ENHANCED IN VITRO TRANSLATION FOR FUNCTIONAL ANALYSIS OF PROTEINS

PhD thesis outline

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1. Introduction

The availability of proteins of interest is a prerequisite for protein functional studies. The proteins investigated in this work were produced by the wheat germ cell-free proteins synthesis (CFPS) system.

To create an efficient *in vitro* translation system the cell extract containing the translation apparatus must be supplemented with the protein of interest coding nucleic acid template, amino acids, energy supplying components, cofactors, and other reagents. The pEU3-NII vector family developed for this system provides the mRNA by T7 polymerase-based *in vitro* transcription reaction and ensures the inevitable elements of eukaryotic mRNA, a tobacco mosaic virus omega enhancer sequence replacing the 5' cap and a 1626 nucleotide long 3' untranslated sequence instead of the poly(A) sequence. Sequences coding either for hexahistidine (His_6) or glutathione-S-transferase (GST) were inserted into the vectors to aid affinity purification and detection of the expressed proteins. Although the His_6- and GST-tag are two of the most generally used tags, they both have their own shortcomings in certain applications. To meet specific demands, we introduced novel affinity tags into the pEU3-NII vector and two of them, i.e. the FLAG- and the double-His_6-tag (His_{12}-tag) holding plasmid are presented in this thesis.

Most proteins are post-translationally modified to fine-tune their functions, and phosphorylation is probably the most widely studied of these modifications. The negative charges which are introduced by protein kinases can affect conformation, interactions and in many cases can directly influence the activity of the modified protein. Extracellular signals frequently trigger the consecutive activation of the mitogen-activated protein kinase (MAPK) cascade components resulting in the activation of the MAPK, the effector protein of the tier which is responsible for specific phosphorylation of the appropriate protein substrates. In our work, we set out to investigate a putative *Arabidopsis thaliana* (At) – the main model organism of dicots – MAPK substrate, the AtPIN1 transmembrane protein which is a member of the 'long' pin-formed (PIN) protein subfamily of auxin transporters and modulates the directionality of auxin flow. The AtPIN1 central hydrophilic loop (HL) holds several Ser/Thr kinase phosphorylatable amino acids, and their phosphorylation status determines the localization of the protein.

The sequential progression of cell cycle phases is controlled by the complex formation of various cyclin-dependent kinases (CDK) with specific cyclins. CDK activities are controlled at different levels and the main regulation mechanism is the

availability of appropriate cyclin partners. The E2 promoter binding factors (E2Fs) are the core components of the transcriptional control of various cyclins and are also involved in centrosome duplication, therefore control cell cycle progression and cell proliferation.

The animal activator E2F transcription factors E2F1-E2F3 are equipped with an N-terminally located DNA-binding domain followed by the dimerization partner 1 and 2 (DP1, DP2) binding heterodimerization domains. Formation of heterodimers strengthens binding of E2Fs to the *cis*-acting elements of the target genes hence increases transactivation activity of E2Fs. The retinoblastoma (Rb) interacting pocket binding domain is localized in the transactivation domain; therefore, Rb interaction masks the E2F transactivation domain and consequently represses expression of the respective E2F target genes. Upon hyperphosphorylation of Rb, the interacting E2F transcription factors are released and initiate transcription of the relevant genes. Plants also have E2F-DP-Rb pathway that is active at both G_1/S and G_2/M. AtE2FA/B/C have a similar domain organization, and they can only bind to DNA if they heterodimerize with the plant dimerization partners AtDPA or AtDPB. The *Arabidopsis* retinoblastoma-related protein 1 (AtRBR1) is phosphorylated and negatively regulated through phosphorylation by the complexes formed by plant D-type cyclins and AtCDKA;1 at G_1/S transition. All typical plant E2Fs were shown to interact with AtRBR1, but the complexes are involved in different regulatory networks. Expression of AtE2FA is highest in late G_1 phase, whereas AtE2FB was shown to have a role in the regulation of the G_2/M transition; although, AtE2FA and AtE2FB seem to have overlapping functions as well. AtE2FC has a shortened transactivation domain and its function in cell cycle regulation has not been fully revealed yet.

Almost one-quarter of *Arabidopsis* genes contain the experimentally verified E2F-binding site WTTSSCGSS (W=A/T, S=C/G) which is slightly longer than the mammalian consensus sequence, and certain genes require further *cis*-acting elements for activation by AtE2Fs. Of note, multiple E2F target genes do not encompass the mentioned consensus sequence and their recruitment is mediated by interactions with other transcription factors. Recently, a microtubule (MT) nucleation related protein, γ-tubulin, was shown to interact both with the E2F1 protein and E2F1 binding site of the DNA in mammalian cells.

Human and plant γ-tubulin preserved the filament and fibrillar network forming capability of prokaryotic tubulins and several large complexes of γ-tubulin that do not have a function in MT nucleation were found in different eukaryotic organisms. The

oligomerization and polymerization of γ-tubulin was shown both in the cytosol and the nuclear compartment. In mammals, γ-tubulin can interact with lamins and this interaction might be connected to nuclear functions of γ-tubulin. Although plant cells do not have lamin encoding genes, functionally analogue proteins referred to as plamina were found. γ-tubulin present in the nucleus was shown to associate with proteins associated with DNA damage response (e.g., Rad51, ATR, BRCA1) and stress response MAPKs [98].

γ-Tubulin C-terminal comprises a helix-loop-helix domain which is common in DNA-binding proteins and a conserved bipartite nuclear localization signal (NLS) was also identified in the loop. In animal cells, binding of γ-tubulin to E2F1 transcription factor competes out DP and leads to the repression of E2F target genes at G_1/S checkpoint. The γ-tubulin/E2F complex can also bind to the promoters of γ-tubulin and Rb, and γ-tubulin and Rb reciprocally negatively regulate their expression. Depletion of γ-tubulin is lethal for Rb deficient cells which makes γ-tubulin a potential cancer therapy target. Moreover, mammalian γ-tubulin binds to mitochondrial DNA, and it was suggested that γ-tubulin might aid synchronization of mitochondria-related gene expression with mitochondrial replication. In early S phase, human brain-specific serine/threonine-protein kinase 1 (SadB) interacts with and phosphorylates γ-tubulin on Ser131 that it is presumed to control centrosome homeostasis. Phosphorylation of Ser131 facilitates further SadB-dependent phosphorylation of Ser385, an amino acid residue found in the proximity of the NLS of γ-tubulin, that leads to relocation of γ-tubulin to the nuclear compartment.

Plant γ-tubulin is present in the nucleus at G_1/S and G_2/M transitions during the cell cycle, accumulates at the centromere region before the disappearance of the nuclear membrane and was also shown to interact with DNA. Based on sequence homology and co-localization of AtKIN10 and γ-tubulin, it is presumed that AtKIN10 could phosphorylate γ-tubulin Ser131 in plant cells. However, the Ser385 corresponding phosphorylatable amino acid could not be found in plant γ-tubulin, hence double phosphorylation by AtKIN10 is unlikely.

Under *in vitro* circumstances, BRCA1 can ubiquitinate K48 and K344 residues of purified mammalian γ-tubulin and K48 ubiquitination is required to prevent centrosome reduplication in living cells. Knocking out BRCA1 results in aberrant γ-tubulin accumulation at the mitotic centrosomes. BRCA1 homologue was found in *Arabidopsis thaliana* and shown to have a role in cell cycle regulation and DNA repair as in the case of the mammalian BRCA1, but its connection with γ-tubulin has not been described.

2. Objectives

The goal of my doctoral studies was the functional analysis of diverse *Arabidopsis thaliana* proteins synthesized by wheat germ-based *in vitro* translation and enhancement of the available translation vectors to produce proteins of various tags for specific needs.

AtPIN1 protein is a plasma membrane-localized auxin efflux carrier with two transmembrane domains. These two domains encompass a hydrophilic loop which is highly divergent between the PIN proteins. The subcellular localization and auxin transport polarity of AtPIN1 is regulated by phosphorylation of the cytoplasm-oriented hydrophilic loop by several kinases, but our knowledge regarding the involvement of MAPKs in this phosphorylation is limited.

The original pEU3-NII vector has gone through several improvements to increase its applicability in the wheat germ-based *in vitro* translation system, such as the addition of a ligation-independent cloning (LIC) site, affinity tags – the His_6- and GST-tag –, and a tobacco etch virus (TEV) protease recognition site for tagless protein release. Further improvements of the LIC harbouring vectors were required for its diversified application in techniques such as pull-down assays, electrophoretic mobility shift assay (EMSA) and Amplified Luminescent Proximity Homogenous Assay Screen (AlphaScreen).

γ-Tubulin is an intriguing protein. Initially, it was only considered a protein with essential roles in MT nucleation from the centrosome as a component of the well-known γ-tubulin ring complex (γTuRC). In the last 20 years, it has become clear that γ-tubulin is rather a multifunctional protein with roles in the regulation of plus-end MT dynamics, DNA damage, S phase progression and mitotic exit.

The specific aims of my PhD thesis were:

1) Characterization of *Arabidopsis* PIN1-HL phosphorylation by AtMPK4 and AtMPK6.
2) Modification of LIC-harbouring pEU3-NII vectors to encode for additional affinity tags.
3) Functional analysis of *Arabidopsis* γ-tubulin; particularly, analysing its role in the cell cycle regulation through interaction with E2Fs.

3. Methods

Ligation-independent cloning

The coding sequences of the proteins used in this work were inserted into the appropriate vectors by LIC. The LIC method leverages the 3' exonuclease activity of T4 DNA-dependent DNA polymerase for producing 12-15 nucleotide long complementary overhang sequences in the appropriate nucleotide sequence holding linearized vectors and PCR products. Upon mixing the T4 DNA polymerase treated vector and PCR product, numerous hydrogen bonds form and stabilise the newly created circular construct. Following transformation into competent cells the missing phosphodiester bonds are created by the endogenous bacterial ligase.

In vitro protein translation

To maintain the productivity of the wheat germ-based *in vitro* translation reaction for an extended time and achieve a higher protein yield the bilayer method was used. The higher density translation mixture containing the ribosomal subunits, translational factors (initiation, elongation, termination), tRNAs, aminoacyl-tRNA-synthetases and the *in vitro* transcribed mRNA was underlaid to the feeding solution with amino acids, cofactors, energy supplying components (ATP, GTP). The amino acids and energy components could diffuse freely via the formed boundary layer and continuously supply the translation. Energy regeneration was ensured by the addition of creatine kinase to the translation mixture and creatine-phosphate to the feeding solution. Co-translations were achieved by mixing the respective mRNAs in appropriate ratios.

In vitro kinase assay

Due to the open nature of *in vitro* translation, active MAPKs can be obtained by co-translating the respective upstream gain-of-function mutant MAPK kinase with the MAPK in question thus the MAPK can be purified in an enzymatically active form. Myelin basic protein is a commonly used artificial substrate for *in vitro* MAPK activity measurement because its numerous Ser and Thr residues are readily phosphorylated by members of this protein kinase family. On the other hand, the bead-bound putative substrates are λ-phosphatase treated prior to the kinase assay to eliminate the phosphorylation that was caused by endogenous kinases of the wheat germ extract. The *de novo* phosphorylation events were detected by the incorporation of radiolabelled phosphate from [γ-^{32}P]ATP.

Pull-down assay

The pull-down assay is an *in vitro* technique used to determine the interaction between two or more proteins. Compared to immunoprecipitation a tagged "bait" protein is used instead of an antibody. Consequently, either the bait-protein coated affinity beads are incubated with the total translation mixture containing the putative "prey" proteins, or preformed complexes can be isolated through the affinity tag of the "bait" protein. Unbound proteins are removed by washing steps with appropriate buffers. Finally, the protein-protein complexes can be eluted from the beads and separated by gel electrophoresis, transferred to a membrane, and specific detection is accomplished by using antibodies. Non-specific binding of the "prey" protein is also assessed by using the affinity beads as a negative control. We used GST pull-down assay, i.e. the "prey" proteins were GST-tagged.

Electrophoretic mobility shift and super-shift assay

EMSA is a method that is used to determine protein-DNA interactions. The principle of the assay is the alteration of native gel electrophoresis mobility of DNA upon protein binding. The super-shift assay approach is an antibody complemented version of EMSA in which a complex-forming protein-specific antibody also binds to the DNA-protein complex that results in a decreased mobility. The consensus and mutated E2F-binding sites were generated by hybridizing complementary oligonucleotides synthesized with Cy5 labelling on the upper strand and 3' inverse dT modification on the lower strand. The Cy5-labelled dsDNAs were incubated with total translation mixtures in buffer supplemented with a non-specific competitor, the synthetic poly(dI-dC). The gels were imaged using a Typhoon scanner.

AlphaScreen

We used AlphaScreen to determine protein-DNA interactions. The studied DNAs were generated by hybridizing complementary oligonucleotides synthesized with biotin labelling on the upper strand and 3' inverse dT modification on the lower strand. The DNA and diverse variations of *in vitro* translated proteins were captured by their biotin labelling and His_6-tag onto streptavidin donor and Ni-chelate acceptor beads, respectively. The samples were supplemented with BSA and salmon sperm DNA as non-specific competitors. The DNA-protein interactions were determined by luminescence measurement.

4. Results

We set out to identify the amino acid residues of the hydrophilic loop of *Arabidopsis* PIN1 auxin transporter (AtPIN1-HL) which could be phosphorylated by pre-activated AtMPK4 and AtMPK6 kinases. Activities of the purified MPKs were confirmed by *in vitro* kinase assay using the myelin basic protein artificial substrate. The translated putative substrates, AtPIN1-HL and its non-phosphorylatable mutant (PIN1-3A) – in which three Thr amino acid residues were mutated to alanines (T227A, T248A, T286A) – were purified and λ-phosphatase treated prior to the kinase assay. The dephosphorylated substrates were incubated with activated AtMPK4 and AtMPK6 in the presence of [γ-^{32}P]-ATP and incorporation of radiolabelled phosphate was analysed by autoradiography. Although both MPKs phosphorylated the wild type AtPIN1-HL, the extent of phosphorylation was slightly lower in the presence of AtMPK6. Phosphorylation by AtMPK4 and AtMPK6 was equally diminished when the non-phosphorylatable mutant was used as a substrate indicating that these three residues are indeed the target sites of the applied kinases. Next, a larger amount of AtMPK6-phosphorylated AtPIN1-HL was produced and analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to confirm the specific phosphorylation of the three Thr amino acid residues.

We aimed to investigate the cell-cycle functions of γ-tubulin by analysing *in vitro* protein-protein and protein-DNA interactions. Because these experiments required several putative interacting partners, we set out to expand the repertoire of wheat germ CFPS vectors to allow the translation of target proteins with His_{12}-tag and FLAG-tag.

We constructed a vector suitable for *in vitro* translation of proteins with N-terminal His_{12}-tag, comprising two His_6 tags separated by a linker sequence, designated pEU3-NII-HxHLICNot. The His_6-tag is an extensively used tag, but the coordinating histidine residues have a relatively low binding affinity to metal ions and the histidine-selective antibodies are notoriously promiscuous. In comparison to the His_6-tagging, we observed only a slightly improved binding of the His_{12}-tagged protein to Co-nitrilotriacetic acid beads, and – contrary to our expectations – no increase in protein purity. For detection of the proteins by peroxidase-conjugated anti-polyHistidine antibody, we prepared five-fold dilutions of the target protein-containing total translation mixtures. Our results indicated at least an order of magnitude increased sensitivity with double labelling in comparison to the conventional His_6-tagging.

The other tag that was introduced into the wheat germ CFPS vectors is the merely 8 amino acid long FLAG epitope tag that is rarely found in nature; therefore, the antibodies produced against the tag are greatly discriminating and make this tag suitable for highly specific detection. Moreover, its high aspartic acid content confers hydrophilicity; thus, the tag is usually found on the surface of the fusion protein and facilitates antibody binding. Accordingly, by using anti-FLAG antibody, we did not detect any protein non-specifically in the wheat germ extract. Thus, we constructed the pEU3-NII-FLAGLICNot vector and by using FLAG-tagged protein-containing translation mixtures we confirmed the specific detection of the proteins of interest.

It has been described that γ-tubulin can be present at different polymerization levels. To assess the ratio of monomeric γ-tubulin in case of the *in vitro* translated γ-tubulin, we produced the GST-, His_6-, His_{12}-, and FLAG-tag variants of the protein. The highest precipitate:soluble ratio was detected in the case of His_6- and His_{12}-tagged γ-tubulin, whereas the use of FLAG-tag and GST-tag enhanced γ-tubulin solubility. Furthermore, we have found that co-translation of His_{12}-γ-tubulin with its putative interaction partner AtE2FA also increased its solubility.

To study interactions of γ-tubulin with AtE2Fs, we co-translated the proteins and we showed by GST pull-down assay that all three E2Fs (AtE2FA, AtE2FB and AtE2FC) could bind γ-tubulin. Addition of AtDP proteins in excess could not displace γ-tubulin from the preformed γ-tubulin-AtE2F complexes suggesting that a heterotrimer complex is formed. Because γ-tubulin is reported to be a sticky protein, in a similar pull-down assay setup we compared its interaction with AtE2FA and another transcription factor, the cell fate-determining AtMYB66 under stringent washing condition. We found that the γ-tubulin-AtE2FA interaction is highly specific.

To elucidate further details of ternary complex formation we intended to determine the E2F segment responsible for γ-tubulin binding. We truncated E2Fs up to their DNA-binding domains and carried out GST pull-down assay by using the mutant E2F proteins. Accordingly, in absence of their dimerization domains, the E2F mutants were unable to bind AtDPB. On the contrary, γ-tubulin could bind even to the truncated E2Fs implying that the E2F dimerization domain is not required for interaction with γ-tubulin. Altogether, these data hint that γ-tubulin and AtDPB can simultaneously associate with AtE2Fs and form a ternary complex.

Following the demonstration of γ-tubulin-AtE2F interactions, we aimed at investigating whether γ-tubulin could bind DNA directly or through the putative ternary

E2F-DP-γ-tubulin complex by using EMSA and AlphaScreen. For these assays we used consensus or mutant E2F-binding site containing DNA sequences.

First, we tested DNA-binding capacity of the different proteins with EMSA, and we have found that the consensus E2F-binding site holding DNA did not present mobility shift with any of the single proteins or mixtures of γ-tubulin with AtDPB or AtE2FB, but all AtDPB and AtE2FB containing mixtures, including the γ-tubulin containing ones, showed a clear extra band on the PAGE indicating DNA-protein interaction. Surprisingly, the same shift was detected whether the putative heterodimer or higher molecular weight heterotrimer was bound to the DNA. DNA binding of the E2F-DP-γ-tubulin complex was unambiguously confirmed by performing a super-shift EMSA with His_6-labelled γ-tubulin-binding anti-polyHistidine antibody.

Finally, we tested the protein-DNA interactions by using AlphaScreen. In agreement with our EMSA results, we detected only a background signal intensity with the mutated DNA sequence in either protein combination. The single protein combinations and the AtE2FB and γ-tubulin containing mixture could not bind the wild type DNA. As expected, the mixture of AtE2FB-AtDPB heterodimer and wild type DNA produced a distinctively increased signal. Interestingly, the ternary complex also provided strong but slightly decreased signal in comparison to the AtE2FB-AtDPB, suggesting that γ-tubulin might modulate DNA binding of the heterodimer.

5. Conclusions

The environmentally activated MAPK signalling pathways regulate several processes in all eukaryotes. Despite their importance, our knowledge of plant MPK substrate phosphorylation is limited. In our investigation, we aimed to reveal MPK phosphorylation sites in AtPIN1-HL. Based on our results, the conclusions are:

- AtPIN1-HL is phosphorylated *in vitro* by two environmentally activated MPKs, AtMPK6 and AtMPK4.
- According to the LC-MS/MS results, T227, T248 and T286 residues were phosphorylated by AtMPK6.
- The above findings were confirmed by using non-phosphorylatable mutant AtPIN1-3A as the substrate of the *in vitro* kinase reaction. The results indicated that none of the studied kinases could phosphorylate the mutant variant of AtPIN1.

The wheat germ-based *in vitro* translation is generally suitable for the translation of eukaryotic proteins in a native form, and the yield is satisfactory for most of the *in vitro* functional assays. We have further developed the already available pEU3-NII vector set to synthesize proteins with FLAG-tag and His_{12}-tag besides the previously available His_6-tag and GST-tag. Our results are:

- We have created the pEU3-NII-FLAGLICNot (#140184) and pEU3-NII-HxHLICNot (#140183) vectors that were deposited to Addgene plasmid stock.
- Both FLAG- and His_{12}-tagged proteins could be expressed successfully, and the FLAG-tagged proteins could be detected with high specificity, whereas the His_{12}-tagged proteins with high sensitivity in comparison to His_6-tagged proteins.

Although there are thousands of published studies about E2Fs, not all of their protein interaction partners have been revealed yet. Recent findings indicated that γ-tubulin, a classically MT nucleation-related protein, could interact with mammalian E2Fs and affect their transcriptional activities by competing out DPs from the E2F-DP complexes. We aimed at unravelling if such interactions exist in *Arabidopsis* too. Based on the results, our conclusions are:

- Plant γ-tubulin interacts with AtE2FA/B/C, but not with AtMYB66 transcription factor and the interaction is mediated by the N-terminus of AtE2Fs.
- AtE2Fs can simultaneously bind AtDPs and plant γ-tubulin thus forming a ternary complex.
- The ternary complex binds to the E2F consensus site through E2Fs.

Abbreviations:

Alpha: Amplified Luminescent Proximity Homogenous Assay Screen, **At:** *Arabidopsis thaliana*, **ATR:** Ataxia telangiectasia-mutated and Rad3-related homolog, **BRCA1:** breast cancer type 1 susceptibility protein, **CDK:** cyclin-dependent kinase, **CFPS:** cell-free protein synthesis, **E2F:** E2 promoter binding factor, **EMSA:** electrophoretic mobility shift assay, **GST:** glutathione-S-transferase, **His_6:** hexahistidine-tag, **His_{12}:** double His_6-tag, **LC-MS/MS:** liquid chromatography-tandem mass spectrometry, **LIC:** ligation-independent cloning, **MAPK:** mitogen-activated protein kinase, **MT:** microtubule, **NLS:** nuclear localization signal, **PIN1:** pin-formed 1, **PIN1-HL:** pin-formed 1 hydrophilic loop, **Rb:** retinoblastoma protein, **RBR1:** retinoblastoma-related protein 1, **SadB:** human brain-specific serine/threonine-protein kinase 1 (BRSK1), **TEV**: tobacco etch virus.

6. Bibliography of the candidate's publications

Publications related to the thesis:

Szilvia K Nagy*, **Brigitta M Kállai***, Judit András, Tamás Mészáros. (2020) A novel family of expression vectors with multiple affinity tags for wheat germ cell-free protein expression. BMC BIOTECHNOLOGY, 20:1–9
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