barely changed throughout the cell cycle, while phosphorylated forms of MsRBR1/OsRBR1 proteins showed clearly cell cycle dependent changes.

Since OsRBR1 associates with the OsPP2A B'' regulatory subunit both *in vivo* and *in vitro*, we were wondering about the segment of the binding domain in OsRBR1 necessary for the formation of the complex. Yeast two-hybrid experiments between a set of deletion mutants of OsRBR1 and the OsPP2A B'' regulatory subunit demonstrated that an intact B pocket domain is essential and sufficient for the interaction. The C terminus of OsRBR1 seems to be unnecessary for interaction.

The sequence analysis of the identified OsPP2A B'' (*Os10g476600*) revealed that it contained two EF-hand domains which may potentially regulate its function by Ca<sup>2+</sup>-binding. To determine if the EF-hand domain is necessary for the formation of the OsRBR1/OsPP2A B'' complex, yeast two-hybrid experiments were carried out with the wild type OsRBR1 and a series of delete mutants of OsPP2A B'' (just minus C-terminal, minus C-terminal and EF2, minus C-terminal and two EF-hand,

fragment only with C-terminal). It was clearly shown that none of the truncated mutants can bind to OsRBR1. This result confirms the importance of the C-terminal region of OsPP2A B' but also verified that the C-terminal itself does not suffice for combination. The result showed that the full length OsPP2A B' is strongly self-activated, which may be related to the acidity character of the C-terminal region.

On the basis of all the yeast two-hybrid data we can safely conclude that the interaction between the OsRBR1 and OsPP2A B' proteins requires an intact B pocket domain of the RBR protein and the integrity of B' regulatory subunit. Such a finding supports the hypothesis that the dephosphorylation of plant RBR proteins is influenced by the increase in intracellular  $\text{Ca}^{2+}$ ; responding in this way to extracellular stimuli, like e.g. environmental stress factors. Impairment of the interaction between the B' regulatory subunit and OsRBR1 by low concentration of  $\text{Ca}^{2+}$  concentrations provides another piece of evidence in support of this hypothesis.

Co-immunoprecipitation and recombinant protein phosphorylation experiments demonstrated that the OsPP2A B' regulatory subunit could be phosphorylated by PSTAIRE motif containing CDKs. Computer-assisted analysis revealed the presence of three putative CDK phosphorylation sites in the N-terminal region of the protein. A proteomic method proved that Ser95 and Ser119 can be phosphorylated but could not prove the Ser102 because of technique problem; phosphorylation studies on a series of deletion mutants confirmed the Ser95 and Ser119, and verified Ser102 at the same time.

Elimination of protein phosphorylation by serine to alanine exchange is a quite popular method used to verify putative phosphorylation sites. A series of site-directed mutants of the OsPP2A B' regulatory subunit was created. The results of mutants' phosphorylation showed that single mutants, double mutants, and triple mutant phosphorylated in apparently different levels. The experiment confirmed three predicted phosphorylation sites, and also revealed that there are no more potential phosphorylation sites within the OsPP2A B' regulatory subunit.

The triple alanine mutant (TMutA) and phosphorylation mimicking mutant (TMutE) were used to check the PP2A holoenzyme complex assembly and the interaction with OsRBR1. The results agreed with those of Davis (2005): mutations of the phosphorylation sites, by either ablating the phosphorylation site or producing a phosphorylation mimic, had no apparent effect on binding of



PR70 to the PP2A core dimer (A and C subunits). Data from pull down assays and yeast two-hybrid interaction assays between pBD-Gal4-OsPP2A B'' mutants and pAD-Gal4-OsRBR1 also supported the observation that these site-directed mutations did not affect the association between B'' and the catalytic subunit, but non-phosphorylation of B'' does impact on the interaction to OsRBR1. It seems that phosphorylation of B'' regulatory subunit does not play a role in the combination of PP2A holoenzyme but influence the affinity of OsRBR1.

Under standard condition and low concentration of free  $\text{Ca}^{2+}$  condition, TMutA contained PP2A holoenzyme showed lower phosphatase activity to PP2A<sub>Twt</sub> while PP2A<sub>TmE</sub> showed higher phosphatase activity than the PP2A<sub>Twt</sub>, which means that the phosphorylation of B'' indeed elevates the activity of the PP2A heterotrimeric holoenzyme. This experiment also indicated the effect of  $\text{Ca}^{2+}$  on the activity of PP2A, e.g. proved the importance of post-transcriptional regulation of the B regulatory subunit.

## 9. ÖSSZEFOGLALÁS

### Bevezetés

A retinoblasztóma fehérjének (pRB) a sejtosztódásban játszott gátló szerepét többek között a ciklinfüggő kinázok által végrehajtott foszforiláció befolyásolja. Nyugvó (G0) és korai G1 fázisú sejtekben a pRB hipofoszforilált állapotú és képes az E2F-DP transzkripciós faktor heterodimerek kötésére, ami gátolja az E2F-függő transzkripciót. A pRB fehérje a sejtciklus S fázisába történő progressziójával foszforilálódik, ez a poszttranszlációs módosítás felszabadítja az E2F transzkripciós faktorokat a gátlás alól és lehetővé teszi a sejtciklus továbbhaladásához szükséges célgének átíródását. Emlős sejtekben a kezdeti foszforilációt a ciklin D-CDK4 és ciklin D-CDK6 kináz komplexek végzik, míg ezt követően a ciklin E-CDK2 kináz tovább növeli a pRB foszforiláltságát. A mitózis alatt a foszfo-RB gyorsan defoszforilálódik specifikus foszfatáz(ok) által, visszaállítva a nem- vagy hipofoszforilált pRB túlsúlyát az új G1 fázis kezdetére.

A növényi RB-szerű (RBR, RB-related) foszforilációja ugyancsak sejtciklus függő módon történik, a hiperfoszforilált RBR fehérjék megjelennek a G1/S átmenetkor és ez a magas foszforiláltság jellemző a G2 fázis végéig. Tekintettel a sejtciklust szabályozó enzimek és folyamatok jelentős konzerváltságára elmondható, hogy ez az RBR foszforiláció a növények esetében is az E2F kötési képesség csökkenését eredményezi, felszabadítva az E2F-DP transzkripciós faktorokat aktív formájukban.

Az 1 és 2A típusú foszfoprotein foszfatázok (PP1 és PP2A) szabályozó szerepe jól ismert az élesztő és emlős sejtek sejtciklusában és néhány ilyen foszfatáz spéciesz foszfo-RB és foszfo-RBR defoszforilációs képességgel bír. Yan és mtsai (1999) kimutatták, hogy a PP1 szerepet játszik a pRB közvetlen defoszforilációjában, míg a PP2A foszfatázok indirekt módon szabályozzák a G1 fázisú CDK komplexek aktivitását. A PP2A foszfatázoknak fontos szerepük van a mitotikus kinázok szabályozásában és a mikrotubulusok szerveződésében, ahogy ezt Ayaydin és mtsai (2000) lucernában kimutatták.

A PP2A típusú foszfatázok heterotrimer fehérjekomplexek, melyek egy szerkezeti alegységből (PP2Aa/PR65), egy katalitikus alegységből (PP2Ac) és egy B típusú regulátor alegységből (B, B', B'' vagy B''') állnak. Az A és C alegységek asszociációjával alakul ki az

úgynevezett mag ("core") komplex, ehhez kötődnek a különböző B regulátor alegységek széles körű szubsztrátspecifitással bíró holoenzimeket létrehozva. A PP2A holoenzimek foszfatáz aktivitásának specifikussága nagyban függ nemcsak a szubsztrát iránti affinitásuktól, de a sejten belüli lokalizációjuktól is, ezeket a tulajdonságukat túlnyomó mértékben a B regulátor alegység természete határozza meg.

Egy előzetes élesztő két-hibrid könyvtár szűrési kísérletben a *Medicago sativa* MsRBR1 fehérjéjének erős kölcsönhatóját azonosítottuk. Ez a kölcsönható fehérje aminosav sorrendjében 39 %-os azonosságot és 59 %-os hasonlóságot mutatott az egér PR59 PP2A regulátor alegységgel és erős kölcsönhatást mutatott a rizs OsRBR1 fehérjével, de nem asszociált az OsRBR2 homológgal (Lendvai és mtsai, 2007). Hasonlót tapasztalhatunk az egér RB spécieszek esetében is: csak a p107 fehérje képes erős kölcsönhatásra a PR59 PP2A B" regulátor alegységgel, a pRB nem mutat ilyen asszociációt (Voorhoeve és mtsai, 1999b). Ez a hasonlóság alátámasztja azt a feltevést, hogy a növényi RBR változatok (lásd 1.2.1. fejezet) különböző szereppel bírnak a növényi sejtosztódás és differenciálódás során (Dudits és mtsai, 2011).

Munkám során az előzetesen azonosított MsPP2A B" gén rizs homológját, az OsPP2A B"-t izoláltam és jellemeztem a kódolt protein OsRBR1-el történő kölcsönhatását és ennek következményeit.

### **Célkitűzések**

Első feladatom volt a *Medicago sativa* és *Oryza sativa* RBR fehérjéket (foszforilált és nem foszforilált formájukban) specifikusan felismerő ellenanyagok vizsgálata, mivel ezek segítségével követhettük az egyes fehérje spécieszek mennyiségének és a foszforilációs szintjüknek változását a sejtciklus progressziója során.

A következőekben az OsPP2A B" regulátore alegység és az OsRBR1 fehérje közötti specifikus kölcsönhatást vizsgáltam. Kíváncsi voltam arra, hogy a két fehérje mely doménjei szükségesek ehhez az asszociációhoz, valamint arra, hogy az OsPP2A B" feltételezett poszttranszlációs módosítása milyen hatással van a fehérje funkciójára.

Munkám során az alábbi fő célkitűzésekre koncentráltam:

1. Megfigyeltem az MsRBR1 és OsRBR1 fehérjék foszforilációjának változását a sejtciklus különböző fázisaiban, különös figyelemmel arra, hogy mutat-e ez valamilyen különbséget az egyszikű és kétszikű modellnövényeinkben.

2. Analizáltam az OsRBR1 és OsPP2A B" fehérjék *in vitro* és *in vivo* kölcsönhatását.

3. Megvizsgáltam, hogy a fenti fehérjék mely doménjei szükségesek a kölcsönhatáshoz.

4. A komputeres foszforilációs hely azonosítást követően megkezdtem az OsPP2A B" foszforilációjának kísérletes bizonyítását, elemeztem ennek hatását az OsRBR1 fehérje kötésére és a PP2A holoenzim aktivitására. Ez utóbbi tulajdonságokat még nem foszforilálható és konstitutív foszforilációt utánzó mutáns fehérjék segítségével is megvizsgáltam.

### **Felhasznált anyagok és módszerek**

- *Oryza sativa* var. japonica cv. UNGGI-9 növényekből és szuszpenziós kultúrából cDNS könyvtár létrehozása az élesztő két-bibrid rendszer vektorában.

- A könyvtár szűrése az élesztő két-hibrid rendszerben, páronkénti kölcsönhatási vizsgálatok, a kötési erősség kvantitálása  $\beta$ -galactozidáz méréssel.

- A szuszpenziós kultúrák sejtciklus szinkronizálásának optimalizálása, áramlásos citometriás mérések.

- GST és His<sub>6</sub> fúziós rekombináns fehérjék termeltetése.

- a deléciós és helyspecifikus mutáns OsPP2A B" fehérjék foszforilációjának vizsgálata.

- Ko-immunoprecipitáció.

- A különbözőképpen módosított OsPP2A B" fehérjét tartalmazó holoenzimek foszfoprotein foszfatáz aktivitásának vizsgálata.

### **Eredmények és rövid megvitatásuk**

Munkám a MsRBR1/OsRBR1 fehérjéket és ezek foszforilált formáit felismerő ellenanyagok jellemzésével kezdődött. Az anti-AtRBR1 ellenanyag az Arabidopsis AtRBR1 fehérjéjének C-terminális 236 aminosavas régiója ellen készült (Horváth mtsai, 2006). Az antigén kompetíciós kísérleteink alátámasztották ennek az ellenanyagnak lucerna rendszerben történő használatát. Az

MsRBR1 fehérje C-terminálisán található NVYVSPLRS motívum megfeleltethető az emberi pRB fehérje NIYISPLKS motívumának, mely foszforilált változatát felismerő ellenanyag (foszfo-hsRb Ser(807/811) kereskedelmi forgalomban van. Kísérleteket végeztünk, melyekben az MsRBR1 His<sub>6</sub>-C-terminális fragmentjének foszforilációját követtük radioaktív <sup>32</sup>P jelöléssel és a rendelkezésre álló foszfo-pRB ellenanyaggal. Az egybevágó eredmények alapján megállapítottuk, hogy az ellenanyagunk jól használható a foszfo-MsRBR1 szintjének detektálására. Hasonló kísérleteket végeztünk a rizs OsRBR1 fehérjével és ennek foszforilált C-terminális szakaszával is, megállapítván, hogy a tisztított monoklonális anti-OsRBR1 ellenanyag specifikusan csak ezt a rizs retinoblasztóma fehérje fajtát ismeri fel, illetve a foszfo-pRB ellenanyag a rizs rendszerben is jól használható.

Ezek után megvizsgáltuk az RBR és foszfo-RBR szintek változását szinkronizált lucerna és rizs szuszpenziós kultúrákban. Megállapítottuk, hogy míg az MsRBR1 és OsRBR1 fehérjék szintje alig változik a sejtciklus progressziója során, addig a foszfo-RBR szintje erőteljes, az elmélet alapján elvárható sejtosztódási fázis függőséget mutat.

Miután kimutattuk, hogy az OsRBR1 és OsPP2A B" fehérjék *in vitro* és *in vivo* is kölcsönhatnak, kíváncsi voltam, hogy az OsRBR1 mely doménja felelős az asszociációért. A deléciós mutánsokkal elvégzett élesztő két-hibrid kísérlet megmutatta, hogy a fehérje B "zseb" doménje szükséges és elegendő ehhez a kölcsönhatáshoz, míg az OsRBR1 C-terminális régiójának jelenléte nem szükséges.

Az OsPP2A B" regulátor alegység aminosavsorrendjének analízise megmutatta, hogy a fehérje két, valószínűleg kalciumkötési képességgel rendelkező EF-hand domént tartalmaz. Annak meghatározására, hogy ezek az EF-hand domének szükségesek-e az OsRBR1 kötéséhez, egy sorozat B" deléciós mutánsokkal kötési kísérletet végeztem az élesztő két-hibrid rendszerben. Mint az kiderült, egyetlen csonka változat sem tudta az OsRBR1-et megkötni. Ez a kísérlet jól demonstrálta az OsPP2A B" C-terminálisának fontosságát a kölcsönhatásban, de azt is bebizonyította, hogy önmagában ez a domén nem elegendő az asszociációhoz. Az élesztő két-hibrid rendszer másik vektorába klónozva kiderült, hogy az OsPP2A B" erősen transzaktivál, ami magyarázható a fehérje C-terminálisának savas karakterével.

A rendelkezésre álló élesztő két-hibrid adatok alapján megállapítottam, hogy az OsRBR1 és az OsPP2A B" fehérjék kölcsönhatásához az előbbi intakt B doménje és az utóbbi teljes hosszúságú

proteinje szükséges. Az EF-hand motívumok jelenléte a kalcium ionok szerepét mutatja az RBR defoszforilációjában (erre a dolgozatomban kísérletes bizonyítékot is megadok), ami összeköti a sejtciklus szabályozását a környezeti stresszfactorokkal.

Ko-immunoprecipitációs és rekombináns fehérje foszforilációs kísérletekkel bizonyítottuk, hogy az OsPP2A B" regulátor alegység PSTAIRE motívumot tartalmazó CDK-kal foszforilálható. A számítógépes predikció alapján mindhárom foszforilációs hely a fehérje aminoterminálisára volt térképezhető. A proteomikai megközelítés technikai problémák miatt csak a Ser95 és Ser119 aminosavak foszforilációját mutatta ki, míg deléciós mutánsokkal mindhárom (Ser95, Ser102 és Ser119) szerinen történő foszforilációt bebizonyítottuk.

A betérképezett szerin aminosav maradékok alaninra történő cseréje a foszforilálhatósági vizsgálatok jól bevált szköze, így hasonló változtatást az OsPP2A B" regulátor alegységen is elvégeztem. A rekombináns fehérjéken elvégzett kináz reakciók bizonyították az egyszeres, kétszeres és háromszoros cserét tartalmazó fehérjeváltozatok eltérő foszforilálhatóságát, bebizonyították az előző vizsgálatok eredményét és azt, hogy a feltételezett három foszforilálható helyen kívül nincs több a fehérje teljes hosszán.

A háromszoros alanin mutánst (TMutA) és a konstitutív foszforilációt utánzó háromszoros glutamát mutánst (TMutE) használtuk a foszforiláció hatásának vizsgálatára mind a PP2A holoenzim összeszerelődése, mind az OsRBR1 fehérje kötése szempontjából. Az eredmények jó egyezést mutattak a Davis által publikáltakkal (2005): a foszforilációs helyek megváltoztatása nem okoz számottevő különbséget a B" regulátor alegység és a PP2A mag asszociációjában. Élesztő két-hibrid mérések és "pull-down" vizsgálatok azt mutatták, hogy a mutációk nem változtatták meg számottevően a B" és a katalitikus alegység kapcsolatát, de hatással voltak a regulátor alegység és az OsRBR1 kölcsönhatására.

Alacsony szabad  $\text{Ca}^{2+}$  jelenlétében a TMutA változatot tartalmazó holoenzim mind a vad típusú enzimhez képest, mind a TMutE formához képest alacsonyabb defoszforilációs aktivitást mutatott foszfo-OsRBR1 szubsztrát felé, a TMutE formát tartalmazó holoenzimmal pedig magasabb foszfatáz aktivitást mérhettünk. A kísérlet másik része rávilágított a  $\text{Ca}^{2+}$  szerepére a folyamatban, kapcsolatot létesítve a  $\text{Ca}^{2+}$  jelátvitel és a sejtciklus szabályozása között.

## 10. APPENDICES

**Table 1. Primers used in this thesis**

Number	Oligo	Sequence of the oligo (5'-3') *
1	OsRBR1 BamHI Fw	CAGGATCCATGGAGGGTGCCGCGCC
2	OsRBR1 BamHIA-Fw	TTGGATCCATGGCTCCTATCACCCCAGTGA
3	OsRBR1 EcoRIB-Fw	GCGAATTCAGGAATGTGCTGGTGGACAAC
4	OsRB1 EcoRI C-Fw	CGGAATTCCTTGCTGGTTGAGCTAAGG
5	OsRBR1 SalI Rw-1	CGGTCGACTATGTTGGCCTGGCAACAAT
6	OsRBR1 SalI Rw-2	CGGTCGACTACTGATCGATGTGCCGATT
7	OsRBR1 SalI Rw-3	CGGTCGACTATGGACCATCAGCAGCACA
8	OsRBR1 SalI Rw-4	CGGTCGACCTAGCAGTCTGGCTGCTC
9	OsRBR1EcoRI –Fw	TCGAATTCATGGAGGGTGCCGCGCCGCCA
10	OsRBR1SalI N-Rev	CCGTCGACGCTCACTGGGGTGTGATAGG
11	OsRBR2EcoRI –Fw	CGGAATTCATGGCGTCACAGCCTCC
12	OsRB2 SalI C-Rev	CGGTCGACTCAAGAGTCCGTTTTGCT
13	OsPP2A FL-EcoFw	TTGAATTCATGGAGGTGGAGGCGGCGA
14	OsPP2A FL-SalIRev	GCGTCGACTTCAGCCAGCAAGAGATAT

15	OsPP2A DelN1Fw	CC <u>GAATTC</u> ATGAGGCGAGGATCT
16	OsPP2A DelN2Fw	CC <u>GAATTC</u> ATGGTTAGTGAACCAGTG
17	OsPP2A DelC1Rev	TAG <u>TCGACT</u> CATCCTCGCCTCATAAC
18	OsPP2A DelC2Rev	TAG <u>TCGACT</u> CACTCCCTCACTGGTTCACT
19	OsPP2A SamFw	TT <u>CCCGGG</u> TTATGAGGCGAGGATC
20	OsPP2A SamRev	TAC <u>CCCGGG</u> GTGAGCCAGAAGTGCTTC
21	OsPP2A Mut1Rev	TAC <u>CCCGGG</u> GTGCGCCAGAAGTGCTTC
22	OsPP2A Mut2Re	TAC <u>CCCGGG</u> GTGAGCCAGAAGTGCTTCTAGGA GCAAGGGGAGGAG
23	OsPP2A Mut3Rev	TAC <u>CCCGGG</u> GTGCGCCAGAAGTGCTTCTAGGA GCAAGGGGAGGAG
24	OsPP2A Mut4Fw	TT <u>CCCGGG</u> TTATGAGGCGAGGATCTGGAGCT GGACCATCATCCTTGGAGCTCCACTAAAAGT AG
25	OsPP2A DMutERev	TAC <u>CCCGGG</u> GTTCGCCAGAAGTGCTTCTAGGT TCAAGGGGAGGAG
26	OsPP2A MutEFw	TT <u>CCCGGG</u> TTATGAGGCGAGGATCTGGAGCT GGACCATCATCCTTGGGAGAACCACTAAAAG TAG
27	OsPP2A-EF Rev	AC <u>GTCGAC</u> CAGCTGCTGCATCGCAGC



28	OsPP2A(EF1) Rev	CG <b><u>GTCGACT</u></b> CACTCGGATGATTTGTCTTCCTC AG
29	OsPP2A(EF2) Rev	TT <b><u>GTCGACT</u></b> CACATGCACTCCATCCGGTGCA A
30	OsPP2A C Fw	GC <b><u>GAATTC</u></b> ATGCAATTTTCTATGAGGAGCA
31	OsRBR2 EcoRI C-Fw	CG <b><u>GAATTC</u></b> TTGCTGGTGGCTCTAATG
32	OsRBR2SalI N-Rev	CC <b><u>GTCGAC</u></b> CATGGCAGAAGTAACTGGTA
33	AD Forward	AGGGATGTTTAATACCACTAC
34	AD Reverse	GCACAGTTGAAGTGAAGTTGA

\* The engineered restriction sites in the primers are in bold face and underlined.

**Table 2. Combinations of the primers and description of the fragments.**

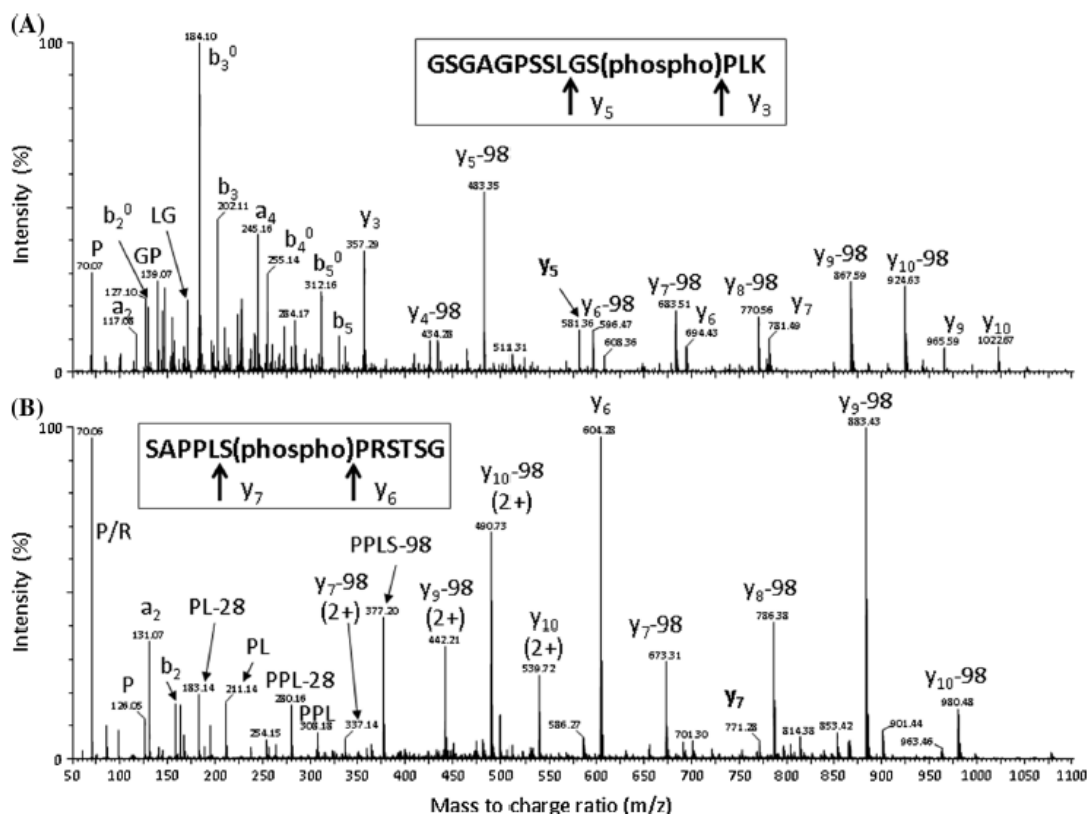
Primer combinations	Vector	Expressed protein
1+5	pGBT9	OsRBR1-fragment, containing the N-terminal region and A domain
1+6	pGBT9	OsRBR1-fragment, containing the N-terminal region, A domain and spacer region
1+7	pGBT9	OsRBR1-fragment, containing the N-terminal region, A domain, spacer region and B domain
1+8	pBD-GAL4	OsRBR1 full length

	2.1	
1+8	pGBT9	OsRBR1 full length
1+8	pET28a	OsRBR1 full length
2+7	pGBT9(+2)	OsRBR1 fragment, containing the A domain, spacer region, and B domain
3+7	pGBT9	OsRBR1 fragment, containing the B domain
9+10	pGBT9	OsRBR1 N-terminal region
4+8	pET28a	OsRBR1 C-terminal region
11+12	pBD-GAL4 2.1	OsRBR2 N-terminal region
13+14	pAD-GAL4 2.1	OsPP2A B'' regulatory subunit full length
13+14	pGDAT7	OsPP2A B'' regulatory subunit full length
13+14	pET28a	OsPP2A B'' regulatory subunit full length
13+14	pGEX-4T-1	OsPP2A B'' regulatory subunit full length
13+17	pET28a	OsPP2A B'' regulatory subunit fragment, containing the first and second CDK phosphorylation sites
13+18	pET28a	OsPP2A B'' regulatory subunit fragment, containing all three CDK phosphorylation sites, but just 375bp long.
15+14	pET28a	OsPP2A B'' regulatory subunit fragment, just containing the

		third CDK phosphorylation site. It is 1311bp long.
16+14	pET28a	OsPP2A B'' regulatory subunit fragment which does not contain any CDK phosphorylation sites. It is 1260bp long.
13+27	pGDAT7	OsPP2A B'' regulatory subunit lacking both EF hands
13+20&19+14	pET28a	OsPP2A B'' full length (nucleotide sequence changed, but amino acid sequence same as wild type)
13+21&19+14	pET28a	OsPP2A B'' M102 (second CDK phosphorylation site was mutated from serine into alanine)
13+21&19+14	pGAD424	OsPP2A B'' M102 (second CDK phosphorylation site was mutated from serine into alanine)
13+22&19+14	pET28a	OsPP2A B'' M95 (first CDK phosphorylation site was mutated from serine into alanine)
13+22&19+14	pGAD424	OsPP2A B'' M95 (first CDK phosphorylation site was mutated from serine into alanine)
13+23&19+14	pET28a	OsPP2A B'' DM95,102 (first and second CDK phosphorylation sites were mutated from serine into alanine)
13+23&19+14	pGAD424	OsPP2A B'' DM95,102 (first and second CDK phosphorylation sites were mutated from serine into alanine)
13+20&24+14	pET28a	OsPP2A B'' M119 (third CDK phosphorylation site was mutated from serine into alanine)
13+20&24+14	pGAD424	OsPP2A B'' M119 (third CDK phosphorylation site was mutated from serine into alanine)

13+22&24+14	pGAD424	OsPP2A B'' DM95,119 (first and third CDK phosphorylation sites were mutated from serine into alanine)
13+21&24+14	pGAD424	OsPP2A B'' DM102,119 (second and third CDK phosphorylation sites were mutated from serine into alanine)
13+23&24+14	pET28a	OsPP2A B'' TMutA (all three CDK phosphorylation sites were mutated from serine into alanine)
13+23&24+14	pGAD424	OsPP2A B'' TMutA (all three CDK phosphorylation sites were mutated from serine into alanine)
13+25&26+14	pET28a	OsPP2A B'' TMutE (all three CDK phosphorylation sites were mutated from serine into glutamate)
13+25&26+14	pGAD424	OsPP2A B'' TMutE (all three CDK phosphorylation sites were mutated from serine into glutamate)
13+28	pGDAT7	OsPP2A B'' regulatory subunit containing EF1 motif
13+29	pGDAT7	OsPP2A B'' regulatory subunit containing EF1 and EF2 motifs
30+14	pGDAT7	OsPP2A B'' regulatory subunit C-terminal region
31+12		OsRBR2 C-terminal region
33+34		Used for checking the fragments which inserted into pAD-Gal4

&: The insert was generated by the ligation of two PCR fragments.



**Figure 18. Characterization of CDK phosphorylation sites in OsPP2A B' subunit by LC-MS/MS analysis.**

(A) Collision induced dissociation (CID) spectrum of the precursor m/z 647.884(2+) representing GSGAGPSSLGS(phospho)PLK. The peptide is a tryptic fragment of His<sub>6</sub>-tagged OsPP2A B' phosphorylated by rice PSTAIRE-kinases. (B) CID spectrum of the precursor m/z 618.783(2+) representing SAPPLS(phospho)PRSTSG obtained by acidic treatment of the His<sub>6</sub>-tagged N-terminal fragment of OsPP2A B' phosphorylated by rice PSTAIRE-kinases. Peptide fragments are labeled according to the nomenclature (Biemann, 1990). b<sup>0</sup> stands for water loss of the corresponding b ions.