ENHANCED IN VITRO TRANSLATION FOR FUNCTIONAL ANALYSIS OF PROTEINS

PhD thesis

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List of abbreviations

AlphaScreen	Amplified Luminescent Proximity Homogenous Assay Screen
ATR	Ataxia telangiectasia-mutated and Rad3-related homolog
BAP	BRCA1-associated protein-1
BRCA1	breast cancer type 1 susceptibility protein
C53	CDK5 regulatory subunit-associated protein 3
CDK	cyclin-dependent kinase
CFPS	cell-free protein synthesis
cps	counts per second
DP	dimerization partner
DEL	DP-E2F-Like protein
E2F	E2 promoter binding factor
EMSA	electrophoretic mobility shift assay
Glu/E	glutamic acid
Gly/G	glycine
GOF	gain-of-function
GST	glutathione-S-transferase
His_{12}	double hexahistidine
His_6	hexahistidine
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LIC	ligation-independent cloning
MAPK	mitogen-activated protein kinase
MKK	*Arabidopsis thaliana* mitogen-activated protein kinase kinase
MPK	*Arabidopsis thaliana* mitogen-activated protein kinase
MT	microtubule
MTOC	microtubule organizing centre
MYB66	Myb-related protein 66
NES	nuclear export signal
NLS	nuclear localization signal
NTA	nitrilotriacetic acid
PAGE	polyacrylamide gel electrophoresis
PIN	pin-formed protein

PIN1-HL	pin-formed protein 1 hydrophilic loop
POD	peroxidase
PTM	post-translational modification
Rb	retinoblastoma protein
RBR1	retinoblastoma-related protein 1
SadB	brain-specific serine/threonine-protein kinase 1
SDS	sodium dodecyl sulfate
Ser/S	serine
SnRK1	sucrose nonfermenting-1 (SNF1)-related protein kinases
TEV	tobacco etch virus
Thr/T	threonine
Tyr/Y	tyrosine
WGE	wheat germ extract
γTuRC	γ-tubulin ring complex

1. Introduction

1.1. *In vitro* protein translation

The availability of proteins of interest is a prerequisite for protein functional studies that is generally ensured by the application of recombinant DNA technology and heterologous protein overexpressing systems. The protein-coding sequence could be conveniently manipulated at the DNA level to add affinity purification and detection facilitating fusion tags and investigate the functional consequences of amino acid substitutions.

The protein of interest can be produced by various means such as chemical synthesis, and *in vivo* by cell-based and *in vitro* by cell-free protein synthesis (CFPS) systems. It has been known since the 1950s that intact living cells are not required for protein translation, i.e. protein synthesis can be accomplished in a test tube if all required translational components are provided. The application of *in vitro* translation reduces the hurdles that come with the maintenance of living organisms and enables the expression of toxic proteins. The translation apparatus could be isolated from living cells and the obtained extract contains ribosomal subunits, translational factors (initiation, elongation, termination), tRNAs, and aminoacyl-tRNA-synthetases. The cell extract must be supplemented with the protein of interest coding nucleic acid template, amino acids, energy supplying components, cofactors, and other reagents to create an efficient *in vitro* translation system. The template is either DNA when coupled transcription-translation approach is used, or *in vitro* transcribed mRNA when these two steps are implemented separately. The protein extracts can derive from prokaryotic (*E. coli*) or eukaryotic (insect, yeast, Chinese hamster ovary, rabbit reticulocyte lysate, wheat germ) cells [1]. Of note, the so-called PURE system which is based on using recombinant elements of *E. coli* translation apparatus instead of a cell extract is also available but it is not cost-effective and provides low protein of interest yields [2].

The CFPS systems are limited by the energy supply, the mRNA accessibility, and the accumulation of by-products that inhibit the translation. When higher protein yields are required, the traditional batch reaction scheme could be replaced by continuous formats such as the continuous flow and continuous exchange reactions to ensure constant reactant supply and by-product removal by either active or passive transport through a semi-permeable membrane [3].

Ranking the batch format CFPS platforms according to their yields indicated that the most productive prokaryotic *E. coli* extract (2.3 mg/ml) was followed by the eukaryotic wheat germ extract (WGE) (1.5 mg/ml) [1]. Although the continuous development in this field resulted in several effective cell-free extracts, such as the protozoan *Leishmania tarantolae* [4], the tobacco BY-2 cell lysate [5], etc., only the WGE will be discussed since this platform was leveraged in our experiments. The applicability of the WGE-based system was elegantly illustrated in 2008 through the creation of a 'human protein factory' by *in vitro* translating more than 10,000 human proteins and creating a microarray with the produced proteins [6]. A microarray applying screening method that was developed to identify E3 ubiquitin ligases of a panel of substrates also testified the power of WGE-based translation [7]. Moreover, the WGE CFPS system was used to express high-quality malaria parasite proteins for producing antigens of vaccine development [8].

Yaeta Endo and co-workers have established a highly efficient WGE CFPS system by eliminating endogenous translational inhibitors (tritin, thionin) and ribonucleases from wheat embryos [9]. Furthermore, they created the pEU3-NII and pEU0-E01 vectors for the eukaryotic WGE based *in vitro* translation which could provide the mRNA by T7 and SP6 RNA polymerase based *in vitro* transcription reactions, respectively. Both vectors ensure that the inevitable elements of eukaryotic mRNA, i.e. 5' methylguanosine cap and poly(A) tail are substituted by a GAA triplet followed by the tobacco mosaic virus omega enhancer sequence and a 1626 nucleotide long 3' untranslated sequence, respectively. These improvements eliminated the hurdles that came with 5' capping of mRNAs and the difficult propagation of poly(A) sequence containing plasmids in bacterial cells [10].

Another important improvement achieved by the same research team was the development of a novel continuous exchange system, known as the bilayer method. In this arrangement, the higher density cell-free extract is underlaid to the feeding solution to create a boundary layer; thus, similarly to the physical semi-permeable membrane amino acids and energy components can diffuse freely and are supplied continuously to maintain the productivity of the translation reaction for an extended time and achieve a higher protein yield [11]. This simple setup is more cost-effective than the membrane-based continuous systems and more suitable for robotization, hence a rational choice for high-throughput proteomics studies.

To ease the creation of target protein-coding vectors and assure a single method for

the parallel production of different protein-encoding templates, our research group replaced the pEU3-NII vector multicloning site with a ligation-independent cloning (LIC) site [12]. 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.

Beside the LIC site, the sequences coding for either hexahistidine (His_6) or glutathione-S-transferase (GST) affinity tags were inserted into the original plasmids to aid affinity purification and detection of the expressed proteins. Furthermore, the obtained pEU3-NII-HLIC and pEU3-NII-GLIC vectors possess a tobacco etch virus (TEV) protease recognition site following the N-terminally localized affinity tags; thus, tagless target protein could be released by the protease treatment [12].

Although the His_6- and GST-tag are two of the most commonly used tags and their applications are supported by many commercially available products, they both have their drawbacks: polyhistidine stretches could be found in several endogenous proteins, hence co-purify with the target protein, whereas the relatively large GST-tag can affect the protein function; therefore, its cleavage is often required before downstream processes. To tackle these shortcomings and meet specific demands, several affinity tags were designed [13]. Due to their various advantages, the applied novel tags in this work are the FLAG-tag [14] and the double-His_6 tag (His_{12}) [15].

1.1.1. *In vitro* kinase assays

Most proteins are post-translationally modified to fine-tune their functions, and phosphorylation is one of the most abundant from 431 types of reported modifications [16]. Furthermore, due to its multiple roles, such as regulation of cell proliferation, differentiation, and DNA-repair mechanisms, phosphorylation is probably the most widely studied post-translational modification (PTM). Phosphorylation is a fast, simple, and reversible PTM, generally, the terminal phosphate of ATP is covalently attached to the hydroxyl group on serine (Ser, S), threonine (Thr, T), or tyrosine (Tyr, Y) amino acid residues by kinases and removed by phosphatases. The introduced negative charge can

affect conformation, interactions and in many cases can directly influence the activity of the modified protein [17]. Several protein kinases are themselves activated by other proteins kinases, e.g. the members of mitogen-activated protein kinase (MAPK) signal transduction pathways. MAPKs belong to the Ser/Thr kinase superfamily and contrary to their name they are not only activated by mitogens, but also by diverse biotic and abiotic stresses. Extracellular signals frequently trigger the consecutive activation of the MAPK cascade components (MAPK kinase kinase – MAPK kinase – MAPK), 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 *Arabidopsis thaliana (At)* – the main model organism of dicots –, there are 60 MAPK kinase kinases, 10 MAPK kinases (AtMKK) and 20 MAPKs (AtMPK). According to their sequence similarities plant MPKs can be divided into four groups (A, B, C and D), the two main MPK subtypes can be distinguished by the phosphoacceptor site of their activation loop, i.e. members of the A, B and C group have a TEY, whereas the D group representatives possess a TDY amino acid residue in this location [18]. Members of group A and B MPKs are intensively studied, e.g. the A group representing AtMPK6 has a role in environmental and hormonal stress response, while the group B member AtMPK4 takes part in cell cycle regulation and environmental stress response. On the contrary, our knowledge of the physiological role of Group C and D kinases is very limited. The several high-throughput screens that were performed to identify MPK substrates identified hundreds of proteins testifying the importance of MPKs in cell signalling, but many plant MPK target proteins are still awaiting identification [19–21].

In our work, we set out to investigate a putative plant MPK 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. In general, the long PINs are targeted to the plasma membrane through their conserved N- and C-terminal membrane-embedded hydrophobic domains that encompass the divergent central hydrophilic loop (HL) which holds several Ser/Thr kinase phosphorylatable amino acids [22]. Besides AGC kinases, phosphorylation by auxin transport-regulated plant development involved D6 protein kinases and PINOID kinases is required for appropriate AtPIN1 localization. Moreover, it was recently shown that AtPIN1 is phosphorylated by MPKs as well, e.g. Ser337 is phosphorylated by the AtMKK7-AtMPK6 pathway to

control AtPIN1 polarity [23–25].

Phosphorylation sites can be validated by creating the mutant variant of the protein, i.e. the putatively phosphorylated amino acid residue is changed to a non-phosphorylatable one such as alanine, and the phosphorylation level of wild type and mutant proteins are compared following an *in vitro* kinase reaction. On the other hand, protein phosphorylation can be mimicked by replacing the putatively phosphorylated residue with a negatively charged amino acid such as glutamate, e.g. S/TxxxxxS/T consensus phosphorylation site in the case of most plant MKKs. Of note, constitutively active MPKs cannot be expressed by using phosphomimetic mutants, because MPKs have a specific TxY motif in their catalytic domain, and the Thr and Tyr residues must be phosphorylated sequentially to activate the MPKs [26]. Due to the open nature of *in vitro* translation, the WGE could be used to obtain active MPKs by co-translating the respective upstream GOF mutant MKK with the MPK in question thus the MPK is purified in an enzymatically active form [27].

Although radioactivity is health hazardous and its application demands special laboratories, *de novo* phosphorylation events are most often detected by the incorporation of radiolabelled phosphate from [γ-^{32}P]ATP since no other method can outperform this approach in terms of sensitivity [28]. 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 [29].

1.2. Cell-cycle regulation

The formation of new cells is the result of a cyclic process with two main phases: interphase (G_1, S, G_2) – when the cell grows and replicates DNA, and mitosis (M) – when the division of the cell into two daughter cells is accomplished. Endoreplication (also known as endoreduplication) is a cell cycle variation characterized by several DNA replications in the absence of mitosis leading to polyploidy. In plants, endoreduplication is a regular process in various cell types [30].

The sequential progression of cell cycle phases is controlled by the complex formation of various cyclin-dependent kinases (CDK) with specific cyclins. CDKs are activated by extracellular regulatory molecules (mitogens) and inhibited during cell cycle checkpoints. When the progression of the cell cycle is disturbed (e.g. DNA damage, erroneous mitotic spindle assembly) a transient cell cycle arrest is required to enable processes that could

correct the mistakes [31].

The highly organized compartmentalization and delicately regulated communication and transport between the compartments are essential in all eukaryotic organisms. The cytoskeleton and nucleoskeleton are highly dynamic networks that – besides ensuring the form and mechanical stability of the cell – play role in intracellular transport, genome organization, signal transduction, cell movement, and cell-cell interactions.

In non-dividing cells, the centrosomes organize the microtubular network; however, in mitotic cells, the two centrosomes ensure the assembly of the bipolar mitotic spindle and the segregation of the sister chromatids between daughter cells. Normally, centrosome duplication happens once during the cell cycle in synchrony with genomic DNA replication, thus the daughter cells inherit one centrosome and one genome set [32].

The centrosomes are embedded in an amorphous material, the so-called pericentriolar material. The size of the pericentriolar material constantly changes during the cell cycle and in cell division acts as a signalization hub by recruiting different checkpoint proteins. Centrosomal localization of the cyclin E-CDK2 complex is required to synchronize centrosome duplication and DNA replication. [33]. The abnormal function of the centrosome keeps the cells in G_1 phase by initiating the p38-p53-p21 signalization pathway [34]. Excess centrosome number can be caused by S phase centrosome amplification or mitotic errors and is frequently associated with DNA segregation defects and DNA instability. Abnormal centrosome number can be observed in almost all solid tumours [32].

The E2 promoter binding factors (E2Fs) are the core components of the transcriptional control of various cyclins and are also involved in centrosome duplication, thus control cell cycle progression and cell proliferation [35]. 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 [36].

1.2.1. E2F transcription factors

The animal E2F transcription factors can be activators (E2F1, E2F2, E2F3a, E2F3b) or repressors (E2F4-E2F8) of the cell cycle progression (Figure 1). They are all equipped with an N-terminally located DNA-binding domain which in the case of E2F1-E2F6 is followed by the dimerization partner 1 and 2 (DP1, DP2) binding heterodimerization domains. The formation of heterodimers strengthens the binding of E2Fs to the *cis*-acting

elements of the target genes, hence increase the transactivation activity of E2Fs. E2F7 and E2F8 have E2F-like DNA-binding domains and act as repressors independently of DP interaction. DPs alone barely have affinity towards DNA and their binding specificity is determined by E2Fs. Activator E2Fs have nuclear localization signal (NLS) close to their cyclin A binding site that facilitates their nuclear localization, whereas E2F4 and E2F5 can be exported to the cytoplasm via their nuclear export signal (NES). Isoform-specific mRNAs of E2F3a/E2F3b and E2F7a/E2F7b are transcribed by using alternative promoters and produced by alternative splicing, respectively [35, 37].

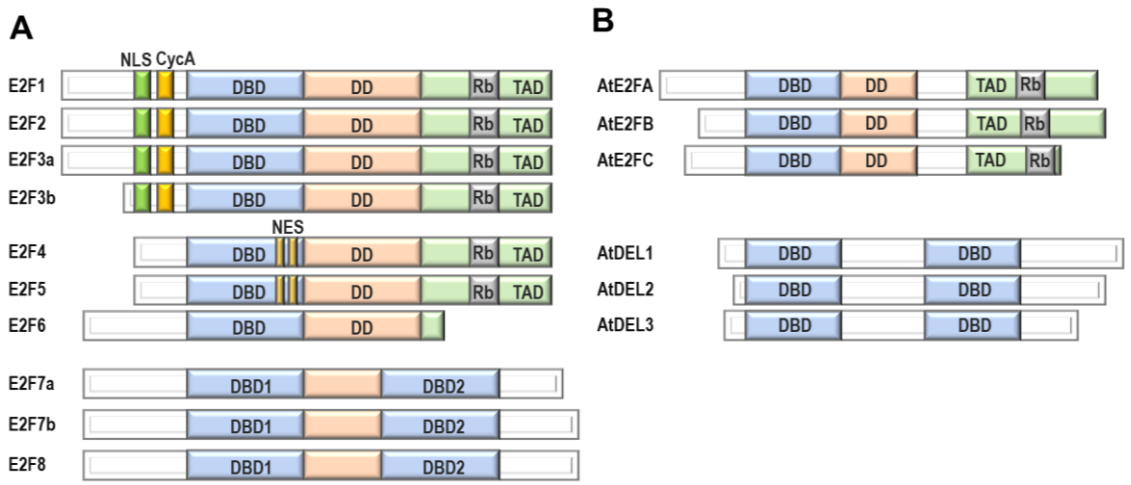

Figure 1. Schematic representation of the domain organization of human and *Arabidopsis thaliana (At)* E2F transcription factors. The DNA-binding domain (DBD), the dimerization domain (DD), the transactivation domain (TAD), and the pocket protein binding domain (Rb) are boxed. E2F1-3 have an N-terminal NLS sequence beside the cyclin A-binding site (CycA); E2F4 and E2F5 have bipartite NES. Human E2F7-E2F8 and AtDEL1-DEL3 atypical E2Fs have a duplicated DNA-binding domain and lack heterodimerization domains and pocket protein binding domains. Modified from [35, 38].

The pocket protein binding domain can bind the retinoblastoma (Rb), p107 and p130 pocket proteins and is localized in the transactivation domain of E2F1-5 (Figure 1); therefore, Rb interaction masks the E2F transactivation domain and consequently represses expression of the respective E2F target genes [35]. The protein level of activator E2Fs fluctuate during the cell cycle and reach their highest expression at the G_1/S transition point. In contrast, the protein level of repressor E2Fs are nearly constant in the different phases of the cell cycle and are also expressed in quiescent cells. Furthermore, the C-terminus of E2F6-E2F8 does not encode for a pocket binding domain, thus their repressor activity is independent of pocket proteins [35].

In animal cells, Rb is unphosphorylated in quiescent cells, gets hypo-phosphorylated

by cyclin D-CDK4/6 complexes at the restriction point, and hyper-phosphorylated by cyclin E-CDK2 in the late G_1 phase. Unless Rb is hyper-phosphorylated it can bind E2F transcription factors and recruit histone-modifying enzymes (e.g. histone deacetylase, HDAC) that condense the chromatin and repress E2F target genes. Upon hyperphosphorylation of Rb, the interacting E2F transcription factors are released and initiate transcription of the relevant genes. The accumulating released activator E2Fs recruit coactivators (e.g. histone acetyltransferase, HAT) to specific promoters, therefore enable the transcription of S phase promoting genes by loosening the chromatin structure [35, 39].

The expression of E2F target genes is oscillatory and is regulated at several levels to prevent abnormal cell cycle progression. Transcriptionally they are negatively regulated by feedback mechanisms involving E2Fs and c-Myc, post-transcriptionally by microRNAs, and post-translationally by phosphorylation in several organisms. Transcriptional activity of the synthesized pool of E2Fs is regulated either indirectly through phosphorylation of Rb by cyclin-CDK complexes or more drastically by degradation in the proteasome. During S/G_2 E2Fs are polyubiquitinated by SKP-CUL1 complexes, while in mitosis the degradation is achieved by the anaphase-promoting complex [40].

1.2.1.1. Plant E2Fs

Plants also have E2F-Rb pathway and in *Arabidopsis thaliana* six E2F family members, two DPs (AtDPA and AtDPB) and one Rb-related protein (AtRBR1) have been described [41]. AtE2FA/B/C (also known as AtE2F3/1/2) have a similar domain organization as human E2F1-5 with their DNA-binding and dimerization domains, whereas E2FD/E/F (also known as DP-E2F-Like, DEL proteins) can be considered as orthologs of mammalian atypical E2F7/8 with their duplicated DNA-binding domain and lack of dimerization domain (Figure 1). These atypical AtE2Fs autonomously bind DNA, but are not able to transactivate, and play role in cell elongation and endocycle regulation [42, 43].

AtDPA and AtDPB are closely related to human DP2 and DP1 and have a similar domain organization; although, based on the phylogenetic analysis they form a separate branch. In contrast to animal DPs, the plant DP dimerization domain, but not the DNA-binding domain is required for heterodimerization with E2Fs [41]. AtE2FA/B/C can only

bind to DNA if they heterodimerize with AtDPA or AtDPB [44]. Maize studies resulted in similar findings, i.e. E2F transcription factors that form heterodimers with AtDPB have a higher affinity for DNA [45]. According to yeast two-hybrid experiments, AtE2FA and AtE2FB preferably interact with AtDPA and heterodimerization increases transactivation activity, moreover AtDPA binding masks the NES located in the E2F heterodimerization domain. In case of AtE2FC, interaction neither with AtDPA nor AtDPB influenced transactivation or affect AtE2FC localization [43].

Remarkably, plants have at least 92 different cyclin-CDK complex variants. 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 [46]. All typical plant E2Fs were shown to interact with AtRBR1, but the complexes are involved in different regulatory networks [47].

Plant E2Fs were shown to have functions in a wide range of processes and in contrast to animal counterparts the *Arabidopsis* E2Fs cannot be classified as activators or repressors [48]. Moreover, the E2F-DP-RBR pathway is active at both G_1/S and G_2/M transitions in plants. This finding was supported by the identification of mitosis-specific activator motif involved in G_2/M transition in AtDPA and AtE2FB and the co-purification of the mitotic AtCDKB1;1 with AtRBR1 [46, 48].

Expression of AtE2FA is highest in late G_1 phase and is required for S phase progression [49]. AtE2FA/AtDPA heterodimer overexpression activates cell division and endocycle and inhibits plant development and growth [43, 50, 51]. In accordance, AtE2FA silencing results in decreased mitosis and endocycle activity and polyploidy [47]. The AtE2FA-AtRBR1 complex represses genes required for endoreduplication and cell differentiation [47]. Furthermore, AtE2FA is also involved in the maintenance of genomic integrity since upon DNA damage AtRBR1 and AtE2FA are recruited to γH2AX foci [52].

Although AtE2FA and AtE2FB are mainly present in dividing cells and postmitotic cells, respectively, the two transcription factors seem to have overlapping functions since single E2F mutant plants are viable, but double knock out plants cannot survive [53]. Overexpression of AtE2FB leads to the expression of mitosis related AtCYCB1;1 and AtCDKB1;1, indicating its role in the regulation of the G_2/M transition [48]. These data are corroborated by the finding that AtE2FB/AtDPA overexpression stimulates mitosis

and inhibits endocycle. AtE2FB-AtRBR1 complexes prevent endoreplication and cell differentiation in young leaves [53].

AtE2FC has a shortened transactivation domain and its function in cell cycle regulation has not been fully revealed yet. It was described that AtE2FC downregulates the early S phase gene AtCDC6 and its increased and decreased expression activates the endocycle and mitosis, respectively [54]. Somehow contradictory, the AtE2FC/AtDPA complex is involved in photomorphogenesis and metabolism but not in cell cycle regulation [51].

1.2.1.2. DNA binding

It was recently reported by chromatin immunoprecipitation experiments that mammalian E2Fs can also regulate genes with promoter sequences other than the strict consensus TTTSSCGC (S=G/C) motif. Strikingly, Rabinovich et al. demonstrated that E2F1, E2F4 and E2F6 bind primarily to non-consensus motifs *in vivo*, and transactivation of target genes is assumed to be mediated in cooperation with other transcription factors. In contrast, under *in vitro* circumstances, E2F1-6 heterodimerization with DPs are required to strongly bind to the consensus E2F motif, and the binding is abolished if key residues are changed in these sequences. The difference in binding specificity between *in vivo* and *in vitro* experiments could be explained by regulation through interactions with other proteins or certain PTMs, thus in some cases *in vitro* E2F site binding experiments might not be biologically relevant. E2F7 and E2F8 can form homodimers or heterodimers between each other to bind DNA without DPs [55].

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. As was the case with mammalian findings, multiple E2F target genes do not encompass the mentioned consensus sequence and their recruitment is mediated by interactions with other transcription factors [51, 56].

1.2.2. γ-Tubulin

γ-Tubulin was first discovered in 1989 in *Aspergillus nidulans* by Oakley and Oakley [57] and its involvement in MT function as a MT minus-end nucleator and nuclear division (M/G_1 transition) regulation was soon suggested [58, 59]. α-, β- and γ-tubulin evolved from a common ancestor and are found in all examined eukaryotes, whereas the

δ, ε, ζ, and η tubulin isoforms represent another branch of the tubulin phylogenetic tree and are not found in plants and yeast [60]. The two γ-tubulin genes found in *Arabidopsis thaliana* share 98% amino acid sequence identity [61] and have redundant functions [62]. In comparison to the human counterpart, plant and yeast γ-tubulin contain a disordered domain forming 20 extra amino acids at the C-terminus (Figure 2) [63].

The N-terminally located GTPase domain comprises the conserved GDP/GTP-binding site (GGTGSG amino acid sequence motif) in α-, β- and γ-tubulin (Figure 2) [60]. During polymerization of α/β-tubulin dimers into MTs, the GTP bound to β-tubulin but not to α-tubulin is hydrolysed. GTP bound by α-tubulin is non-exchangeable and the conserved glutamic acid residue (Glu254) of α-tubulin is essential for GTP hydrolysis in β-tubulin. This residue is lysine in β-tubulin and either Gly or Ser in γ-tubulins implying that γ-tubulin monomer contacts might not involve GTP hydrolysis [64–66].

The X-ray crystal structure determination of GTP- and GDP-bound monomeric human γ-tubulin showed that – alike other GTPases – the protein conformation is not changed in response to nucleotide-binding and both GTP and GDP are bound with similar nanomolar affinity [67, 68]. GTPase activity of γ-tubulin has not been demonstrated, but data obtained by yeast experiments suggest that GTP-bound γ-tubulin has a higher affinity for α/β-tubulin dimers and α-tubulin might induce GTP hydrolysis in γ-tubulin [69].

γ-Tubulin comprises a helix-loop-helix domain which is common in DNA-binding proteins and a conserved bipartite NLS was also identified in the loop. The NLS comprises two surface-exposed stretches of basic and acidic amino acids (RKx(D/E)xFx(D/E)xF, residues 399-418, Figure 2) and the mutational analysis revealed that it is essential for nuclear localization of γ-tubulin [70]. Although putative NES is also predicted in γ-tubulin [71, 72], the export mechanism is currently unknown, e.g. treatment with a nuclear export inhibitor did not lead to human γ-tubulin accumulation in the nucleus [73].

Ran GTPase and exportin co-purification with γ-tubulin in *Arabidopsis* extract hints that transport of MT-associated proteins and chromatin-associated factors on the nucleus-cytoplasm pathway might be important in the coordination of MT dynamics and chromatin organization [74].

1.2.2.1. Microtubule nucleation and polymerization

In eukaryotes, γ-tubulin and several γ-tubulin complex proteins (GCPs) form the

cytosolic γ-tubulin ring complex (γTuRC) which could be found in microtubule organizing centres (MTOCs) and regulate the association of α/β-tubulin dimers with the growing MTs [75].

Besides MT nucleation from canonical MTOCs such as the centrosome, MT nucleation also occurs on existing MTs, membranes and chromatin and contributes to the assembly of the mitotic spindle in centrosomal and acentrosomal cells. A key player that recruits γTuRC to nucleate new MTs from pre-existing MTs is the eight-subunit augmin complex that is responsible for the mitotic spindle localization of γ-tubulin and formation of centrosome-independent MTs in mitotic and meiotic spindles [76–78]. Golgi is a membrane-directed MT nucleation site [79] and interaction of γ-tubulin with the Golgi membrane protein p115 is required for the maintenance of Golgi structure and late mitotic spindle [80].

In acentrosomal plant cells nucleation of MTs takes place at scattered sites in the spindle, phragmoplast and interphase cortical arrays, and on the outer nuclear membrane. γ-Tubulin enables the formation of membrane-bound and MT-bound MTOCs and the large membrane-associated γ-tubulin complexes play a role in MT nucleation from dispersed sites [63, 81–83].

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 and the oligomerization and polymerization of γ-tubulin was shown both in the cytosol and the nuclear compartment [63, 84–88].

In mammalian cells, the smaller molecular weight γ-tubulin strings (γ-strings) connect the nuclear and cytosolic pools of γ-tubulin across the nuclear envelope. At high γ-tubulin concentrations during the G_1 phase, large molecular weight γ-tubules can form in a GTP-dependent manner and are assumed to serve as the cytosolic γ-tubulin pools. At the G_1/S transition, this pool is depleted and γ-tubulin starts to accumulate in the nucleus and simultaneously enriches in the pericentriolar material to assist in the centriole duplication [36, 86, 89, 90]. Rosselló et al. proposed that cellular γ-tubulin forms a meshwork that regulates the G_1/S transition, mitotic progression, and cytokinesis and might aid the synchronization of cytosolic and nuclear events during cell division [85].

In comparison to centrosomal animal cells, the acentrosomal plant cells have a larger

amount of γ-tubulin which is distributed at disperse sites and form high molecular mass assemblies (filaments, fibrillar structures) in a cell-cycle-dependent manner. Discrete tubular formations of γ-tubulin were seen in the nucleus and perinuclear regions of non-dividing *Arabidopsis* cells. Chumová et al. proposed that polymerization is an intrinsic property of γ-tubulin, short filaments might function as seeds for MT nucleation and the double-stranded stable filaments might have scaffolding or sequestration functions [63].

1.2.2.2. Nuclear functions

In mammals, γ-tubulin can interact with lamins and this interaction might be connected to nuclear functions of γ-tubulin [36, 84]. Lamins and the associated proteins are localised to the inner surface of the nuclear membrane, characterized by dynamic interactions with chromatin, and play role in chromatin organization, transcription, replication, and DNA repair. Although plant cells do not have lamin encoding genes, functionally analogue proteins referred to as plamina were found in land plants. Similarly to animal lamins, the plamina can form filaments and mediate nuclear processes such as the size and form determination of the nucleus and organization of heterochromatin [74, 91].

It is known that MTs are involved in sequestering DNA damage responsive proteins in the cytoplasm. Upon DNA damage, the needed proteins use MT motor proteins as cargo vehicles for transportation into the nucleus [70]. The centrosomes are also involved in the DNA damage response pathway and genotoxic stress highly influences centrosomal organization [92]. γ-tubulin is a truly multifunctional protein, it seems to participate in the nuclear targeting of microtubular motor proteins transported signalling molecules [77]. Accordingly, γ-tubulin present in the nucleus was shown to associate with proteins associated with DNA damage response [70, 73, 93–97] and stress response MAPKs [98]. It interacts with DNA repair protein Rad51 during S phase and in response to DNA damage which implies its function in DNA repair by homologous recombination [93]. γ-Tubulin could translocate into nucleoli as well to interact with CDK5 regulatory subunit-associated protein 3 (C53) DNA damage G_2/M checkpoint protein [73]. Furthermore, it co-immunoprecipitates with Ataxia telangiectasia-mutated and Rad3-related homolog (ATR) and breast cancer type 1 susceptibility protein (BRCA1) in the nucleus and accumulates at the centrosome following DNA damage [94, 99].

In animal cells, nuclear γ-tubulin forms a complex with E2F1 transcription factor and

C-terminal DNA-binding domain (residues 334-452, Figure 2) of γ-tubulin is required for this interaction. The binding of γ-tubulin competes out DP and leads to the repression of E2F target genes at the G_1/S checkpoint. Hence, γ-tubulin regulates S phase progression by ensuring that the centrosomes and DNA are replicated only once during the cell cycle [36]. Furthermore, 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. Lack of Rb results in high levels of γ-tubulin, while Rb overexpression was observed in γ-tubulin mutants. Depletion of γ-tubulin is lethal for Rb deficient cells which makes γ-tubulin a potential cancer therapy target [95, 100].

Moreover, mammalian γ-tubulin binds to mitochondrial DNA and this binding was increased by mutating the NLS of γ-tubulin. It was suggested that γ-tubulin might aid synchronization of mitochondria-related gene expression with mitochondrial replication [101]. Mutation of GTPase domain localized Cys13 to Ala disrupts the γ-tubulin meshwork and decreases the mitochondrial respiratory capacity. It was also proposed that the intact GTPase domain is essential for the nuclear functions of γ-tubulin [100]. Reduced levels of γ-tubulin cause increased levels of the mitochondria-specific DNA polymerase γ during S phase progression [101].

The relatively high amount of γ-tubulin in acentrosomal plant cells makes easier the characterization of nuclear pools of this protein [81, 87]. Plant γ-tubulin is present in the nucleus at G_1/S and G_2/M transitions during the cell cycle and accumulates at the centromere region before the disappearance of the nuclear membrane and interacts with DNA [87, 88].

γ-Tubulin is important for the coordination of late mitotic events and cytokinesis in *Schizosaccharomyces pombe*, *Aspergillus nidulans* and *Arabidopsis thaliana* [82, 102, 103]. It also has a role in the inactivation of the anaphase-promoting complex after mitotic exit and between late mitosis and S phase [104].

1.2.2.3. Post-translational modifications

The PTMs of γ-tubulin such as phosphorylation and monoubiquitination contribute to its cell-cycle dependent regulation.

The chromatin and centrosome localized mammalian brain-specific serine/threonine-protein kinase 1 (SadB) takes part in the cell cycle by regulating mitotic entry and centrosome biogenesis. In the early S phase, the human SadB interacts with and

phosphorylates γ-tubulin on Ser131. The phosphorylated Ser is part of the S-X-S motif (S129-D130-S131, Figure 2) that is required for the γ-tubulin lateral interactions and might stabilize γ-tubulin in a polymerized form at the original centriole to inhibit the *de novo* centriole formation in other parts of the cell; thus, it is presumed that Ser131 phosphorylation controls centrosome homeostasis. Accordingly, a transient increase of phosphorylated Ser131 levels can be observed in the late G_1 phase, G_1/S transition and S phase [105].

Phosphorylation of Ser131 facilitates further SadB-dependent phosphorylation of Ser385, an amino acid residue in the proximity of the NLS of γ-tubulin (Figure 2). Ser385 phosphorylation leads to the relocation of γ-tubulin to the nuclear compartment. It was hypothesized that Ser385 phosphorylation might induce a conformational change resulting in the release of γ-tubulin from the γTuRC complex and unmasking its NLS [36, 106].

The closest plant homologues of human SadB kinases are the AtKIN10 and AtKIN11 kinases of the sucrose nonfermenting-1 (SNF1)-related protein kinases (SnRK1) subfamily and are involved in the regulation of cell division [107]. Based on γ-tubulin sequence homology, it is presumed that AtKIN10 could phosphorylate γ-tubulin Ser131 in plant cells (Figure 2), therefore might also regulate the centrosome duplication [108]. This hypothesis is supported by the finding that AtKIN10 and γ-tubulin are colocalized [109]. However, the Ser385 corresponding phosphorylatable amino acid could not be found in plant γ-tubulin, hence double phosphorylation by AtKIN10 is unlikely (Figure 2) [107].

The region around human γ-tubulin Ser364 is highly conserved and holds a CDK1 phosphorylation consensus motif (S/T-P, Figure 2) [110]. It was found in *Tetrahymena thermophila* that the corresponding Ser360 phosphorylation state influences γ-tubulin nuclear and cortical functions and mutations that prevent Ser360 dephosphorylation are lethal [111]. In budding yeast, γ-tubulin is hyperphosphorylated during G_1 phase, and dephosphorylation of the conserved S360 CDK1 phosphorylation site and Tyr445 is required for mitosis [112, 113]. Tyr445 is localized in the disordered C-terminal region of *Arabidopsis* (Tyr446) and yeast γ-tubulin (Figure 2). Disordered regions are known to be general hubs of protein interaction networks and susceptible to charge-altering PTMs [114].

MT nucleation activity is increased in early mitosis during mitotic spindle assembly and gradually declines as cells exit mitosis. The MT nucleation activity is regulated by a panel of different mechanisms, e.g. ubiquitination of γ-tubulin by BRCA1 [97] and deubiquitination by BRCA1-associated protein-1 (BAP) [115]. Under *in vitro* circumstances, BRCA1 can ubiquitinate K48 and K344 residues of purified mammalian γ-tubulin (Figure 2). Centrosomal localization of BRCA1 can be observed throughout the cell cycle and γ-tubulin K48 ubiquitination is required to prevent centrosome reduplication in living cells and both BRCA1 inhibition and γ-tubulin K48R mutation lead to centrosome amplification [97].

γ-tubulin activation by CDK1 and association with BRCA1 is required for transportation of γ-tubulin and BRCA1/γ-tubulin complexes to the MTs and into the nucleus, respectively [70].

It was also described that knocking out BRCA1 results in aberrant γ-tubulin accumulation at the mitotic centrosomes [116]. Another E3 ubiquitin ligase, Cullin-4A also co-localizes with γ-tubulin at the centrosome region and polyubiquitinates γ-tubulin *in vitro* [117]. 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 [118].

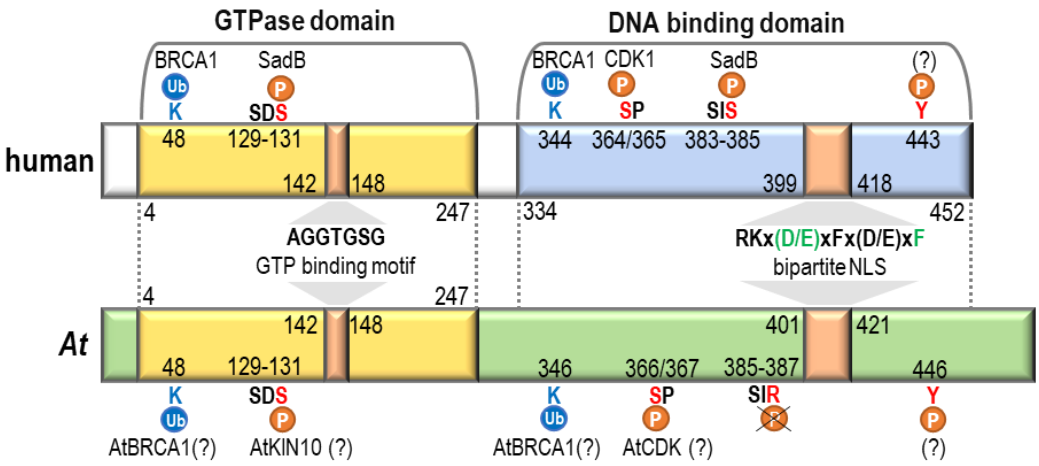

Figure 2. Schematic representation of the domain organization and important PTMs of human and *Arabidopsis thaliana (At)* γ-tubulin. The GTPase domain, GTP binding motif, DNA binding domain and bipartite NLS are boxed. Amino acid residues in the bipartite NLS that differ in plant γ-tubulin are marked with green letters, 'x' stands for any amino acid. Phosphorylated and ubiquitinated amino acid residues are marked by red and blue letters, respectively. Numbering represents the conserved amino acid position in the two sequences. The domains and PTMs were mapped based on UniProt [119] and [36, 70, 97, 105, 106, 108, 110, 114].

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 PTMs 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, such as the addition of a LIC site, affinity tags – the His_6- and GST-tag –, and a 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 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 hydrophilic loop (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

The coding sequence of γ-tubulin (AtTUBG1) was inserted into the pEU3-NII-GLICNot, pEU3-NII-HLICNot, pEU3-NII-HxHLICNot, and pEU3-NII-FLAGLICNot vectors by LIC as described previously for translation of γ-tubulin with GST-, His_6-, His_{12}-, and FLAG-tag, respectively [120]. His_{12}-γ-tubulin was co-translated with GST or GST-AtE2FA by using 1:1 mRNA ratio in the translation mixtures.

Bilayer translation reactions were carried out according to Nagy et al. [120]. The translation reaction was miniaturized for screening purposes, i.e. mini-translations were prepared in a 384-well plate (AlphaPlate – 384 SW, Perkin Elmer). 1 mg/ml creatine-kinase (Roche) stock solution was diluted 7.25 times in 1X SUB-AMIX (CellFree Sciences). The translation mixture consisting of 0.9 µl WEPRO, 0.4 µl mRNA and 0.5 µl diluted creatine-kinase was underlaid to 18 µl 1X SUB-AMIX. After 20-hour incubation at 22°C, the target protein containing total translation mixtures were transferred to Eppendorf tubes and complemented with water to the initial 19.8 µl volume to compensate for the modest evaporation. 8 µl aliquots of the translation and co-translation mixtures were subjected to 13000 rpm centrifugation for 15 minutes, the supernatants were transferred to new tubes and the precipitates were dissolved in PBS. All samples were heated in sodium dodecyl sulfate (SDS) sample buffer complemented with dithiothreitol for 5 minutes at 95°C. The samples were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride membrane, and probed with antibodies specific for the affinity tags (1:2000 rabbit anti-GST, Upstate Biotechnology; 1:5000 goat anti-rabbit, Cell Signaling; peroxidase (POD)-conjugated 1:2000 mouse anti-polyHis-POD, Sigma; 1:1000 mouse anti-FLAG M2, Sigma). Expected molecular weights of expressed proteins: His_6-γ-tubulin 55.3 kDa, His_{12}-γ-tubulin 57.7 kDa, FLAG-γ-tubulin 55.4 kDa, GST 26 kDa, GST-AtE2FA 78.6 kDa, GST-γ-tubulin 79.9 kDa.

4. Results

4.1. Kinase assay with *in vitro* translated wild type and mutant AtPIN1-HL

The pEU3-NII wheat germ expression vector was previously modified in our laboratory to encode for LIC site, TEV protease cleavage motif and N-terminal His_6-tag and GST-tag [12]. These vectors were successfully used in several *in vitro* plant and human protein functional assays by our research group [27, 52, 121, 122]. Various *in vitro* kinase assays were also implemented by using pre-activated MPKs.

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 active AtMPK4 and AtMPK6 kinases. Phosphorylation-dependent activation of both MPKs is a two-step process; thus, constitutively active MPK mutant cannot be generated by replacing the Thr and Tyr residues found in the catalytic domain TEY motif with phosphomimetic amino acids [26]. Because MKKs have a different phosphorylation site, it is possible to create their constitutively active gain-of-function variant (GOF-MKKs). Due to the open nature of *in vitro* translation, MPK activation can be achieved by co-translation with the appropriate upstream GOF-MKK.

To this end, His_6-labelled AtMPK4 and AtMPK6 were co-translated with GOF AtMKK1 and AtMKK4, respectively, by mixing the mRNAs coding for the respective MKK:MPK in a 1:10 ratio. Next, activities of the purified MPKs were confirmed by *in vitro* kinase assay using myelin basic protein artificial substrate (data not shown). 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) – hold an N-terminal GST-tag, therefore were purified by using glutathione-coated beads. The bead-bound AtPIN1 substrates were λ-phosphatase treated prior to the kinase assay to eliminate the phosphorylation that was caused by endogenous kinases of the WGE [123]. The dephosphorylated substrates were incubated with activated AtMPK4 and AtMPK6 in the presence of [γ-^{32}P]-ATP. Incorporation of radiolabelled phosphate was analysed by autoradiography. We did not detect any background phosphorylation when the bead-bound substrates were incubated without activated MPKs (Figure 3). 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 (data not shown).

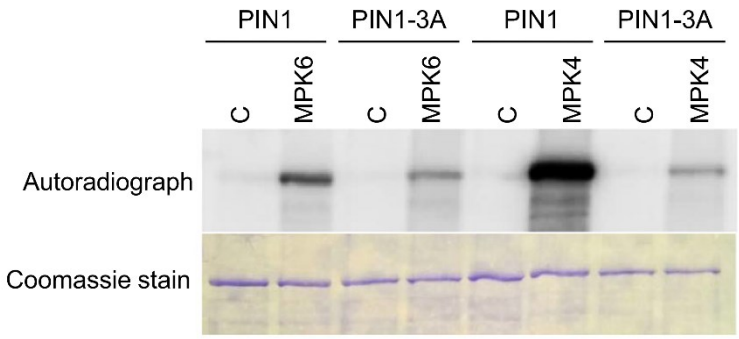

Figure 3. *In vitro* kinase assay with wild type and non-phosphorylatable mutant of PIN1-HL. GST-tagged PIN1-HL (PIN1) and T227A, T248A, T286A mutant GST-PIN1-HL (PIN1-3A) were *in vitro* translated and affinity purified by glutathione-coated beads. The bead-bound substrates were either incubated without kinases (C, control) or with affinity-purified, activated AtMPK6 and AtMPK4, respectively, in the presence of [γ-^{32}P]-ATP. The samples were separated with SDS-PAGE, visualized by Coomassie Blue staining and incorporation of radiolabelled phosphate was detected with autoradiography.

4.2. Modified pEU3 vectors for wheat germ cell-free protein expression

We aimed to investigate the cell-cycle functions of γ-tubulin by analysing *in vitro* protein-protein and protein-DNA interactions. Because these experiments demand several putative interacting partners, it was a prerequisite to possess a toolbox with several affinity tags from which we could choose the most appropriate when required. We set out to expand the repertoire of WGE CFPS vectors to allow the translation of target proteins with His$_{12}$-tag, FLAG-tag, HALO-tag, or double-tags (N-terminal GST-tag and C-terminal AviTag or His$_6$-tag). My work was to design and construct the modified His$_{12}$-tag and FLAG-tag coding vectors and to test the expression of the tagged proteins. In the following, only the His$_{12}$-tag and FLAG-tag harbouring vectors will be presented.

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. Khan et al. presented an *E. coli* His$_{12}$-tagging expression vector that provided improved binding and purification of tagged proteins on nickel-nitrilotriacetic acid (Ni-NTA) surfaces and detection [15]. Since sensitive and specific

detection is a prerequisite in pull-down assays, 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 (Figure 4A), designated pEU3-NII-HxHLICNot. The vector was tested by insertion of the coding sequence of an *Arabidopsis thaliana* MPK (AtMPK9) by LIC. Both His_6-AtMPK9 and His_{12}-AtMPK9 were produced by WGE and subsequently purified by immobilized metal affinity chromatography with Co-NTA beads. The affinity-purified proteins were separated on SDS-PAGE and analysed by Coomassie Blue staining (Figure 4B) and Western blot (Figure 4C). Both proteins were successfully synthesized and purified with Co-NTA beads; we observed only a slightly improved binding of His_{12}-tagged AtMPK9 to Co-NTA beads, and – contrary to our expectations – no increase in protein purity (Figure 4B).

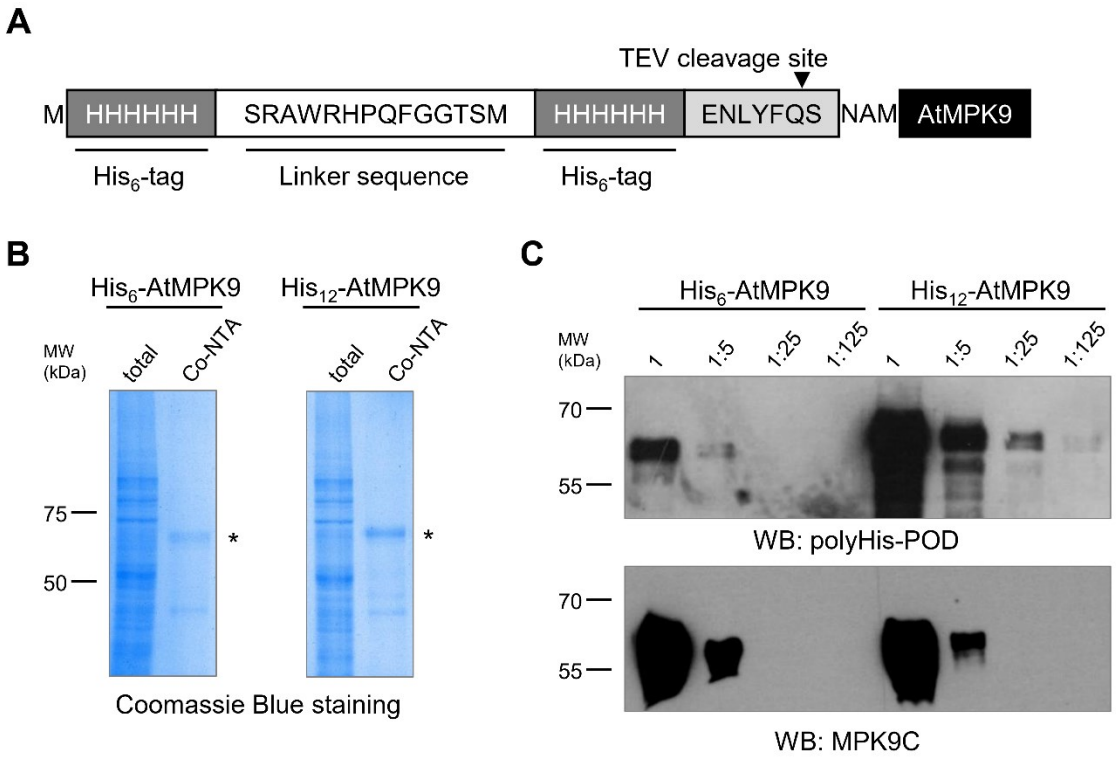

Figure 4. Immobilized metal affinity chromatography purification and Western blot of His_6-AtMPK9 and His_{12}-AtMPK9. (A) The schematic amino acid sequence of the His_{12}-tagging vector coding region. **(B)** *In vitro* translated and Co-NTA purified His_6-AtMPK9 and His_{12}-AtMPK9 proteins were separated with SDS-PAGE and visualized by Coomassie Blue staining. Purified AtMPK9 proteins are shown with asterisks. **(C)** Serial five-fold dilutions of total translation mixtures were studied by Western blot. The membranes were probed with anti-polyHis-POD and anti-MPK9C antibodies, respectively. **(B,C)** Expected molecular weights of expressed proteins: His_6-AtMPK9 60.4 kDa, His_{12}-AtMPK9 62.8 kDa. The molecular weight markers are shown in kDa.

For Western blot analysis, we prepared five-fold dilutions of the AtMPK9 containing total translation mixtures. Detection of the proteins by anti-polyHis-POD antibody resulted in at least an order of magnitude increased sensitivity with double labelling in comparison to the conventional His_6-tagged AtMPK9. The equal loading of the AtMPK9 was demonstrated by using anti-AtMPK9C antibody (Figure 4C).

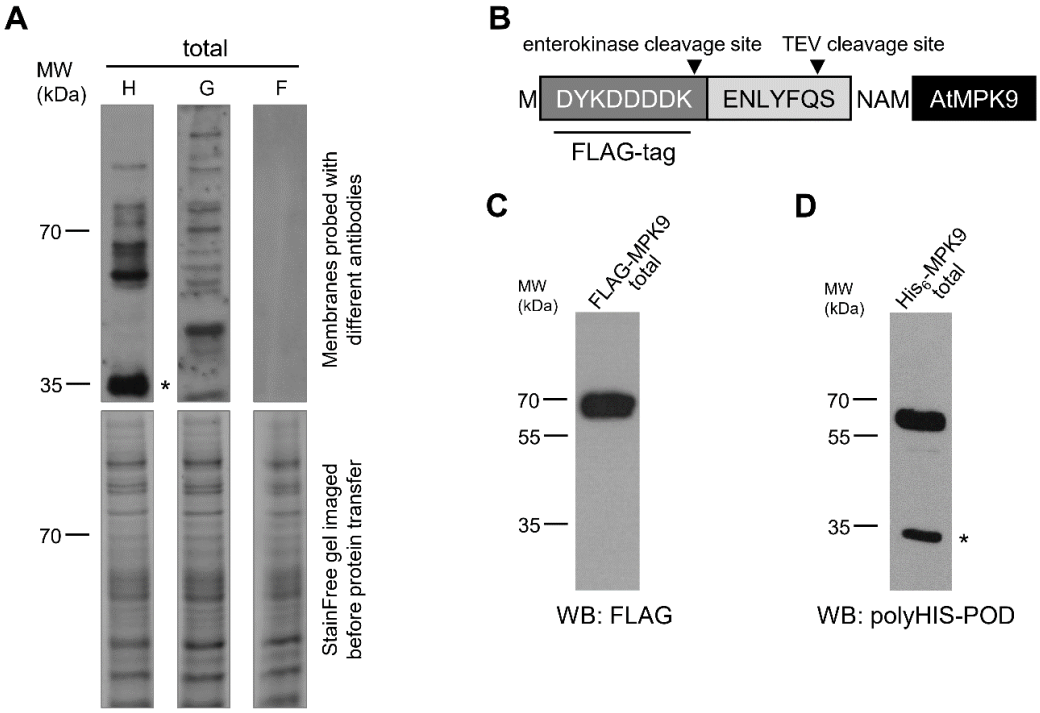

Figure 5. Western blot analysis of crude WGE and FLAG-tagged AtMPK9. (A) An aliquot of WGE (W7240) was run on TGX Stain-Free gel and imaged before transfer by UV light (bottom). The proteins transferred to the membrane were probed with anti-polyHis-POD (lane H), anti-GST (lane G), and anti-FLAG (lane F) antibodies (upper). **(B)** The schematic amino acid sequence of the FLAG-tagging vector coding region. **(C)** Total translation mixtures containing FLAG-AtMPK9 and His_6-AtMPK9 were separated on SDS-PAGE and analysed by Western blot with anti-FLAG and anti-polyHis-POD antibodies. The most prominently detected endogenous wheat germ protein by anti-polyHis-POD is indicated with an asterisk. Expected molecular weights: FLAG-AtMPK9 60.6 kDa, His_6-AtMPK9 60.4 kDa. The molecular weight markers are shown in kDa.

The other tag that was introduced into the WGE CFPS vectors is the merely 8 amino acid long FLAG epitope tag (Figure 5B). Its high aspartic acid content confers hydrophilicity; thus, the tag is usually found on the surface of the fusion protein and facilitates antibody binding. This amino acid stretch is rarely found in nature; therefore, the antibodies produced against the tag are greatly discriminating and make this tag suitable for highly specific detection. First, we separated crude total translation mixtures on SDS-PAGE and compared the Western blot signals of anti-polyHis-POD, anti-GST,

and anti-FLAG antibodies, respectively. Equal loading was demonstrated by StainFree imaging of the gel before transfer (Figure 5A, bottom). ECL analysis has shown that the anti-polyHis-POD and anti-GST antibodies could decorate several proteins in the WGE, while the anti-FLAG antibody did not generate a signal, not even after extended exposure (Figure 5A, upper). This observation prompted us to construct the pEU3-NII-FLAGLICNot vector with N-terminal FLAG-tag and we tested it by insertion of AtMPK9 as before. We translated and compared Western blot signals of His_6- and FLAG-tagged AtMPK9 containing total translation mixtures with anti-polyHis-POD and anti-FLAG antibodies, respectively. In agreement with our previous results, the anti-FLAG antibody was highly specific and detected a single band of FLAG-AtMPK9, whereas with anti-polyHis-POD we detected an approximately 35 kDa endogenous wheat germ protein beside His_6-AtMPK9 (Figure 5C).

4.3. *In vitro* translation of γ-tubulin with different affinity tags

It has been described that γ-tubulin can be present at different polymerization levels; thus, to assess the ratio of monomeric γ-tubulin in the case of the WGE translated γ-tubulin, we produced the GST-, His_6-, His_{12}-, and FLAG-tag variants of the protein. Following centrifugation, the supernatant containing the soluble proteins was separated from the precipitate containing the insoluble proteins. The samples were separated by SDS-PAGE and analysed by Western blot with the appropriate antibodies. 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 (Figure 6).

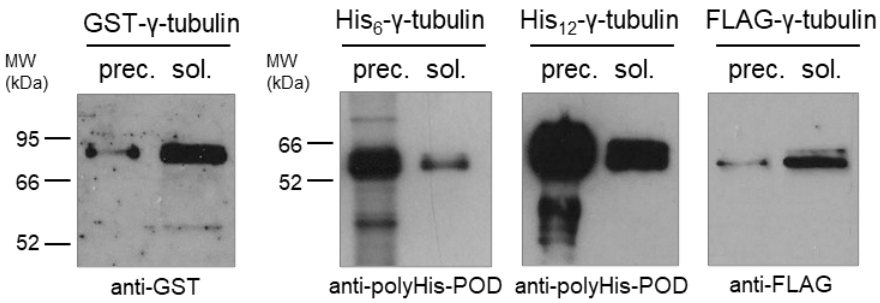

Figure 6. Effect of various affinity tags on *in vitro* translated γ-tubulin solubility. Total translation mixtures containing GST-, His_6-, His_{12}- and FLAG-γ-tubulin were centrifuged and the precipitated (prec.) and soluble (sol.) fractions were separated by SDS-PAGE and analysed by Western blot with anti-FLAG, anti-polyHis-POD and anti-GST antibodies. Expected molecular weights: GST-γ-tubulin 79.9 kDa, His_6-γ-tubulin 55.3 kDa, His_{12}-γ-tubulin 57.7 kDa, FLAG-γ-tubulin 55.4 kDa. The molecular weight markers are shown in kDa.

Of note, similarly to our results with AtMPK9, we have seen improved detection of His_{12}-tagged γ-tubulin compared to its His_6-tagged form under the same conditions (data not shown).

In another experiment, we co-translated His_{12}-γ-tubulin with GST and GST-AtE2FA, respectively, by adding equal amounts of *in vitro* synthesized mRNA to the translation reactions. When γ-tubulin was co-translated with the GST-tagged putative interaction partner AtE2FA, γ-tubulin solubility was increased compared to when was translated alone or co-translated with the negative control GST (Figure 7). The yield of the expressed His_{12}-γ-tubulin was decreased with the increased size of the protein co-translated, probably due to the engagement of the translational machinery in the co-translation of a larger protein (Figure 7).

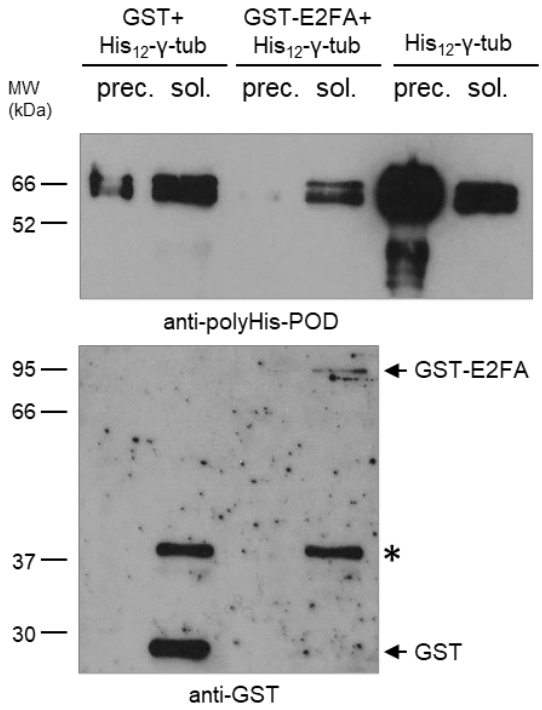

Figure 7. Effect of co-translation on His_{12}-γ-tubulin solubility. Total translation mixture containing His_{12}-γ-tubulin and co-translations containing His_{12}-γ-tubulin and GST or GST-E2FA were centrifuged and the precipitated (prec.) and soluble (sol.) fractions were compared. The samples were separated by SDS-PAGE and analysed by Western blot with anti-polyHis-POD and anti-GST antibodies. The non-specifically decorated endogenous wheat germ protein is indicated with an asterisk. Expected molecular weights: GST 26.0 kDa, GST-E2FA 78.6 kDa, His_{12}-γ-tubulin 57.7 kDa. The molecular weight markers are shown in kDa.

These results encouraged us to use *in vitro* translated γ-tubulin for our interaction experiments and to use co-translation whenever possible. In most cases, following the

translation reaction, the translation mixtures were centrifuged and only the soluble protein fractions containing supernatants were used.

4.4. γ-Tubulin interaction with E2Fs

Animal and plant γ-tubulin nuclear localization was shown by several research groups and cell cycle-related functions of γ-tubulin were also suggested. In animal cells, it is known that γ-tubulin interacts with E2F1 to regulate cell proliferation [36]. We aimed to test plant γ-tubulin interactions with the three classical E2F transcription factors AtE2FA, AtE2FB and AtE2FC. To this end, His_6-tagged γ-tubulin was co-translated with GST-tagged E2Fs and GST pull-down assay with glutathione beads showed that all three E2Fs can bind γ-tubulin (Figure 8A, left block). Next, we confirmed that all three *in vitro* translated E2Fs can interact with AtDPA and AtDPB (data not shown), then we used His_6-AtDP-containing total translation mixtures to check whether they can disrupt the previously formed γ-tubulin-AtE2F complexes. The addition of AtDP proteins did not result in the detection of non-complex forming unbound γ-tubulin, suggesting that even an excess of AtDPs could not displace γ-tubulin from the preformed γ-tubulin-AtE2F complexes (Figure 8A, right block). Although the data is not shown, we also confirmed that His_6-AtDPs do not bind to glutathione beads non-specifically and only interact very weakly with γ-tubulin in comparison to AtE2Fs. Based on these results, bead binding of AtDPs can be explained either by heterotrimer complex formation, i.e. γ-tubulin-AtE2F-AtDP complexes, or interaction with bead-bound E2Fs which are not bound by γ-tubulin. This latter scenario could be assumed since it cannot be excluded that following co-translation, the E2F proteins are in molar excess compared to γ-tubulin.

Because γ-tubulin is reported to be a sticky protein, in a similar pull-down assay setup we assessed its interaction with another transcription factor, the cell fate-determining Myb-related protein 66 (AtMYB66). For this assay, we used His_{12}-tagged γ-tubulin that can be detected with high sensitivity, as shown previously. We demonstrated that the His_{12}-γ-tubulin-GST-AtE2FA complex is not disrupted even under stringent (500 mM NaCl) washing conditions, while His_{12}-γ-tubulin was not found to be associated with AtMYB66-coated glutathione beads (Figure 8B). Collectively, these results indicate that γ-tubulin interaction with the studied E2F proteins is highly specific and the interaction with DPs does not disrupt the E2F-γ-tubulin complexes.

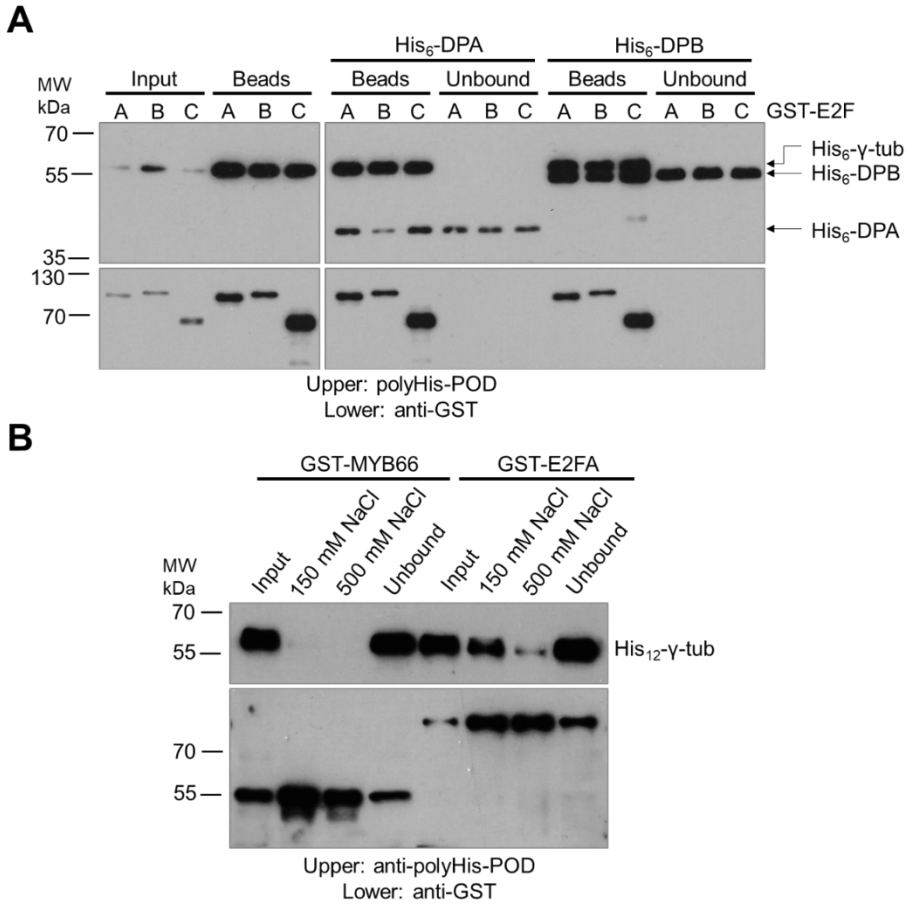

Figure 8. γ-Tubulin specifically interacts with E2Fs and this interaction is not affected by DPs. (A) His_6-γ-tubulin was co-translated with GST-tagged E2FA, E2FB, and E2FC, and GST pull-down assay using glutathione beads was performed. Translation mixtures containing His_6-DPA and His_6-DPB were added to the isolated complexes to investigate whether DPs can disrupt the preformed E2F-γ-tubulin interactions. **(B)** Translated His_{12}-γ-tubulin was incubated with GST-MYB66 and GST-E2FA transcription factors, and the complexes were isolated by glutathione beads. The non-specifically bound proteins were washed off with 150 mM or 500 mM NaCl. **(A,B)** The input, bead-bound and unbound proteins were probed with anti-polyHis-POD (upper) and anti-GST (lower). Expected molecular weights of expressed proteins: GST-E2FA 78.6 kDa, GST-E2FB 77.6 kDa, GST-E2FC 70.5 kDa, GST-MYB66 49.6 kDa, His_6-DPA 35.0 kDa, His_6-DPB 44.7 kDa, His_6-γ-tubulin 55.3 kDa. The molecular weight markers are shown in kDa. Of note, the apparent molecular weight of GST-E2FB is higher than the calculated one, thus signal specificity and successful protein translation were also confirmed by anti-E2FB antibody (data not shown).

To elucidate further details of ternary complex formation we aimed at determining the E2F segment responsible for γ-tubulin binding. We truncated E2Fs up to their DNA-binding domains (Figure 1B) and carried out the previously described GST pull-down assay by using the mutant E2F proteins. According to our expectations, in absence of their dimerization domains, the E2F mutants were unable to bind AtDPB (Figure 9A). On the

contrary, γ-tubulin could bind to the truncated E2Fs, implying that the E2F dimerization domain is not required for interaction with γ-tubulin (Figure 9B). Altogether, these data hint that γ-tubulin and AtDPB can simultaneously associate with AtE2Fs and form a ternary complex.

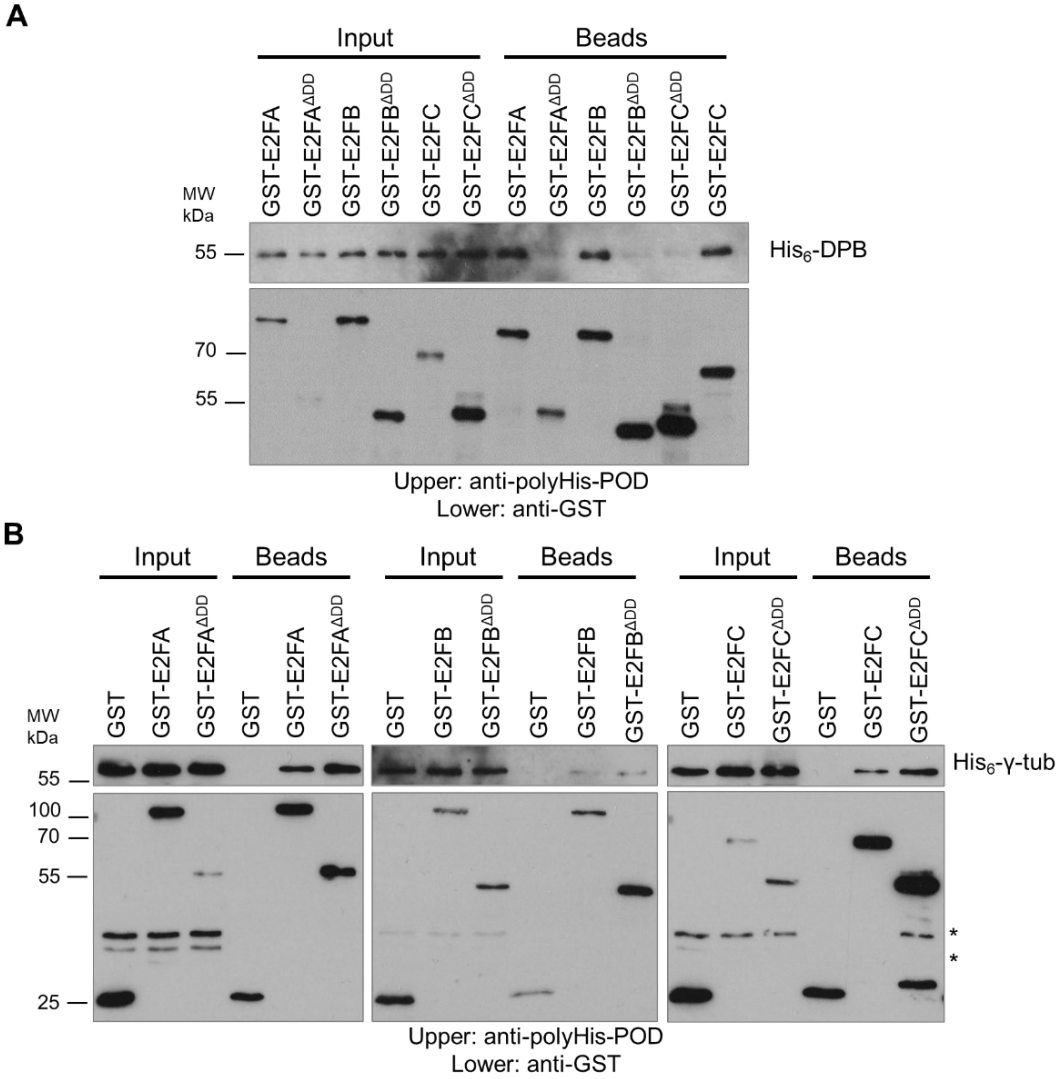

Figure 9. Dimerization domain (DD) of E2Fs is not required for γ-tubulin binding. (A) The wild type and truncated (ΔDD) GST-E2Fs were translated *in vitro* and incubated with His_6-DPB-containing translation mixtures. The complexes were isolated by GST pull-down assay using glutathione beads to study if the truncated E2Fs can bind DPB. **(B)** GST, wild type GST-E2Fs and truncated GST-E2FΔDDs were translated and incubated with His_6-γ-tubulin. The complexes were isolated by glutathione beads to test if any of the truncated E2Fs can bind γ-tubulin. **(A,B)** The input and bead-bound proteins were probed with anti-polyHis-POD (upper) and anti-GST (lower). Expected molecular weights of expressed proteins: GST-E2FA 78.6 kDa, GST-E2FAΔDD 51.9 kDa, GST-E2FB 77.6 kDa, GST-E2FBΔDD 47.6 kDa, GST-E2FC 70.5 kDa, GST-E2FCΔDD 52.4 kDa, His_6-DPB 44.7 kDa, His_6-γ-tubulin 55.3 kDa. The molecular weight markers are shown in kDa.

4.5. Analysis of DNA binding capacity by EMSA and AlphaScreen

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. The proteins were *in vitro* translated and the consensus or mutant E2F-binding site containing DNA sequences were labelled with Cy5 and biotin for EMSA and AlphaScreen, respectively.

First, we tested the DNA-binding capacity of the different proteins with EMSA by incubating individual *in vitro* translation mixtures containing His_6-AtDPB, His_6-AtE2FB or GST-γ-tubulin or their combinations with Cy5-labelled DNA sequences. We did not detect gel electrophoretic mobility shift of the mutated DNA with any of the proteins or protein combinations. 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 (Figure 10A). Furthermore, the co-incubation order of the three protein-containing mixtures before DNA was added did not affect the previously discussed mobility shift (Figure 10A, last three lanes). Surprisingly, the same shift was detected whether the putative heterodimer or higher molecular weight heterotrimer was bound to the DNA. To unambiguously confirm DNA binding of the E2F-DP-γ-tubulin complex we performed a super-shift EMSA (Figure 10B). To this end, combinations of translation mixtures containing untagged AtDPB, GST-AtE2FA and His_6-γ-tubulin were added to the E2F-binding site possessing DNA and the mixtures were complemented by anti-polyHistidine antibody prior to electrophoresis. Separation of the diverse mixtures provided a strong band for the sample containing all three proteins, implying DNA binding of the heterotrimer.

Finally, we tested the protein-DNA interactions by using AlphaScreen with Streptavidin Donor beads and Nickel chelate Acceptor beads. We used biotinylated DNAs of consensus and mutated E2F-binding sites and the same target protein containing mixtures as described in our EMSA experiment, i.e. His_6-AtDPB, His_6-AtE2FB and GST-γ-tubulin. The strength of the interaction is characterized by the measured luminescence signal intensity in counts per second (cps). 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 (Figure 11). Interestingly, the ternary complex also provided a strong but slightly decreased signal in comparison to the AtE2FB-AtDPB, suggesting that γ-tubulin might modulate DNA binding of the heterodimer (Figure 11).

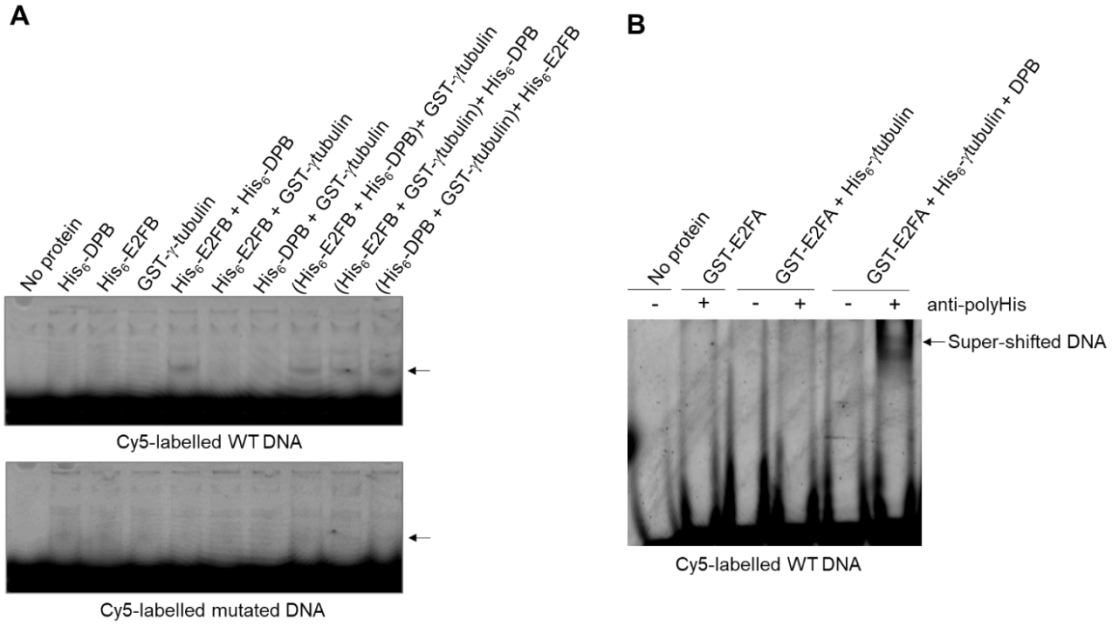

Figure 10. DNA binding capacity of various combinations of His6-DPB, His6-E2FB and GST-γ-tubulin by EMSA. (A) The target protein containing total translation mixtures were incubated with Cy5-labelled DNA coding for wild type (WT) or mutated E2F-binding site as specified, and the protein-DNA interactions were analysed by native PAGE. In the case of heterotrimers, two proteins were pre-incubated before adding the third protein, as marked by the brackets. The position of the shifted band is indicated by an arrow. **(B)** To demonstrate the E2F-binding site binding capacity of the putative heterotrimer, the target protein containing total translation mixtures were incubated with Cy5-labelled WT DNA, and some samples were supplemented with anti-polyHis antibody. The protein-DNA interactions were analysed by native PAGE. The position of the super-shifted band is indicated by an arrow.

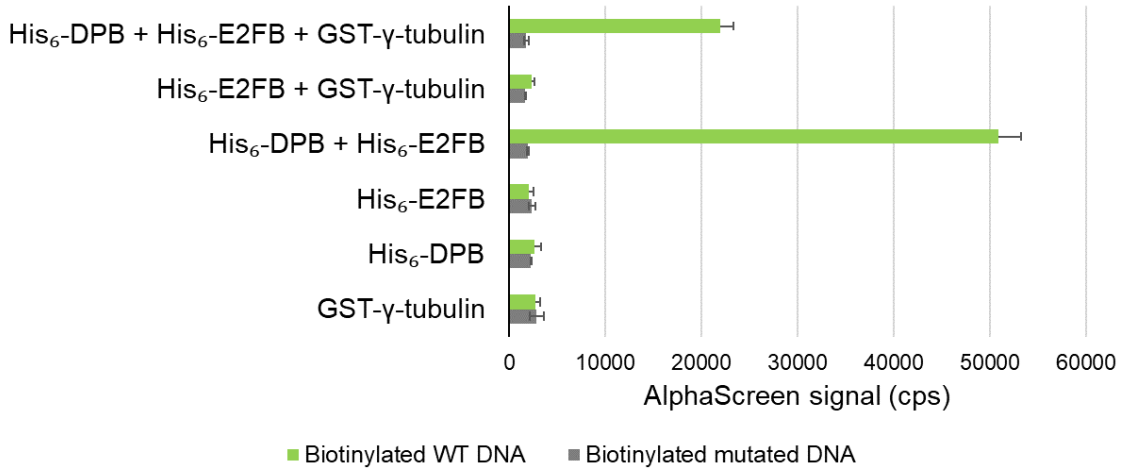

Figure 11. Analysing DNA binding capacity of various combinations of His_6-DPB, His_6-E2FB and GST-γ-tubulin by AlphaScreen. The target protein containing total translation mixtures were mixed in the indicated combinations and incubated with biotin-labelled DNA coding for wild type (WT) or mutated E2F-binding site in the presence of AlphaScreen Streptavidin Donor and Nickel chelate Acceptor beads. The AlphaScreen signal was measured in counts per second (cps).

5. Discussion

Wheat germ cell-free translation of individual eukaryotic proteins with native structure and their application in various assays (e.g. protein-protein and protein-DNA interaction assays, kinase assays, ubiquitination assays) were shown by several research groups, including ours.

Bardóczy et al. demonstrated that the WGE-based *in vitro* translation of plant MPKs outperforms *E. coli* overexpression in terms of *in vitro* kinase activity [12]. Enzymatically active MPKs can be purified from co-translation mixtures of the upstream GOF-MKK with the MPK of interest. Using the established system, we have carried out *in vitro* kinase assays with GST-tagged plant PIN1-HL and its non-phosphorylatable mutant by using activated and affinity-purified His_6-tagged AtMPK4 (Group B) and AtMPK6 (Group A) kinases. We showed by radiolabelling that both MPKs – although AtMPK4 to a higher extent – could phosphorylate AtPIN1-HL. LC-MS/MS analysis confirmed three novel (T227, T248, T286) and a previously reported (S337) phosphorylation [23] by AtMPK6. Replacement of the three Thr residues with non-phosphorylatable alanine residues (PIN1-3A) leads to the diminished phosphorylation by AtMPK6 and AtMPK4 *in vitro*.

Phosphorylation by PINOID kinases is known to influence the subcellular localization of PIN proteins [25]. The importance of MPK-mediated phosphorylation of the previously mentioned Thr residues was investigated *in planta* in root-derived protoplasts by our co-authors. They have found that the expression of the AtPIN1 phosphomimetic mutant results in protein aggregate-formation with altered subcellular localisation, whereas the non-phosphorylatable mutant appears in a soluble form and associates with the RFP:ER marker. These data collectively indicate that phosphorylation of the conserved Thr sites by the two environmentally activated MPKs might regulate the subcellular localization of AtPIN1 and plant organ growth.

The wider applicability of the WGE-based *in vitro* translation for protein functional studies was limited by the small number of available affinity tags. Therefore, we have developed further our LIC site and His_6- and GST-tag harbouring vectors to synthesize proteins with FLAG- and His_{12}-tag. The FLAG-tag is a small peptide tag (DYKDDDDK), which is rarely found naturally in proteins, especially in comparison to the relatively abundantly occurring histidine composed amino acid stretches of eukaryotic proteins. FLAG-tagged proteins can be purified by bead-coupled anti-FLAG antibodies, making it

an expensive purification technique. In consequence, the FLAG-tag is generally used when specific detection rather than the purification of the target protein is required [13]. Khan et al. demonstrated that two His_6 tags connected by a linker sequence had improved binding to Ni-NTA surfaces [15]. With an ambition to improve the purification of WGE expressed proteins, we created the His_{12}-tagging vector. According to our results, the binding was not enhanced significantly compared to the His_6-tagged protein, although further investigations with other affinity beads and buffer conditions are required to address this issue unambiguously. On the other hand, we could see a striking sensitivity difference between His_6- and His_{12}-tagged proteins if they are detected on Western blots, i.e. at least 25x less target protein could be visualized by using the same Western blot conditions. This result makes the application of His_{12}-tagged proteins the method of choice when in pull-down assays the detection of small amounts of prey proteins is required. Of note, FLAG-tag detection sensitivity could be improved by using three FLAG tags in tandem, i.e. a frequent practice in the case of epitope tags [13].

Although most studies focus on γ-tubulin function as a centrosome and MT organizer, γ-tubulin was shown to have functions in the coordination of cytosolic and nuclear events during the cell cycle in both animal and plant cells. For a long time, the chromatin-associated γ-tubulin amounts were underestimated because commercially available antibodies or tag-specific anti-GFP antibodies did not recognize this pool, possibly due to the PTMs and different conformations of different task performing γ-tubulins [81, 84, 87]. Nuclear localization of γ-tubulin in animal and plant cells in different stages of the cell cycle triggered several studies to investigate the non-canonical roles of γ-tubulin [87, 93]. Recent findings indicated that mammalian γ-tubulin has a role in G_1/S transition through interaction with E2Fs in the nucleus and modulates their transactivation activity [36]. In a collaboration, we aimed at investigating under *in vitro* circumstances whether *Arabidopsis* γ-tubulin has similar roles in cell cycle regulation in plants as well.

Due to the polymerization-prone nature of γ-tubulin [63, 90] we have compared the solubility of tagged γ-tubulins by using our novel vectors. Following centrifugation of the crude translation mixtures, the precipitates and soluble protein-containing supernatants were separated by SDS-PAGE and analysed by immunoblotting. The obtained data demonstrated different solubilities of proteins of different tags. In contrast to the His_6- and His_{12}-labelled varieties, most of γ-tubulin was found in the soluble fractions when

GST- or FLAG-tag was used. In general, a high solubility rate is expected for proteins synthesized by WGE CFPS [124], and this was enhanced further by using the GST-tag – generally regarded as a solubility enhancing tag [13] – and the hydrophilic FLAG-tag [14]. The higher insoluble fraction of His-tagged γ-tubulin might be explained by the observation that polyhistidine tags could negatively alter protein solubility in certain cases [125].

Alleviated by the highly sensitive detection of His_{12}-tagged proteins, we examined the effects of co-translation on the solubility of the His_{12}-tagged γ-tubulin. The decreased translation rate and co-translational folding of complexes on ribosomes are reported to improve folding [126]. Indeed, the fraction of soluble His_{12}-γ-tubulin increased when it was co-expressed with its putative interacting partner GST-AtE2FA instead of GST.

Lindström et al. have observed that in the case of animal γ-tubulin the large molecular weight γ-tubules are formed in a GTP-dependent manner [86], whereas Chumová et al. have shown that GTP does not affect the assembly of higher molecular weight γ-tubulin filaments in plants [63]. GTP-binding, but not GTPase activity of γ-tubulin was described [67–69]. In the case of the bilayer *in vitro* translation used by us, the feeding buffer contains 0.25 mM GTP; thus, it cannot be excluded that the expressed γ-tubulin is co-translationally GTP-bound, as its affinity for GTP is in the nanomolar range.

According to the classical model, E2F transcription factors have a role in preparation for S phase and commitment to cell division. They form a complex with DPs and bind to promoters of genes involved in DNA replication. In animal cells γ-tubulin can interact with activator E2F1, E2F2, or E2F3, but not with repressor E2F6 [36]. The border between activator and repressor activities of plant E2Fs is not that obvious as in the case of animal E2Fs [48]. We provide several lines of evidence that plant γ-tubulin can specifically interact with all E2Fs: (i) co-translation of γ-tubulin with AtE2FA improved γ-tubulin solubility, (ii) not even high salt concentration disrupts the interaction between γ-tubulin and AtE2FA, (iii) γ-tubulin can be detected with GST-tagged AtE2FA, AtE2FB and AtE2FC in GST pull-down assays. Höög et al. mapped the interaction surface between animal γ-tubulin and activator E2Fs to the E2F heterodimerization domain, and accordingly γ-tubulin displaced DP1 from the E2F1-DP1 heterodimer [36]. In contrast, our dimerization-domain containing truncated plant E2Fs (data not shown) and our dimerization domain lacking E2F variants also interact with γ-tubulin, suggesting that the

dimerization domain is not required for plant E2F-γ-tubulin interactions. These results were corroborated by the finding that γ-tubulin is not displaced from AtE2F-γ-tubulin complexes by AtDPs, these three proteins rather form a ternary complex. The interaction between γ-tubulin and AtE2Fs was also confirmed by *in planta* immunoprecipitation reactions implemented by our co-authors.

In mammalian cells, γ-tubulin interaction with activator E2Fs represses their activities and regulates G_1/S transition, e.g. repression of cyclin E expression is assumed to prevent centrosome reduplication. It was also revealed that animal γ-tubulin can bind directly to the E2F binding site, and this binding is mediated by the C-terminal domain of γ-tubulin encompassing its DNA binding domain (Figure 2B) [36]. In our EMSA and AlphaScreen *in vitro* experiments, γ-tubulin alone or in association with AtE2FB did not bind to the E2F consensus DNA sequence, only its ternary complex-forming form could bind to the E2F binding site. We showed that the formation of the putative ternary complex through random pre-incubation of the mixtures does not influence DNA binding; thus, in agreement with our pull-down assays, AtDPB and γ-tubulin binding to AtE2FB do not take place at overlapping sites. Furthermore, our AlphaScreen experiments suggest that the association of γ-tubulin with the AtE2FB-AtDPB heterodimer modulates the strength of DNA binding of AtE2FB-AtDPB, i.e. DNA binding strength is decreased, but confirmation of this observation requires further experiments.

Because γ-tubulin and E2F1 bind to the same DNA sequence in animal cells, Rb and γ-tubulin might complement each other in the regulation of gene expression. The E2F induced genes are required for centrosome duplication and DNA replication. It is also known that Rb is involved in the recruitment of DNA-remodelling factors that indicates γ-tubulin might have similar functions [85]. The expression and localization of γ-tubulin is altered in retinoblastoma; thus, γ-tubulin might be a suitable target in Rb-deficient tumours [95]. Additionally, γ-tubulin has an important role in the proper operation of the nervous system, the spontaneous mutation of *TUBG1* was associated with brain malformations causing intellectual disabilities in children [77].

Although cell cycle regulators are conserved between animals and plants, the latter organisms have developed unique mechanisms owing to their sessile nature [127]. The participation of γ-tubulin in the formation of a repressor complex together with E2Fs at the G_1/S transition seems to be evolutionarily conserved but several plant-specific

regulatory roles are also suggested at the G_2/M transition for this protein. Our co-authors have found that γ-tubulin silencing derepresses certain E2F target genes at both G_1/S and G_2/M transitions, e.g. *CYCD3;1* and *CDKB1;1*, respectively, and causes endoreduplication. In addition to the plant-specific mitotic regulator CDKB1;1, they have found that γ-tubulin also participates in the repression of *CCS52A*, which was shown to be repressed also by the AtE2FA-AtRBR1 complex [47]. AtCCS52A is an activator of the plant anaphase-promoting complex and represses endocycle onset [127]. Mitotic roles of plant γ-tubulin are also supported by its co-localization with AtCYCB1;3 at the nuclear membrane during G_2 phase [46].

In plants, the decision between proliferation and differentiation is tightly regulated. When they stop proliferating and start differentiating, plants often choose to enter the endocycle (endoreplication, endoreduplication), and γ-tubulin seems to be a key player in the coordination of these plant developmental events.

6. 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, and 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, and accordingly, 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.

7. Summary

The effectors of MAPKs, a broad range of substrates, are modulated through their phosphorylation states. AtPIN1-HL produced by *in vitro* translation was subjected to *in vitro* kinase assays with activated AtMPK6 and AtMPK4 by using ^{32}P-labelled ATP. We found that both kinases could phosphorylate AtPIN1. Next, we performed an *in vitro* kinase assay with cold ATP and AtMPK6, and LC-MS/MS analysis of the sample revealed three novel phosphorylation sites. Finally, the obtained results with the non-phosphorylatable PIN1-3A mutant protein confirmed that the mutated amino acid residues are indeed involved in the phosphorylation and AtPIN1-HL is a *bona fide* AtMPK4 and AtMPK6 substrate. Intending to implement sophisticated *in vitro* functional analyses of proteins, it was indispensable to continuously expand and enhance our toolkit; thus, we have improved the available WGE-based *in vitro* translation compatible vectors. The data presented in the thesis testified enhanced detection specificity and sensitivity of FLAG- and His_{12}-tagging vector encoded proteins, respectively. In addition to the classical role of γ-tubulin in MT nucleation, it was also shown to affect the transcriptional activity of E2F1 in mammalian cells during S phase. Considering the *in planta* observations hinting nuclear functions of γ-tubulin, we aimed to investigate the plant γ-tubulin-AtE2F interactions and DNA-binding properties. Knowing that γ-tubulin is a notoriously polymerizing protein, we thoroughly evaluated the properties of differently tagged plant γ-tubulin in the WGE-based translation system. We found that all studied tags provided a certain level of γ-tubulin in a soluble monomeric form. The protein interaction studies showed that γ-tubulin could interact with the analysed plant E2Fs and could not be expelled from the complex even by an excess of DPs. The specificity of these interactions was scrutinized by applying the AtMYB66 transcription factor. These experiments demonstrated that while the AtMYB66 protein could not interact with γ-tubulin, the γ-tubulin-AtE2FA complex could not be disrupted even by rigorous washing with a high salt concentration buffer. We also showed by using the truncated version of E2Fs that their dimerization domain is not required for γ-tubulin binding, and γ-tubulin and DPs can simultaneously bind to E2Fs and form a ternary complex. Finally, we found that the γ-tubulin-AtE2FB-AtDPB ternary complex can bind to the E2F consensus motif holding DNA and – similarly to the AtE2FB-AtDPB heterodimer – this binding takes place through the E2F protein.

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9. 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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Publications not directly related to the thesis:

Veronika Svitkova, Jana Blaskovicova, Monika Tekelova, **Brigitta M Kallai**, Teodora Ignat, Veronika Horackova, Petr Skladal, Pavel Kopel, Vojtech Adam, Dana Farkasova, Jan Labuda. (2017) Assessment of CdS quantum dots effect on UV damage to DNA using a DNA/quantum dots structured electrochemical biosensor and DNA biosensing in solution. SENSORS AND ACTUATORS B: CHEMICAL 243:435–444 **IF: 5.667**

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