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Characterization of RanGTPase pathway proteins in plants

Ph.D. Thesis

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Declaration

I declare that this Ph.D. thesis has been written solely by myself. All sources cited in this work are listed in the "Reference" section. All published results included in this thesis have been approved by the co-authors.

In Olomouc, 27th June, 2014

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Abstract

Accurate segregation of genetic information and therefore complete set of genes to daughter nuclei is one of the key events in cell cycle. Proper timing of cell cycle events is regulated during interphase by nucleocytoplasmic transport of regulatory proteins. During mitosis, chromosome segregation is mediated by mitotic spindle, a microtubular structure present in all eukaryotic cells. RanGTPase pathway is one of the most important signaling pathways involved in the control of nucleocytoplasmic transport and mitotic spindle assembly. Although the regulators of RanGTPase pathway have been well characterized in animals, the information in plants is limited. In order to clarify these processes in plants, I have investigated new and poorly characterized proteins regulated by RanGTPase pathway in a model plant *Arabidopsis thaliana*.

Homologue of human Ran-binding protein in microtubule-organizing center, RanBPM was identified in *Arabidopsis*. *At*RanBPM was cloned to binary vectors and localization of the protein was analyzed using both microscopical techniques and biochemical techniques. In contrast to human homologue, *At*RanBPM protein is localized in nucleus and cytoplasm of interphase cells and is depleted during mitosis. Furthermore, *At*RanBPM is present in high molecular weight complexes. RanGTP was not observed among interactors of *At*RanBPM. Therefore the involvement of *At*RanBPM in RanGTPase pathway is unlikely and probably plays another role, most likely in regulation of protein degradation.

Targeting protein for Xklp2 (TPX2) is key factor involved in mitotic spindle assembly regulated by RanGTPase pathway. *At*TPX2 is localized with spindle microtubules similarly to its animal homologues; however, its exact function is unknown. To elucidate the process of microtubule nucleation in acentrosomal plant cells in more detail, *At*TPX2 was overexpressed in *Arabidopsis* cells. In *At*TPX2 overexpressing cells, the protein decorated microtubule bundles formed in the vicinity of nuclear envelope and in the nuclei. Moreover, colocalization and co-purification experiments provided an evidence for interaction of *At*TPX2 and importinβ and Ran, respectively. This suggests that *At*TPX2 is involved in chromatin-induced microtubule formation regulated by RanGTPase pathway also in acentrosomal plant cells.

It was shown that *At*TPX2 colocalizes with *At*Aurora1, *Arabidopsis* homologue of Aurora kinase A, in cell-cycle specific manner. Human TPX2 is an activator of Aurora kinase A, a mitotic kinase required for spindle formation and cell cycle progression. To prove whether similar regulatory mechanism exists also in plants, *At*TPX2 and *At*Aurora1 were expressed in bacteria and purified. It was shown that *At*TPX2 acts as a substrate for

*At*Aurora1. *At*TPX2 was not only the substrate, but also significantly increased autophosphorylation activity of the kinase. Moreover, the increased *At*Aurora1 activity resulted in increased phosphorylation of histone H3, the important downstream target of *At*Aurora1. Activation of *At*Aurora1 by *At*TPX2 could be a mechanism for translation of RanGTP signaling to phosphorylation cascade performed by Aurora kinases.

Altogether, our findings elucidated the process of chromatin-induced microtubule nucleation in acentrosomal plant cells regulated by *At*TPX2. Furthermore, *At*TPX2 was found to be the activator of AtAurora1, the mitotic kinase involved chromosome segregation and cell cycle progression. The results suggest that *At*TPX2 together with *At*Aurora1 might be involved in proper chromosome segregation during mitosis. Taken together, our findings help to elucidate complicated and still not fully understood phenomenon of chromosome segregation and cell cycle regulation, the key events in cell life.

Abstrakt

Přesné rozdělení genetické informace a tudíž i kompletní sady genů do dceřiných buněk jsou klíčovými událostmi buněčného cyklu. Transport regulačních proteinů mezi jádrem a cytoplazmou v interfázních buňkách reguluje správné načasování jednotlivých fází buněčného cyklu. Samotná segregace chromozomů je pak zprostředkována mitotickým vřeténkem, strukturou z mikrotubulů, která se vytváří během mitózy ve všech eukaryotických buňkách. RanGTPázová dráha je jedna z nejdůležitějších regulačních drah nezbytná pro život buňky, zapojená v regulaci transportu proteinů mezi jádrem a cytoplazmou, stejně jako v tvorbě mitotického vřeténka. Komponenty RanGTPázové dráhy jsou již poměrně dobře charakterizovány u živočišných buněk, nicméně jejich znalost u rostlin je limitována. Tato dizertační práce popisuje neznámé nebo málo charakterizované proteiny RanGTPázové dráhy u modelové rostliny huseníčku rolního (*Arabidopsis thaliana*).

První protein, kterým se tato dizertační práce zabývá je protein RanBPM. Poprvé byl popsán v lidských buňkách, kde byl lokalizován v centrosomech, tj. místech zodpovědných za organizaci mikrotubulárního cytoskeletu u živočichů. Protein se podílí na procesu nukleace mikrotubulů a byla prokázána jeho interakce s regulačním proteinem Ran. Na základě podobnosti sekvencí byl charakterizován jeho homolog u *Arabidopsis*, *At*RanBPM. Sekvence byla klonována do binárního vektoru a lokalizace proteinu byla studována pomocí biochemických technik a mikroskopie. Navzdory podobnosti s živočišným proteinem, *At*RanBPM je během interfáze lokalizován v jádře a cytoplasmě, a v mitóze signál zcela mizí. Protein je součástí vysokomolekulárních proteinových komplexů, nicméně nebyla prokázána jeho interakce s proteinem Ran. Rostlinný homolog proteinu RanBPM zřejmě plní jinou roli než účast v RanGTPázové dráze, pravděpodobně regulaci degradace proteinů.

Klíčovým faktorem zapojeným do tvorby mitotického vřeténka, který je regulovaný RanGTPázovou dráhou, je protein TPX2. Podobně jako jeho živočišný homolog je *At*TPX2 lokalizován s mikrotubuly mitotického vřeténka, nicméně jeho přesná funkce u rostlin není známá. Pro objasnění jeho funkce v rostlinných buňkách, byl *At*TPX2 nadprodukován v buňkách huseníčku, kde byl lokalizován se svazky mikrotubulů okolo jaderné membrány a jádra. Kolokalizační a kopurifikační studie prokázaly interakci AtTPX2 s importinemβ a také s proteinem Ran. Tato zjištění naznačují roli proteinu TPX2 v tvorbě mitotického vřeténka v blízkosti chromozomů regulovaného RanGTPázovou dráhou také u acentrozomálních rostlinných buněk.

Lidský homolog TPX2 je aktivátorem Aurora kinázy A, mitotické kinázy nezbytné pro

tvorbu mitotického vřeténka a průchod buněčným cyklem. Bylo prokázáno, že *At*TPX2 je v průběhu buněčného cyklu lokalizován s *At*Aurora1, homologem Aurora kinázy A u huseníčku. Pro potvrzení přítomnosti podobného mechanismu i u rostlin byly připraveny rekombinantní proteiny *At*TPX2 a *At*Aurora1 v bakteriích *Escherichia coli*. Bylo zjištěno, že *At*TPX2 je fosforylován kinázou *At*Aurora1 *in vitro*. *At*TPX2 významně zvyšoval autofosforylační aktivitu Aurora kinázy, která vedla ke zvýšené fosforylaci histonu H3, důležitého substrátu této kinázy. Tyto výsledky naznačují, že aktivace *At*Aurora1 pomocí *At*TPX2 může být důležitým mechanismem pro přenos RanGTP signalizace na fosforylační kaskádu zprostředkovanou Aurora kinázami. Výsledky publikované v této práci objasňují proces nukleace mikrotubulů v blízkosti chromatinu u acentrozomálních rostlinných buněk regulovaný proteinem *At*TPX2. *At*TPX2 byl charakterizován jako aktivátor *At*Aurora1, mitotické kinázy zapojené v segregaci chromozomů a průchodu buňky buněčným cyklem. *At*TPX2 by tedy mohl být společně s kinázou *At*Aurora1 zapojen v segregaci chromozomů během mitózy. Naše výsledky pomáhají objasnit komplikovanou a stále ne plně objasněnou problematiku segregace chromozomů a regulace buněčného cyklu, klíčové procesy buněčného cyklu.

Contents

1. Introduction

Eukaryotic cells are characterized by enclosure of chromosomes within cell nuclei by a nuclear envelope which forms a semi-permeable barrier separating nucleoplasm and cytoplasm. The nuclear envelope consists of a double membrane, which is permeated with nuclear pore complexes allowing the diffusion of small molecules and active transport of larger proteins and nucleic acids. Specific biochemical environment can be therefore established around chromatin, providing the environment for regulation of gene expression and separating sites of transcription from the sites of protein synthesis and metabolism. Separation of the genome within the nucleus, however, presents a problem for cell division, when replicated chromosomes have to be segregated into two daughter nuclei. Thus, regulation of transport between nucleus and cytoplasm and coordination of mitotic events are critical for maintaining basic cellular functions (Cooper, 2000).

Ran is a small GTPase that belongs to the Ras super-family of GTPases (Wennerberg *et al*., 2005). Members of the family are involved in nucleocytoplasmic transport, mitotic spindle assembly, regulation of cell-cycle progression and nuclear envelope reconstitution after completion of mitosis (Goldfinger, 2008). The function of Ran is well described in metazoans where cycling between RanGTP and RanGDP bound forms is regulated by Ran GTPase activating protein RanGAP1 and guanine nucleotide exchange factor RCC1 (Klebe *et al*., 1995). Despite a broad knowledge of RanGTPase pathway proteins and their regulatory factors in animals and yeast, its members in plants are still poorly characterized. Furthermore, plant cell specifics such as the presence of cell wall and lack of centrosomes, the main microtubule organizing centers in most animal cells, suggests differences between RanGTPase pathway in metazoans and plants (Masoud *et al*., 2013).

2. RanGTPase pathway: an overview

Small GTPases belong to the group of hydrolase enzymes. Similarly to the α-subunit of heterotrimeric G proteins, they can bind and hydrolyze guanosine triphosphate (GTP) to form guanosine diphosphate (GDP). The difference between these two groups of enzymes comes with the regulation – small GTPases hydrolyze its GTP after stimulation by upstream signal, which is followed by its transfer to downstream effector molecule by its binding. They are often called Ras superfamily of small GTPases in respect to the founding member, *Ras* oncogene (Goodsell, 1999). The superfamily comprises over 150 members in human and its

orthologs in other metazoans. In *Arabidopsis* ninety-three small GTPases genes are predicted (Yang, 2002). The superfamily members are divided to distinct subfamilies – Ras, Rad, Rab, Rap, Rho, Rheb, Rit, Arf, Miro and Ran. Ras members are involved in regulation of cell proliferation (Stacey *et al*., 1988), Rad GTPases interact with calmodulin, however, the exact function is not known (Moyers *et al*., 1997), Rab GTPases are involved in membrane trafficking and vesicular transport (Schwartz *et al*., 2007), Rap mediates cell adhesion (Bos, 2005). Rho members are involved in cytoskeleton dynamics and morphology and therefore in cell morphology (Kaibuchi *et al*., 1999). Rheb GTPases regulate mTOR, which controls cell growth, proliferation and mobility (Abruch *et al*., 2006). Rit GTPases probably play role in regulation of signaling pathways (Shao *et al*., 1999), Arf GTPases are involved in vesicle transport and actin remodeling (Boman and Kahn, 1995), and a new group of Miro GTPases participate in mitochondrial transport (Reis *et al*., 2009).

Ras-related nuclear protein (Ran), firstly described in 1993 by Moore & Blobel (1993), is an important regulator of several cell cycle events. The protein is relatively small, with the size of about 25 kDa and contains typical conserved 20 kDa Ran catalytic domain specific for the RanGTPase family, the effector binding site and C-terminal acidic motif. Furthermore, two functional catalytic loops are present: switch I and switch II that surround the γ-phosphate of GTP. The C-terminal motif contacts core of the Ran in GTP-bound form thus allowing switch I and II to interact with nucleotide and effectors. In GDP-bound form, C-terminal part extends away from the core and prevents switch I and II to mimic GTP-bound form, therefore protecting Ran from adopting erroneous RanGTP bound-like conformation (Scheffzek *et al*., 1995; Richards *et al*., 1995; Vetter *et al*., 1999). Ran is a soluble protein participating in protein import to the nucleus during interphase (Stewart, 2007), functioning in spindle assembly (Carazo-Salas *et al*., 2001), and in nuclear envelope assembly during mitosis (Zhang and Clarke, 2000). Such diverse processes are regulated by intracellular gradient between two Rans´ forms. Ran homologues have also been identified in plant species, such as tomato (Ach and Gruissem, 1994), tobacco (Merkle *et al*., 1994) and *Arabidopsis* (Haizel *et al*., 1997). In *Arabidopsis thaliana*, four genes for RanGTPase are present, namely *At*Ran1, *At*Ran2, *At*Ran3, *At*Ran4. *At*Ran1, *At*Ran2 and *At*Ran3 are about 95 % identical and differ mostly in its C-terminal part suggesting that they are true human Ran/TC4 orthologs, while *At*Ran4 which is more diverse probably fulfills another function. *At*Ran1, *At*Ran2 and *At*Ran3 localize to both nucleus and cytoplasm and their expression is highest in meristematic tissues (Haizel *et al*., 1997).

Generally, Ran is present as active GTP-bound form (RanGTP) and inactive GDP-

bound Ran (RanGDP), therefore creating so-called RanGTPase switch (Vetter, *et al*., 1999; Scheffzek, *et al*., 1995). Intrinsic activity of exchange between these two states is low and depends on the association of the GTPase with regulatory proteins. Guanine nucleotide exchange factors (RanGEFs) enhance the formation of RanGTP inside the nucleus during interphase or around chromatin in mitosis. In cytoplasm or distant from chromatin GTPase-activating proteins (RanGAPs) increases the GTPase activity of Ran and RanGDP formation (Figure 1; Lundquist, 2006). Furthermore RanGDP formation is stimulated by other Ran-binding proteins, Ran-binding protein 1 (RanBP1) and Ran-binding protein 2 (RanBP2) through their Ran-binding domain. After hydrolysis of GTP, small GTP binding protein is ready to begin the cycle again. Thus, positioning of these regulatory factors and active transport of Ran into nuclei ensures high concentration of RanGTP in nucleus and lower concentration of RanGDP in cytoplasm.

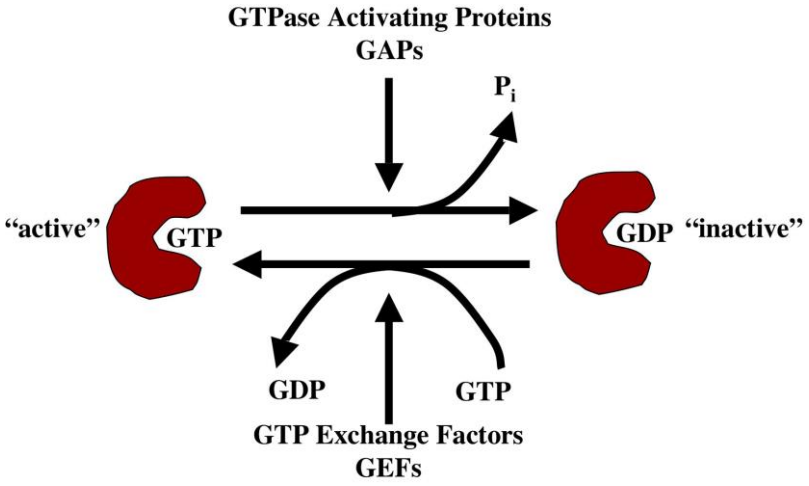

Figure 1: Schematic overview of RanGTPase switch. Ran is shuttling between active, GTP and inactive, GDP-bound forms (Lundquist, 2006)

2.1. Ran effector proteins

Ran-binding proteins are key players that affect RanGTPase pathway through interaction with Ran, therefore regulating both subcellular localization and conversion between GTP- and GDP-bound states of Ran. There are two groups of important effectors of RanGTP: Ran-binding proteins which contain at least one Ran-binding domain (Vetter *et al*., 1999), and nuclear transport receptors (Lonhienne *et al*., 2009). Depending on the order in which proteins were identified as RanGTP interactors, they were usually named as RanBP1, RanBP2 etc. Naming, however, does not reflect categorization to the above mentioned groups.

Arabidopsis RanGTPase pathway-related proteins and its human homologues are listed in Table 1. Ran-binding proteins with RanBP1-type Ran-binding domain act as regulators of Ran guanine nucleotide exchange (Vetter *et al*., 1999). They can either increase the binding affinity of transport receptor to RanGTP and cargo, as is the case of RanBP3 and nuclear transport receptor CRM1 (Englmeier *et al*., 2001), which directly stimulate RanGTP hydrolysis of GTPase activating protein RanGAP (Becker *et al*., 1995), or promote RanGTP hydrolysis, as does the founding member RanBP1, through coactivation of RanGAP (Bischoff and Gorlich, 1997). Ran-binding domain is about 150 amino acids long and binds with high affinity to GTP-bound form of Ran (Vetter *et al*., 1999). Due to its crucial role in RanGTP hydrolysis, RanBP1 and RanBP1-like proteins are among the most important Ran-binding proteins.

Ran- binding proteins with RanBP1-type Ran-binding domain form a small family of three members in human and six in *Arabidopsis*. *Arabidopsis* genome encodes three very similar RanBP1 genes – RanBP1a, RanBP1b (Haizel *et al*., 1997), and RanBP1c (Kim *et al*., 2001) and all of them display cytoplasmic localization. RanBP1a was shown to bind Ran, and RanBP1c acts as a coactivator of RanGAP1 *in vitro* (Kim and Roux, 2003).

Gene for RanGTPase activating protein RanGAP is present in two copies in *Arabidopsis thaliana*: *At*RanGAP1 and *At*RanGAP2. In agreement with data from animals, plant *At*RanGAPs are localized at nuclear envelope in interphase cells and are involved in regulation of nucleocytoplasmic transport (Pay *et al*., 2002). Targeting plant RanGAPs to nuclear envelope involves plant-specific WPP domain (Rose and Meier, 2001).

Table 1: **List of *Arabidopsis* proteins involved in RanGTPase pathway and their human homologues**. AGI locus designation, *Arabidopsis* protein designation and UniProt numbers are given. Plant importins are indicated by green colour, exportins are printed in red. To date uncharacterized proteins are printed in grey. Adapted from Merkle *et al*. (2011).

		Protein	AGI number	UniProt number	Human homologue	UniProt number
Ran		*AtRan1*	*At5g20010*	P41916	Ran	P62826
		AtRan2	*At5g20020*	P41917		
		AtRan3	*At5g55190*	Q8H156		
		AtRan4	*At5g55080*	Q9FLQ3		
Ran-binding effectors	RanBP1 type	*AtRanBP1a*	*At1g07140*	Q9LMK7	RanBP1	P43487
		AtRanBP1b	*At2g30060*	Q8RW68		
		AtRanBP1c	*At5g58590*	P92985		
		AtRanBPL1	*At1g52380*	Q9C829	RanBP3	Q9H6Z4
		AtRanBPL2	*At3g15970*	Q8LG62		
		AtRanBPL3	*At4g11790*	Q9T0E6	Nup50	Q9UKX7
		AtRanGAP1	*At3g63130*	Q9LE82	RanGAP1	P46060
		AtRanGAP2	*At5g19320*	Q9M651		
	nuclear transport receptors	*AtIMPbeta*	*At5g53480*	Q9FJD4	Importin beta	Q14974
		AtTRN1	*At2g16950*	Q8H0U4	TNPO1	Q92973
		AtSAD2	*At3g59020*	F4J738		
		AtCAS	*At2g46520*	Q9ZPY7	CAS, XPO2	P55060
		AtXPO1a	*At5g17020*	F4KFL2	CRM1	O14980
		AtXPO1b	*At3g03110*	F4IZR5		
		AtExportin-T (Paused)	*At1g72560*	Q7PC79	XPOT	O43592
		AtXPO5 (Hasty)	*At3g05040*	Q0WP44	XPO5	Q9HAV4
		AtRanBP7/8	*At2g31660*	F4IRR2	IPO7, IPO8	O95373, O15397
		AtIMP9	*At1g26170*	F4IE40	IPO9	Q96P70
		AtIMP11	*At3g08960*	F4IYK6	IPO11	Q9UI26
		AtRanBP6	*At5g19820*	Q93VS8	RanBP6	O60518
		AtIMP4	*At4g27640*	Q8W498	IPO4	Q8TEX9
		AtIMP13	*At1g12930*	Q94A39	IPO13	O94829
		AtTransportin SR	*At5g62600*	Q8GUL2	TNPO3	Q9Y5L0
		AtRanBP16/17	*At5g06120*	F4K2C1	XPO7, RanBP17	Q9UIA9, Q9H2T7
		AtExportin4	*At3g04490*	Q56Z41	XPO4	Q9COE2
		AtRanBPM	*At1g35470*	F4HYD7	RanBP9/RanBPM	Q96S59

The founding member of Ran binding proteins/nuclear transport receptor was importin β that transports proteins between cytoplasm and nucleus (Gorlich *et al*., 1997). Whole importin β family of RanBPs comprise about 20 members in human, in *Arabidopsis* about 17 RanBPs/NTR coding genes were found (Merkle, 2010) and to date, some of them have been

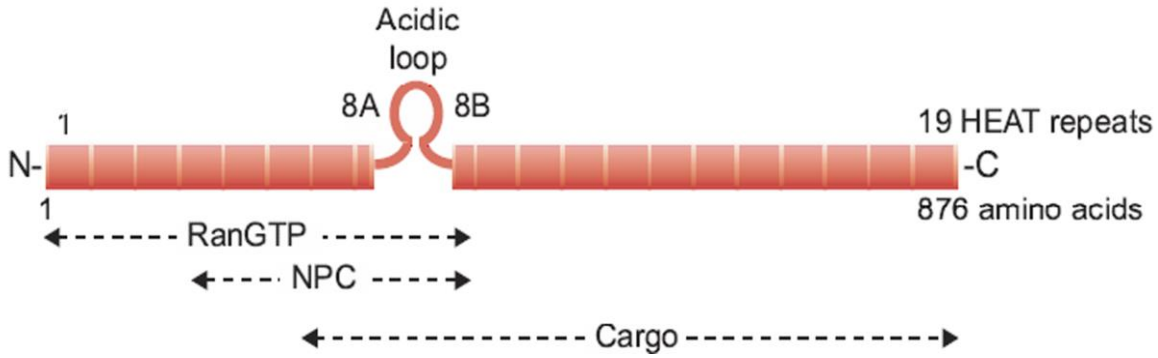

Figure 2: Structure of importin β. General structure of importin β is similar to all other Ran-binding nuclear transport receptors. Protein sequence consists of 19 HEAT repeats, each of them composed of A and B helix connected by a short turn. In HEAT repeat 8, the turn is replaced by an acidic loop involved in regulation of cargo binding and release. HEAT repeats 1 – 8 are responsible for binding of RanGTP. Repeats 4 – 8 bind nucleoporins and regulate NPC binding. Cargo binding occurs at C-terminal part of molecule (Strom and Weis, 2001).

characterized. All of them have relatively large molecular mass about 90 – 130 kDa. They exhibit sequence similarity mainly in its N-terminal RanGTP binding domain and possess variable number of HEAT repeats, tandem repeats of about 40 amino acids, named after four proteins where they were first identified, Huntingtin, Elongation Factor 3 (EF3), the Protein Phosphatase 2A (PP2A), and the yeast kinase Target of Rapamycin 1 (TOR1) (Andrade and Bork, 1995). Cargo binding occurs at C-terminal part of molecule (Figure 2).

2.1.1. Ran-binding protein in microtubule organizing centre

Ran-binding protein in microtubule-organizing center, RanBPM, was originally identified as Ran-binding protein by yeast two hybrid screen using Ran as a bait. The protein was found to be localized in animals´ microtubule-organizing centers, centrosomes (Yokoyama *et al*., 1995; Nakamura *et al*., 1998). Although, RanBPM did not contain typical Ran-binding domain as present in RanBP1 or importin β, further characterization revealed

that it has about 55 kDa, and when overexpressed it is able to nucleate microtubules in human cell cultures. Therefore, its putative role in microtubule nucleation was consistent with the involvement of RanGTPase pathway in chromatin-induced mitotic spindle assembly (Nakamura *et al*., 1998). Later, Nishitani *et al.* (2001) re-evaluated the original findings and found that 55 kDa RanBPM was incorrectly translated version of 90 kDa protein. Furthermore, antibody against 55 kDa truncated RanBPM did not recognize full-length protein and full-length RanBPM did not colocalize with centrosomes. Finally, only short RanBPM was able to nucleate microtubules.

RanBPM comprises four highly conserved domains - SPRY, LisH, CTLH and CRA and is homologous to the human RanBP10, another Ran-binding protein with RanGEF activity (Harada *et al*., 2008). Based on their unique domain composition, RanBPM and RanBP10 were defined as members of Scorpin family of proteins (SPRY-containing Ran-binding protein; Hosono *et al*., 2010). The above mentioned domains are mainly involved in various protein-protein interactions. Complete RanBPM acts as a scaffold protein which links interactions between cell surface receptors and their intracellular signaling pathways (Suresh *et al*., 2012; Murrin and Talbot, 2007). RanBPM is part of large CTLH-complexes, which are composed of LisH, CTLH and CRA domains-containing proteins, transducin/WD40 repeat proteins and armadillo repeat proteins. Similar complexes are present in mammals and budding yeast, their exact biological function remains elusive (Kobayashi *et al*., 2007; Regelmann *et al*., 2003).

3. RanGTPase pathway in nucleocytoplasmic transport

3.1. Organization of nuclear structure: an overview

The main characteristic of eukaryotic cells is the presence of various membrane-detached organelles or compartments in cytoplasm, cell nucleus being one of them. Nuclear envelope forms a physical barrier between nucleoplasm and cytoplasm and separates the sites of DNA replication and transcription from the sites of protein synthesis. Signal transfer between the nucleus and the cytoplasm is handled by different mechanisms, primarily through nuclear pore complexes (NPC), large protein structures embedded in nuclear envelope. Recently, indirect communication based on signal transmission from cytoskeleton to intranuclear component has been described. Nucleocytoplasmic communication is a bidirectional process and allows cells to respond to signals from cellular and organismal

environment (Cooper, 2000).

Nuclear envelope of eukaryotes is composed of two lipid bilayer membranes and is organized similarly (Kite, 1913). The outer nuclear membrane (ONM) is continuous with endoplasmic reticulum and the inner nuclear membrane (INM), forming perinuclear space continuous with endoplasmic reticulum between them (Watson, 1955). The fusion of the two membranes occurs at the NPCs, channels for regulated exchange of large molecules between cytoplasm and nucleoplasm. ONM and INM differ by chemical composition of their lipid bilayer and by associated proteins, which differ between particular cell types, tissues and species. Nuclear envelope provides shape, stability and plasticity for the nucleus and anchors nuclear pore complexes, thus providing a platform for communication between nucleus and cytoplasm. It also provides anchor sites for chromatin and cytoskeleton (Hetzer *et al*., 2005). Moreover, ONM of plants possess the activity to nucleate microtubules and acts as microtubule organizing center (MTOC) (Mizuno, 1993). A list of plant proteins localized at nuclear envelope and their putative animal homologues is given in Table 2.

3.1.1. Nuclear lamina

Nuclear lamina is a mesh of intermediate filaments, which lines inner nuclear membrane in vertebrates. One of the most important functions of lamins is the attachment of chromatin to nuclear envelope during interphase. Mutations and abnormalities in vertebrate's lamin composition cause wide range of laminopathies, syndromes in which one or more tissue is perturbed, therefore pointing to an importance of these proteins for the development of organisms (Worman, 2011).

Although the true lamin homologues have not been identified in plants, NMCP 1 (Nuclear Matrix Constituent 1) in *Daucus carota* and *Arabidopsis* lamin-like protein CRWN 1 – 4 (Crowded nuclei) are the best candidates. Despite the differences between the overall protein structure, these plant proteins show similarities with their putative animal counterparts, including the presence of coiled-coil domains, a role in regulation of nuclear shape and morphology, as well as localization at nuclear periphery (Masuda *et al*., 1997; Dittmer *et al*., 2007; Capell and Collins, 2006; Wang et al., 2013). Recently, AtLINC1 was found to interact with *Arabidopsis* SUN proteins that are responsible for its NE localization and tethering (Graumann, 2014). Therefore, AtLINC1 might be a true component of plant lamin-like structure.

3.1.2. Inner nuclear membrane proteins

Vertebrate nuclear envelope contains over 60 inner and outer nuclear membrane proteins. Almost all of the INM proteins interact directly with lamins (Wilson *et al*., 2010). SUN (Sad1p/Unc-84) domain homology proteins were identified as components of INM, with SUN-domain in perinuclear space and N-terminal domain in the nucleoplasm, interacting with lamins (Hodzic *et al*., 2004).

The only specifically INM-embedded proteins characterized to date in plants are *Arabidopsis* SUN-domain containing proteins *At*SUN1 and *At*SUN2 (Graumann *et al*., 2009). Although significantly smaller than animal SUN homologues, *At*SUN1 and *At*SUN2 possess the same structural composition and are localized at nuclear envelope (Oda and Fukuda, 2011). *At*SUNs interact through perinuclear space with proteins of outer nuclear membrane, forming LINC complexes (Linker of Nucleoskeleton and Cytoskeleton). A new nucleocytoplasmic link was identified by Batzenschlager *et al*. (2013) and Janski *et al*. (2012). Components of γ-tubulin complex (γ-TuCs) involved in microtubule nucleation at nuclear envelope and one of its interacting proteins, GIP (GCP3-interacting protein), may be involved in molecular connections between microtubule-organizing centers at the ONM and chromocenters bound to INM. Therefore there might be a direct link between microtubule nucleation and chromatin organization (Batzenschlager *et al*., 2013).

3.1.3. Outer nuclear membrane proteins

Proteins of the outer nuclear membrane identified in vertebrates belong to the spectrin repeat containing proteins (Syne/Nesprins). They are characterized by the presence of the C-terminal KASH-domain (Klarsicht/ANC-1/SYNE1 homology) that interacts with SUN-domain proteins of INM and forms LINC complexes - molecular bridges that physically link microtubules, actin and intermediate filaments of cytoplasm with surface of the nucleus. They are involved in positioning cell nucleus, anchorage of telomeres to INM and participate in chromatin decondensation during cell division (Starr *et al*., 2010). These complexes are conserved in animals and yeast and function in positioning cell nucleus and in chromosome movement (Crisp *et al*., 2006). They are also involved in tethering microtubule-organizing centers to the nuclear envelope. However, the exact role of SUN/KASH bridges in this process remains unclear (Malone *et al*., 1999).

In plants, WPP domain (WPP for tryptophan/proline/proline motif) is responsible for targeting proteins to NE. The first WPP-domain protein identified was *Solanum lycopersicon* MFP1 (MAR-binding filament-like protein 1) (Samaniego *et al*., 2005). Orthologues of MFP1

are distributed along the whole plant kingdom. Two out of three *Arabidopsis* homologues *At*WPP1-3 were found to associate with ONM and NPC. To complete the list of *Arabidopsis* WPP domain proteins, RanGAP1 and RanGAP2 were characterized by Patel *et al*. (2004).

Table 2: **List of plant nuclear envelope-localized proteins with their putative animal homologues**. *Arabidopsis* proteins are quoted with AGI and UniProt numbers; proteins from *Daucus carota* (*Dc*) and *Solanum lycopersicon* (*Sl*) with UniProt numbers.

	Protein	AGI number	UniProt number	Human homologue	UniProt number
nuclear lamina	*DcNMCP1*		O04390	LMNA	P02545
	AtCRWN1	*At1g67230*	F4HRT5		
	AtCRWN2	*At1g13220*	F4HP35		
	AtCRWN3	*At1g68790*	Q9CA42		
	AtCRWN4	*At5g65770*	F4JXK1		
inner nuclear membrane	*AtSUN1*	*At5g04990*	Q9FF75	SUN-domain containing proteins	
	AtSUN2	*At3g10730*	Q9SG79		
outer nuclear membrane	*SlMFP1*		P93203	KASH-domain proteins - functional homologues, not structural!	
	AtMFP1	*At3g16001*	Q9LW85		
	AtWPP1	*At5g43070*	Q9FMH6		
	AtWPP2	*At1g47200*	Q9C500		
	AtWPP3	*At5g27940*	Q0WQ91		
	AtWIP1	*At4g26455*	Q8GXA4		
	AtWIP2	*At5g56210*	Q9FH18		
	AtWIP3	*At3g13360*	Q94AV5		
	AtWIT1	*At5g11390*	Q8L7E5		
	AtWIT2	*At1g68910*	A8MQR0		
	AtRanGAP1	*At3g63130*	Q9LE82	RanGAP1 not same localization !	P46060
	AtRanGAP2	*At5g19320*	Q9M651		

Two classes of WPP domain-interacting proteins have been identified to localize to ONM, namely *At*WIPs (WPP-domain interacting proteins) and *At*WITs (WPP-domain interacting tail-anchored proteins). WIP proteins have sequence similarities to KASH proteins, particularly the C-terminal PNS-tail which is required for interaction with SUN domain and NE localization. Similarly, all *At*WIP1, *At*WIP2 and *At*WIP3 bind to both *At*SUN1 and *At*SUN2. Interaction occurs between PNS tail of *At*WIPs and SUN domain of

*At*SUNs, and results in localization of *At*WIPs at nuclear envelope. This complex is involved in maintenance of elongated or super elongated shape of *Arabidopsis* nuclei, possibly important for protection against mechanical stress (Zhou *et al*., 2012). Furthermore, WIPs are plant-specific ONM proteins and together with WITs are responsible for RanGAP outer nuclear envelope targeting (Xu *et al*., 2007). Also, WIP-WIT complexes link to myosinXI-I and to actin cytoskeleton on the cytoplasmic side of nuclear envelope (Tamura *et al*., 2013). Therefore a model for nucleocytoplasmic bridges in plants, containing LINC1 as nucleoskeletal component associating INM SUN proteins, which bind to WIPs at ONM and in turn to actin cytoskeleton might be proposed (Graumann, 2014).

3.2. Nuclear pore complex

Nuclear pore complex (NPC) is a large multiprotein channel, the only route for exchange of macromolecules between nucleus and cytoplasm (Callan and Tomlin, 1950), similar in composition among all eukaryotes. NPCs are highly dynamic complexes, which change their structure and composition according to transported cargoes and cell cycle stages. Transfer of molecules across the nuclear envelope is provided by aqueous channel inside NPC. In yeast and animals, NPCs are multiprotein complexes of about 40 to 60 MDa consisting of varying number of about 30 different nucleoporins (Nups) (Alber *et al*., 2007). Critical factor regulating the transport is the molecule size: molecules smaller than 30 kDa can freely diffuse through the NPC, while transport of macromolecules is energy and transporter-dependent. The number of NPCs on nuclear surface depends on the activity and size of the nucleus as well as on the species, and ranges from ~2–4 NPCs/μm^2 in chicken erythrocytes and human lymphocytes (Maul *et al*., 1971) to 60 NPCs/μm^2 in the *Xenopus* oocytes (Gerace and Burke, 1998). In tobacco BY-2 and onion cells the mean density of NPCs is about 40-50 NPCs per μm^2 which is one of highest described in eukaryotes (Fiserova *et al*., 2009). *In vitro* transport studies and single molecule imaging on permeabilized fixed HeLa cells revealed that approximately 1000 molecules are transported across NPC per second (Ribbeck and Gorlich, 2001; Yang *et al*., 2004).

NPC assemble in two phases of the cell cycle. First, during interphase, to double the number of NPCs before cell division, new NPCs are incorporated to nuclear envelope. Second, new NPCs are assembled during nuclear envelope reformation after mitosis at the end of telophase. Coordination of NPC formation is regulated by phosphorylation, RanGAP1 and importin β (Ryan *et al*., 2003; Walther *et al*., 2003; Tran and Wente, 2006).

3.2.1. Nuclear pore complex composition

NPCs of yeast, metazoan and plants have similar basic architecture – a central channel formed by eight subunits is surrounded by a nuclear basket on the nucleoplasmic side and cytoplasmic filaments on cytoplasmic side. Plants NPCs appear smaller than NPCs of *Xenopus* oocytes and seem larger than in yeast (diameter of 105 nm, 110 – 120 nm and 95 nm, respectively) (Goldberg and Allen, 1996; Kiseleva *et al*., 2004; Fiserova *et al*, 2009).

The composition of plant nuclear pore complex morphology was studied in tobacco BY-2 cells by Fiserova *et al*. (2009). Studies on 3-days and 10-days old cells revealed significant differences in the structure and composition of the complexes. Similarly to nuclear pore complexes of *Xenopus* oocytes (Goldberg and Allen, 1996), cytoplasmic side of most NPCs in 3-days old cells was composed of cytoplasmic ring, complex of eight subunits which encompass a cylindrically-shaped channel. In some of the NPCs cytoplasmic filaments emerged. In 10-days stationary cells, the composition of pores was similar; however the central channel was smaller. Furthermore, internal filaments emanating inside NPCs were often observed. The structure of the cytoplasmic side of NPCs was similar in dicots (tobacco) and monocots (onion) in both size and composition (Fiserova *et al*., 2009). The eight-subunit conformation of nuclear basket was clearly observed in some NPCs. Thinner filaments were anchored to nucleocytoplasmic ring. Thicker filaments connected neighboring NPCs, nuclear matrix proteins or chromatin. NPCs were distributed non-randomly over the nuclear surface, organized into rows in stationary cells. Dynamic changes of the components parts and inner pore diameter in dividing and stationary BY-2 cells suggest the regulation of activity and specificity of transport through NPCs (Fiserova *et al*., 2009).

In yeast and vertebrates, nucleoporins were studied by proteomic techniques including isolation of NPCs followed by mass spectrometric identification of particular Nups (Rout *et al*., 2000; Cronshaw *et al*., 2002). Such comprehensive proteomic analysis is missing in plants. Tamura *et al.* (2010) generated transgenic plants overexpressing GFP tagged mRNA export factor1 (RAE1) and used them for immunoprecipitation of complexes using anti-GFP antibody. They identified about 24 proteins classified as Nups. Furthermore, some of the proteins were used as bait for further NPCs member characterization, allowing identification of around 30 putative *Arabidopsis* nucleoporins.

Plant nucleoporins could be subdivided into five distinct classes according to overall shape and predicted similarities within yeast, vertebrates and *Arabidopsis* Nups.

Transmembrane Nups assemble the NPC to the pore membrane and anchor it to the nuclear envelope. Up to now, only two transmembrane Nups were identified in plants, Gp210

(Glycoprotein of 210 kDa) – homologue of yeast Pom152 – and NDC1 (Nuclear Division Cycle1) (Greber *et al.*, 1990; Wozniak *et al*., 1994). *At*Gp210 plays an essential role in embryo development (Tzafrir *et al*., 2008).

The **cytoplasmic filaments** localized at cytoplasmic side of NPCs provide environment for specific interactions with transport receptors. The biggest nucleoporin in vertebrates, Nup358, is responsible for RanGAP localization to NPC, facilitating transportin-dependent nuclear import (Hutten *et al*., 2009). Plants have no Nup358 homologue, however at least in *Arabidopsis*, they use WIP-WIT complex for RanGAP1 localization to the nuclear envelope (Zhao *et al*., 2008).

Nuclear basket is composed of eight filaments protruding inside the nucleus. Each nuclear basket contains one large coiled-coil nucleoporin, NUA in plants, and two phenylalanine-glycine repeat domains (FG-repeat) Nups (Nup136/Nup1 and Nup50). FG-Nups bind directly to transport receptor, therefore mediating active transport through NPC. Mutant phenotype of Nup136/Nup1 RNAi lines of *Arabidopsis thaliana* shows changes in nuclear shape morphology indicating that similarly to its *Xenopus laevis* counterpart Nup153, Nup136/Nup1 might interact with components of plant nuclear lamina (Tamura and Hara-Nishimura, 2011).

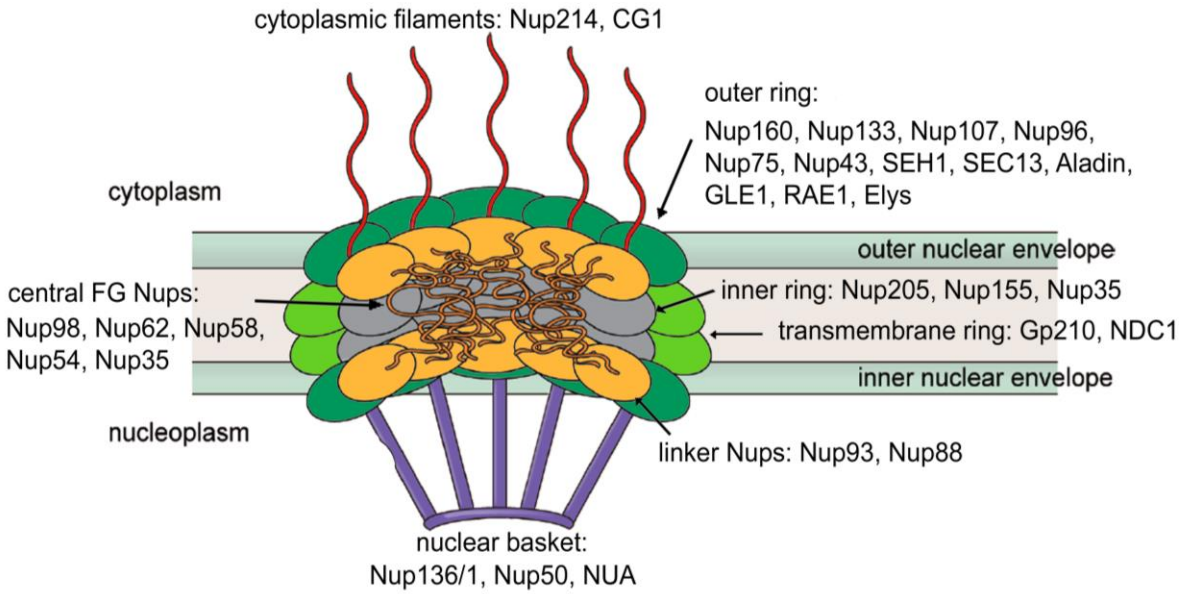

Figure 3: Structure and composition of plant nuclear pore complex. NPC is composed of various combinations of about 30 different types of nucleoporins. Modified from Alber *et al*. (2007).

Central FG Nups are also called barrier Nups. They form selective barrier for nucleocytoplasmic transport. In plants one out of five identified central FG Nups was characterized, Nup62 (Zhao and Meier, 2011). Mutant plants of Nup62 exert dwarf phenotype and early flowering, indicating diverse roles in plant development.

Rigid skeleton of NPCs is formed by **scaffold nucleoporins** which form three separated complexes – the outer ring, inner ring and linker Nups. Structure and composition of nuclear pore complex is depicted on Figure 3. The list of nucleoporins identified in *Arabidopsis* with its human homologue is given in Table 3.

Table 3: **List of nucleoporins identified in *Arabidopsis thaliana***. A majority of *Arabidopsis* nucleoporins were identified by a extensive proteomic analyses by Tamura *et al*. (2010) and proteins were named according to similarities with known human proteins. *Arabidopsis* proteins are quoted with AGI and UniProt numbers, human homologues with UniProt numbers.

		Protein	AGI number	UniProt number	Human homologue	UniProt number
Transmembrane Nups		*Gp210*	*At5g40480*	F4KHD8	NUP210	Q8TEM1
		Ndc1	*At1g73240*	Q8L628	NDC1	Q9BTX1
Cytoplasmic filaments Nups		*Nup214*	*At1g55540*	Q9ZVV0	NUP214	P35658
		CG1	*At1g75340*	Q9FWS3	NUPL2	O15504
Nuclear basket Nups		*NUA*	*At1g79280*	A4GSN8	Nucleoprotein TPR	P12270
		Nup136/Nup1	*At3g10650*	Q9CAF4		
		Nup50a / AtRanBPL1	*At1g52380*	Q9C829	Nup50 / RanBP3	Q9UKX7 / Q9H6Z4
		Nup50b /AtRanBPL2	*At3g15970*	Q8LG62		
Central FG Nups		*Nup35*	*At3g16310*	A04326	NUP35	Q8NFH5
		Nup54	*At1g24310*	Q8GYF7	NUP54	Q7Z3B4
		Nup58	*At4g37130*	Q8RWH9		
		Nup62	*At2g45000*	Q8L7F7	NUP62	P37198
		Nup98a	*At1g10390*	Q8RY25	NUP98	P52948
		Nup98b	*At1g59660*	F4ID16		
Scaffold Nups	Linker Nups	*Nup88/MOS7*	*At5g05680*	Q9FFK6	NUP88	Q99567
		Nup93a	*At2g41620*	O22224	NUP93	Q8N1F7
		Nup93b	*At3g57350*	F4J284		
	Inner ring Nups	*Nup35*	*At3g16310*	O04326	NUP35	Q8NFH5
		Nup155	*At1g14850*	Q9LQU6	NUP155	O75694
		Nup205	*At5g51200*	F4KBW6	NUP205	Q92621
	Outer ring Nups	*Nup43*	*At4g30840*	O65565	NUP43	Q8NFH3
		Nup75	*At4g32910*	Q8RXH2	NUP75	Q9BW27
		Nup96	*At1g80680*	Q8LLD0	NUP98/NUP96	P52948
		Nup107	*At3g14120*	Q8L748	NUP107	P57740
		Nup133	*At2g05120*	F4IGA5	NUP133	Q8WUM0
		Nup160	*At1g33410*	Q9C811	NUP160	Q12769
		SEH1	*At1g64350*	Q93VR9	SEH1	Q96EE3
		SEC13	*At2g30050*	O64740	SEC13	P55735
		Aladin	*At3g56900*	Q8GWR1	Aladin	Q9NRG9
		GLE1	*At1g13120*	Q0WPZ7	GLE1	Q53GS7
		Rae1	*At1g80670*	Q38942	RAE1	P78406
		Elys	*At2g39810*	Q84JU6	Elys	Q8WYP5

3.3. Receptor-mediated nuclear transport

Active receptor-mediated transport through NPCs is dependent on transport molecules, which bind their cargo in one compartment (cytoplasm or nucleus) and release it in the other compartment. Particular recognition motifs present on transported molecules are necessary for either binding to NPC directly or for association with nuclear transport receptors (NTRs), providing a basis for receptor-mediated nuclear transport (Pemberton and Paschal, 2005). A majority of nuclear transport receptors are Ran-binding proteins, more exactly they have a high affinity to GTP-bound form of Ran. Although the knowledge of plant nucleocytoplasmic transport improved during the last few years, most of the information comes from yeast and animal studies. Therefore this chapter focuses mainly on nuclear import and export processes in non-plant species.

Importin β was the first nuclear transport receptor described in 1995 by Görlich and colleagues, and thus whole family of NTRs was named as importin β or β-karyopherin family of transport receptors (Görlich *et al*., 1995). In general, importin family members possess similarity only in its Ran-binding domain, and transport broad range of different cargoes. Transported cargo proteins contain specific recognition signals – nuclear localization signal (NLS) (Kalderon *et al*., 1984), or nuclear export signal (NES) (Richards *et al*., 1996), respectively. NTRs bind directly or indirectly to the localization signals and both cargo and carrier are transported across nuclear envelope through NPC. Importin β family encodes both importins, responsible for import of proteins to nucleus, and exportins, which participate in nuclear export. The affinity to cargo is dependent on RanGTP. Generally, importins have high affinity to transported proteins in the absence of RanGTP (in cytoplasm), whereas in the presence of RanGTP after transport to nucleus, the cargo is dissociated and importin binds to RanGTP. In contrast, exportins bind their cargo in the presence of RanGTP in the nucleus and after transport through NPCs, when RanGTP is dissociated; exportins are separated from cargo and RanGDP. Some NTRs can transport cargoes in both directions. Although, there are some other nuclear transport pathways, importin β family of NTRs is responsible for most of the transport (Rout and Aitchison, 2001). Export of mRNA from nucleus requires RanGTP, but is not dependent on Ran gradient, therefore is not discussed in this chapter.

3.3.1. Nuclear protein import

Proteins subjected to nuclear import carry classical NLS, target sequence which is recognized directly by importin β (Cingolani *et al*., 2002; Lee *et al*., 2003), or in most cases by an adaptor protein importin α. Importin α belongs to the family of armadillo repeat-

containing proteins and act as a receptor for cytoplasmic proteins with NLS. Classical NLS signal of most proteins subjected to nuclear import consist of a short stretch of basic amino acid residues (monopartite NLS) or two stretches separated with a short spacer (bipartite NLS) (Hodel *et al*., 2001). Although, there are some other import pathways, which neither need adaptor protein for transport, nor NLS at transported cargo, import of proteins with classical NLS is the most-studied nuclear import pathway. The process can be divided into four phases: (1) assembly of the transporter/cargo complex in cytoplasm; (2) translocation through NPC; (3) disassembly of transporter/cargo complex; and (4) importin recycling (Figure 4).

In cytoplasm, classical NLS-containing proteins are bound to importin α through series of armadillo repeats. Armadillo repeats form curving structure with two NLS-binding sites. In addition to NLS recognition site, importin α contains N-terminal importin β binding domain (IBB) (Cingolani *et al*., 1999). Adaptor proteins can recognize slightly different NLS sequences and link them to the NTRs, therefore the number of potential cargoes that are transported by one nuclear transport receptor can be significantly increased. Generally, NLS binding occurs within two distinct NLS-binding domains, which span the armadillo repeat 2-4 (major site) and 6-8 (minor site). The major site is considered to be the main NLS-binding site for monopartite NLS and bipartite NLS bind to both major and minor sites (reviewed in Lott and Cingolani, 2011).

Accurate mechanism of importin α recognition and binding to NLS in plants was described by Chang *et al.* (2012). In rice, NLS of cargo protein is preferentially recognized by minor NLS-binding site. The same experiment performed using rice importin α and rice and mouse NLS signals showed preferential binding of importin α to mouse major NLS-binding site, but to rice minor NLS-binding site. Therefore, in plants the minor site does not only play the supplementary role in recognition of bipartite NLS binding as it is the case in animals. This difference might be related to the plant-specific auto inhibitory binding of importin β binding domain to minor NLS binding site (Chang *et al*., 2012). Cargo binding is followed by binding to importin β through the N-terminal importin β binding domain, which also helps to release cargo in the nucleus.

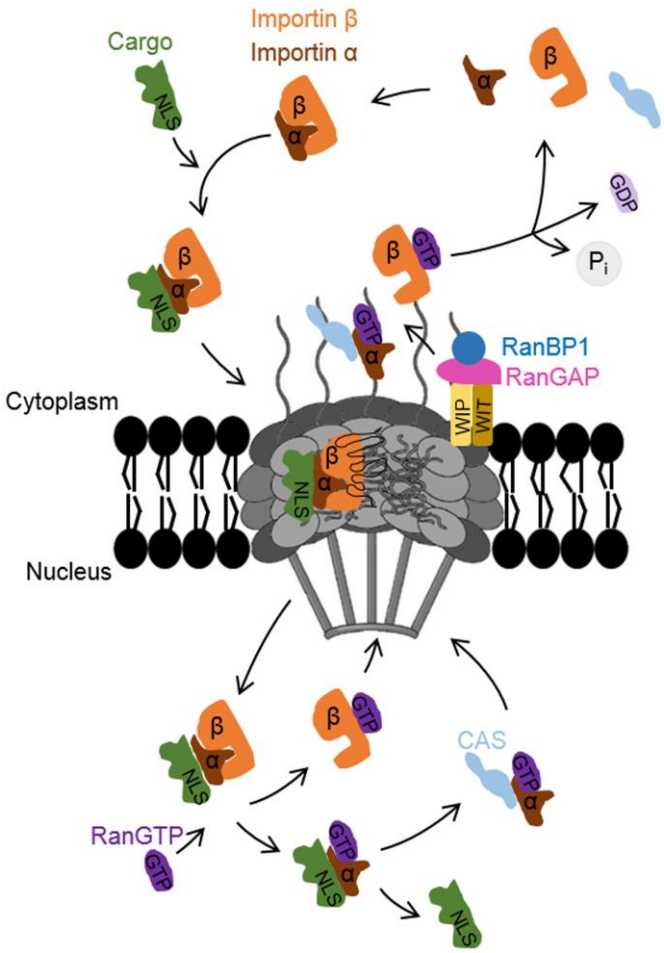

Figure 4: Schematic illustration of nuclear protein import through nuclear pore complex in plants. Nuclear localization signal of import cargo is recognized in cytoplasm by adaptor importin α, followed by importin β binding and translocation through the NPC. FG-nucleoporins are involved in the successful translocation. After entry to the nucleus, RanGTP is bound to both importin α and β, cargo is dissociated and transporters recycled to cytoplasm. In cytoplasm RanGTP hydrolysis is stimulated by RanGAP and its activator RanBP1, resulting in release of importins for next import cycle. E. Tomaštíková (this work).

Once the carrier/cargo complex is formed, it is transported through NPC. As mentioned above, the structure and composition of NPCs is complex, however, FG-repeat nucleoporins seem to be the most important for transport through NPCs. Phenylalanine side chains weekly bind to hydrophobic pockets on importin β family of transporters and allow NPC passage (Bayliss *et al.*, 2000). Different members of the family require slightly different set of FG-repeat Nups (Strawn *et al.*, 2004). Furthermore, only a pool of FG Nups is involved in importin β binding. Crystallographic structures of importin β/RanGTP and importin β/cargo complexes revealed that the outer surface of importin β is exposed to FG-repeat Nups and

inner surface to RanGTP and cargo. In some cases, interaction of importin β with particular Nups is mediated by IBB domain of adaptor proteins, and through importin β flexibility can influence affinity to FG Nups and the speed of transport through NPC (Lott *et al*., 2010).

The cargo/adaptor/transporter complex is dissociated after transport to nucleus. RanGTP binds to importin β with affinity much higher than is importin β affinity to cargo/adaptor complex, therefore releasing cargo in the nucleus (Sun *et al*., 2008). Some of nucleoporins seem to be involved in the process; however, particular Nups have yet not been characterized. The NLS-containing cargo dissociation from importin α probably occurs through the series of interaction of importin α IBB with RanGTP, Nup50 and CAS (Cellular Apoptosis Susceptibility), an importin α export factor belonging to the family of β-karyopherins. Matsuura and Stewart (2005) described the mechanism by which Nup50 displace importin α from binding to NLS of cargo proteins. Furthermore, through Nup50-binding sites in importin α, the disassembly of the complex could be directly coupled to export of adaptor to cytoplasm. Importin α is bound to the CAS and together with RanGTP is transported to the cytoplasm as well. In cytoplasm, the RanGTP/importins complexes are dissociated after hydrolysis of RanGTP to RanGDP which is promoted by RanGTPase activating protein RanGAP and its activator RanBP1. Mammalian RanGAP has specific C-terminal domain necessary for its NPC targeting through interaction with RanBP2/Nup358 therefore RanGAP can directly interact with nucleus-exiting complexes (Matsuura and Stewart, 2005). In *Arabidopsis*, RanBP2 homologue is missing; there are 2 RanGAP homologs RanGAP1, 2 and three RanBP1 homologs RanBP1a, b, c. Targeting mechanism of RanGAP1 and RanGAP2 is provided by plant-specific N-terminal WPP domain, which anchors both RanGAPs to NPC (Rose and Meier, 2001). Free RanGDP is transported back to the nucleus by transporter NTF2 (Ribbeck *et al*., 1998). There are two NTF2 orthologs in *Arabidopsis*, *At*NTF2a and *At*NTF2b, which are functionally redundant (Zhao *et al*., 2006). Nucleotide exchange of RanGDP to RanGTP occurs in nucleus, where the chromatin-bound guanine nucleotide exchange factor RCC1 is present. In plants, however, such exchange factor has not been identified to date.

3.3.2. Nuclear protein export

Nuclear export of proteins is mediated by transport receptors called exportins. They bind to its cargo cooperatively with RanGTP, therefore acting in opposite manner to importins. Exportins vary in a number of substrates, which they can transfer (Köhler et al., 1999). For instance, CAS (exportin 2, Xpo2) is highly specific and exports importin α, thereby recycling it for the next round of protein import. CAS is highly conserved in all

eukaryotes. On the other hand, CRM1 (exportin 1, Xpo1) can transport broad range of structurally unrelated cargoes. Such broad specificity is provided by the presence of leucine-rich nuclear export signal on transported cargoes. NES consensus sequence resembles motif Φ-x_{2-3}-Φ-x_{2-3}-Φ-x-Φ, where Φ are amino acids L, I, V, F, M and x is any amino acid (Kutay and Güttinger, 2005). The presence of hydrophobic amino acids is the most important feature of NES. CRM1 is the best characterized and major nuclear export receptor, which recognizes NES signal peptides within broad range of cargoes (reviewed in Hutten and Kehlenbach, 2007). It belongs to the importin β family of transport receptors. Similarly to other family members, it is formed by 21 HEAT repeats; however, it possesses slightly different mechanism of cargo and RanGTP binding mainly because of cooperative binding of RanGTP and cargo. In contrast to other exportins, it has relatively low affinity to NES cargoes. Efficient binding is increased after interaction with nuclear RanGTP-binding protein RanBP3, a CRM1 cofactor (Lindsay *et al*., 2001). CRM1 binds to the NES of cargo protein through the hydrophobic cleft of HEAT repeats 11 and 12. After binding of RanGTP to N-terminal domain, whole complex is translocated through NPC. The process is energy independent and facilitated by weak interaction with FG Nups. *Arabidopsis* homologue *At*XPO1a and *At*XPO1b exert similar mechanism of cargo and RanGTP binding, however, the precise mechanism is not known. Both proteins are necessary for proper gametophyte development. After transport through NPCs, whole complex must be disassembled. CRM1 binds strongly to Nup214, nucleoporin associated with cytoplasmic ring of NPC, in RanGTP and NES-dependent manner. Nup214 is located closely to Nup358/RanBP2, which possess FG repeats for transport receptor binding, RanBP1-like Ran-binding domain for binding of RanGTP and is associated with RanGAP. Additionally, Ran-binding domain of RanBP1 actively participates in the process (Figure 5). In a simplified way, RanBP1 (or RanBP2) accelerates release of cargo from CRM1-RanGTP complex allosterically by packing the HEAT repeats 11 and 12 against each other, so there is no space for interaction with NES hydrophobic side chain of cargo protein. RanBP1 also binds directly to CRM1 HEAT repeat 15, resulting in change of superhelical conformation. Thereafter contacts between CRM1 HEAT repeat 8, 9 and 19 and RanGTP switch I and II (respectively) are destroyed, which facilitates dissociation of RanGTP from CRM1. After RanGTP is released from the complex, it is accessible for RanGAP and hydrolyzed to RanGDP (Koyama and Matsuura, 2010).

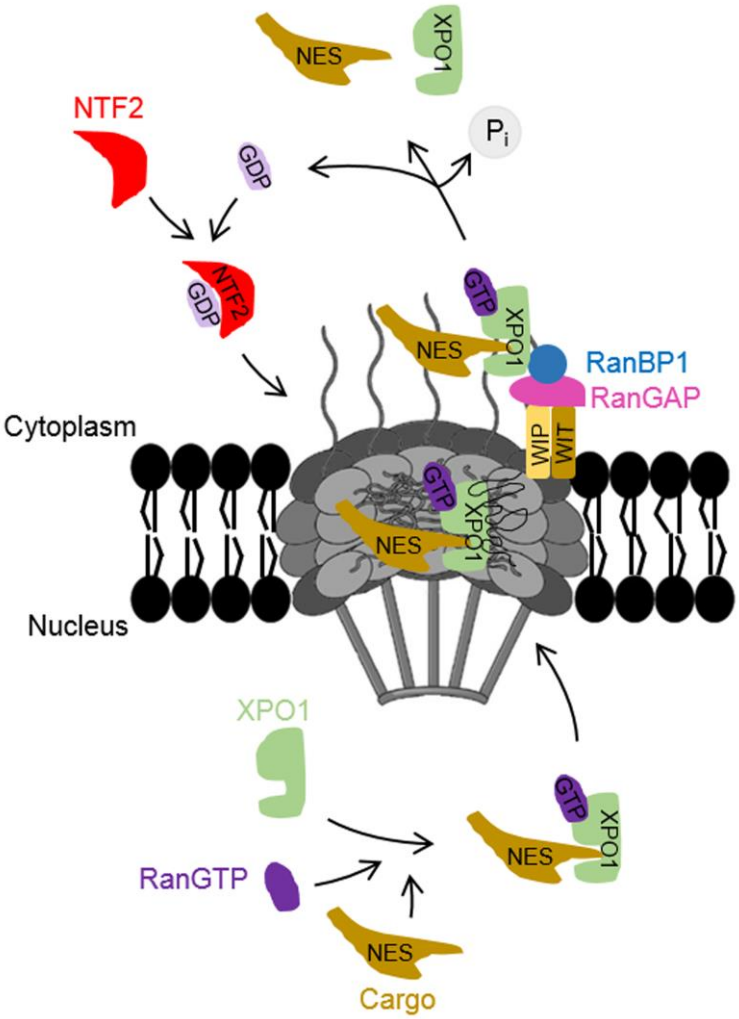

Figure 5: Schematic illustration of nuclear protein export in plants. In nucleus, nuclear export signal of transported protein is recognized by exportin XPO1. After cooperative binding of cargo and RanGTP to transporter, whole complex is translocated through NPC. The process is facilitated by interaction of XPO1 with FG-nucleoporins. On cytoplasmic side of NPC, triple complex is dissociated through interaction with RanBP1 and RanGTP hydrolysis. Cargo and transporter are released in cytoplasm. RanGDP binds to specific transporter NTF2 and is recycled to nucleus. E. Tomaštíková (this work).

4. Ran GTPase pathway in mitotic spindle assembly

Mitosis is the key event in cell cycle, which ensures accurate separation of sister chromatids. When the DNA has been replicated, it condenses into distinct morphological structures - chromosomes. Each chromosome comprises of a pair of chromatids that are connected by a multiprotein complex called cohesin. Mitotic spindle is a bipolar structure that ensures equal separation of the duplicated chromatids to daughter cells. At anaphase onset, when all sister chromatids are bi-oriented, cohesion is removed which allows separation of sister chromatids and equal distribution to the two daughter nuclei. RanGTPase pathway is involved in regulation of mitosis by: I. Providing a spatial signal for spindle assembly around chromosomes, II. Promoting mitotic spindle assembly by releasing spindle assembly factors from inhibitory binding to importins, III. Nuclear envelope reassembly after chromosome separation.

Mitotic spindle is made of microtubule cytoskeleton, which naturally possesses polarity and hundreds of associated factors that ensure its dynamic structure. Microtubule assembly occurs at microtubule organizing centers (MTOC) followed by nucleation, anchoring and releasing of microtubules. The microtubule end where new tubulin subunits are incorporated is called plus end, the other end is known as the minus end. In animal cells, minus end of microtubules is embedded in centrosomes, the main MTOCs (Brinkley *et al*., 1985). The plus end moves outward and undergoes cycles of growth and shrinkage. Once the dynamic ends are captured by kinetochores, the plus end forms stable bundles. As duplicated spindle poles and both kinetochores are present in pairs, forces along the length with an aid of additional proteins form a moving bipolar spindle. Thus, sister chromatids can be precisely separated to daughter cells. The whole process is strictly regulated. Such centrosome-based spindle formation is called "search and capture" model (Kirschner and Mitchinson, 1986). Although this is the main mitotic spindle assembly model, there are cell types lacking centrosomes, such as animal oocytes. In *Xenopus* extracts it was shown that microtubule nucleation can occur also around DNA-coated beads. This system presents an example of a mechanism of spindle assembly called "spindle self-organization" (Heald *et al.*, 1996). All higher plant cells lack centrosomes and have no functional equivalents at the spindle poles. The nucleation of microtubules occurs at nuclear envelope and around chromosomes, with the aid of γ-tubulin (Binarová *et al.*, 2006).

4.1. Microtubules and their dynamics

Microtubules are the essential structural units of mitotic spindle. Cells contain two populations of microtubules: (1) stable, which are involved in cell shape determination, provide platform for intracellular transport and make up the internal structure of cilia and flagella, and (2) short-living unstable microtubules forming mitotic spindle (Schulze and Kirschner, 1987). Heterodimers of α- and β-tubulin subunits are bound head-to-tail forming polarized protofilaments. Thirteen protofilaments are laterally associated and form a hollow tube about 25 nm in diameter – a microtubule (Figure 6).

All protofilaments have the same orientations. Therefore the polymers possess structural polarity – β-tubulin is always oriented toward the fast-growing end (plus-end), whereas α-tubulin is exposed to the slower growing end (minus-end). Microtubules preferentially assemble at their minus ends. The minus end is stabilized by a GTP cap, whereas changes occur mainly on the plus end, where GTP might be hydrolyzed, followed by microtubule polymerization or depolymerization (Mitchison and Kirschner, 1984). The rate of microtubule assembly and disassembly is regulated by critical concentration of α-β dimers. If critical concentration exceeds the limit, microtubules assemble, if it falls below the limit, they disassemble. When the tubulin concentration is near critical concentration, microtubules might exhibit treadmilling by adding subunits on one end and dissociating them on another, and dynamic instability, oscillation between growth and shrinkage. Furthermore, critical concentration differs between plus- and minus-end and GTP- and GDP-bound nucleotide (Desai and Mitchison, 1997). Moreover, microtubule dynamics is regulated by large number of proteins that determine the assembly, stability and association of microtubules with other cell structures.

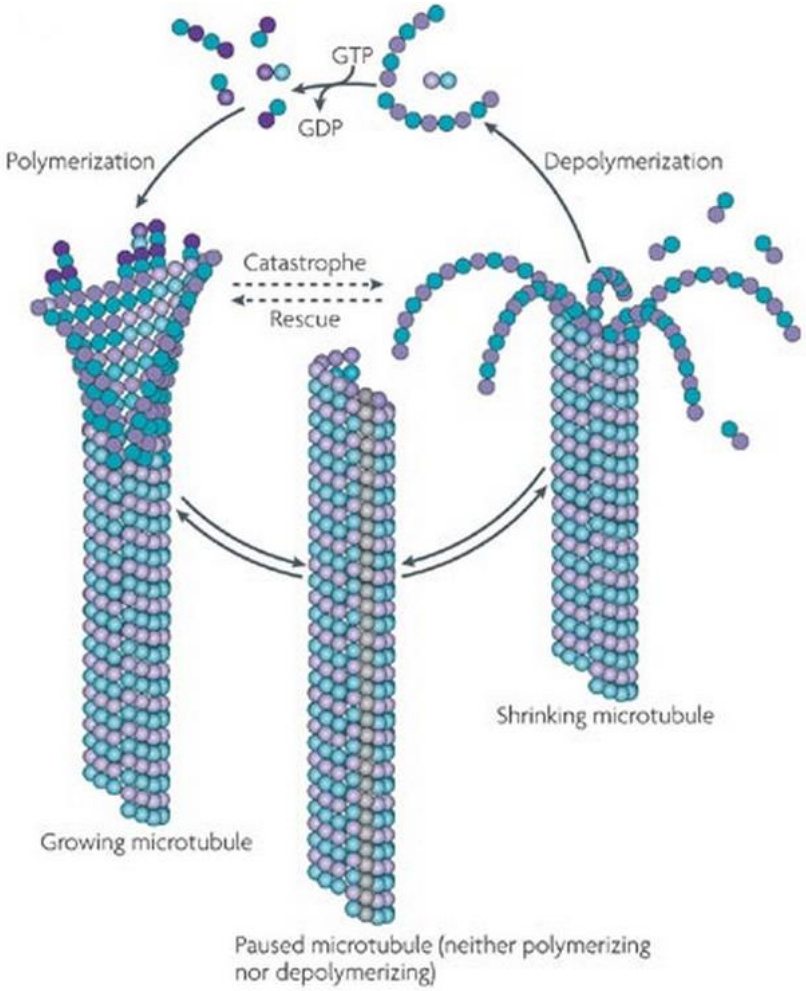

Figure 6: Microtubule structure and dynamics. A microtubule is a hollow tube built from 13 parallel protofilaments (in grey) of αβ-tubulin heterodimers. All heterodimers are oriented in the same direction, giving the microtubule polarity. α-Tubulins are exposed at minus-end, whereas β-tubulins at plus-end. GTP-cap stabilizes the microtubule and maintains its growth. GTP hydrolysis destabilizes microtubule and if it reaches to the GDP-containing tubulins, rapid shrinkage, "catastrophe", occurs. Adapted from Conde *et al.* (2009).

4.1.1. Microtubule-stabilizing proteins

Stabilizing MAPs are composed of microtubule-binding domains and projection loop, which extends from the microtubule structure. Extensive research has been done on brain tissue, as it contains high amount of microtubules. The best known of the microtubule-associated proteins are the members of MAP2/tau family. They are natively unfolded and adopt the secondary structure after binding to their targets (Schoenfeld and Obar, 1994).

MAP4, well-described family member, localizes around microtubules during interphase and mitosis and induces their bundling (West *et al.*, 1991). It stabilizes microtubules by preventing their rapid depolymerization caused by GTP to GDP hydrolysis. EB1 is a highly conserved MAP that concentrates at plus ends of growing microtubules, and regulates the nucleation and elongation of microtubules (Bu *et al.*, 2001). CLIP-170 is the first identified microtubule plus-end tracking protein (+TIP), which binds to C-terminal part of α- and β-tubulin, as well as EB1. It is required for linking endocytic vesicles to MTs (Komarova *et al.*, 2002). CLASPs (CLIP-associated proteins) are plus-end tracking proteins and are responsible for orienting stable microtubule arrays. They are essential for mitotic spindle assembly and involved in mitotic spindle maintenance (Maiato *et al.*, 2003).

Homologs of plant microtubule-stabilizing proteins were identified, including MAP215 homologue microtubule-assembly promoting protein MAP200/MOR1. Members of the family induce incorporation of tubulin dimers to microtubules, increasing the speed of microtubule elongation (Whittington *et al.*, 2000). They are involved in nucleation activity by stabilizing the plus end of microtubules nucleated from γ-tubulin complexes (Gard *et al.*, 2004). Plant MAP65 is a microtubule bundling and stabilizing protein with molecular diversity higher than animal and yeast homologs. The diversity might be required for assembling plant-specific microtubule structures. The bundling activity is dependent on MAP65 dimerization (Smertenko *et al.*, 2004). *Arabidopsis* genome contains +TIPs homologs, for instance EB1 and CLASP. On the other hand, CLIP-170 is not present. Spiral 1 (SPR1) is a plant-specific +TIP (Sedbrook *et al.*, 2004), which localizes to plus ends of microtubules and along microtubules. The protein regulates cortical microtubules and therefore direction of cell expansion (Nakajima *et al.*, 2004).

4.1.2. Microtubule-destabilizing proteins

Katanin was the first microtubule-destabilizing protein identified in animals. It is an ATPase composed of two subunits, p60 which possesses the ATPase activity and causes microtubule severing, and p80 which localizes katanin to MTOCs, and was originally described as protein causing microtubule release from centrosome (McNally *et al.*, 1996). Katanin promotes microtubule depolymerization by severing rapid break down of microtubular fibers (Burk *et al.*, 2001; Bouquin *et al.*, 2003). Another microtubule destabilizer, Op18/stathmin, is phosphoprotein which promotes tubulin sequestration and microtubule catastrophes, rapid microtubule depolymerization caused by GTP do GDP hydrolysis (Howell *et al.*, 1999). Mammalian MCAK, a member of kinesin superfamily, is

another protein with known microtubule-destabilizing function. Unlike other kinesins, it does not move along microtubules, but couples the ATPase activity with microtubule bending.

Less is known about microtubule-destabilizing proteins in plants. The best described is katanin. Its microtubule-severing activity is responsible for alignment, bundling and reorganization of cortical microtubules by releasing them from nucleation sites (Burk *et al.*, 2007). Similarly to animal cell division, microtubule-severing activity is required for division of plant cells (Panteris *et al.*, 2011).

4.2. Microtubule-organizing centers (MTOCs)

Spontaneous assembly of new microtubules is possible, however occurs very slowly and disassembly is favored over assembly. Therefore, cells have evolved special nucleation sites, which are found in microtubule organizing centers. Generally, during interphase, microtubule minus-ends are embedded in MTOCs whereas plus-ends are projected to cytoplasm, while in mitotic spindle plus-ends are targeted toward chromosomes. The centrosome, primary MTOC in animals, was identified nearly a century ago (Wilson *et al.*, 1928). It comprises a pair of centrioles, and microtubules are embedded in them through their minus ends. Spindle pole body is a functional analog of centrosome in fungi. It is a multilayered structure embedded in nuclear envelope, which provides microtubule nucleation in both directions, to nucleus and cytoplasm. Plant cells do not possess such distinct microtubule organizing centers, and microtubule nucleation occurs at nuclear envelope, on membranes and on existing microtubules (Murata *et al.*, 2005; Wasteneys *et al.*, 2002; Binarová *et al.*, 2006). In spite of differences in morphology of MTOCs in different groups of organisms, the main composition remains similar – all MTOCs contain γ-tubulin, homologue of α- and β-tubulin, which is indispensable for nucleation of microtubules. In addition to its microtubule-nucleating activity, γ-tubulin is involved in coordination of late mitotic events (Hendrickson *et al.*, 2001) and regulation of spindle checkpoint control in animal cells (Prigozhina *et al.*, 2004).

Several models for microtubule nucleation through γTuRC – γ-tubulin ring complexes (γ-tubulin complex protein 2 - GCP2, GCP3, GCP4, GCP5, GCP6 and NEDD1; Zheng *et al.*, 1995) have been proposed. A "template model" suggests that γ-tubulin in γTuRC act as template for microtubules. γTuRC interact laterally to form a ring on which γ-tubulin is located and interacting longitudinally with α-tubulin at the microtubule minus end (Zheng *et al.*, 1995). A "protofilament model" hypothesize that γ-tubulins make longitudinal contacts with each other around the ring forming a protofilament. αβ-Tubulin dimers are added to the

γ-tubulin protofilament laterally (Erickson *et al.*, 1996).

Plant γ-tubulin was described to be capable of microtubule polymerization *in vitro* (Dryková *et al.*, 2003; Horio and Oakley, 2003). It is localized in dispersed sites around microtubule arrays in plant cells (Panteris *et al.*, 2000). The central role of plant γ-tubulin in the assembly of acentrosomal mitotic spindle together with preexisting microtubules forming a branched structure was described by Murata *et al.* (2005). Reverse genetic approach was used to elucidate the function of γ-tubulin. Independently, two groups revealed that γ-tubulin plays a central role in the formation and organization of microtubular arrays in *Arabidopsis* (Binarová *et al.*, 2006; Pastuglia *et al.*, 2006).

In plants, γTuRCs are localized in all places of acentrosomal nucleation of microtubules; therefore there is requirement for additional proteins providing correct localization to MTOCs. In *Arabidopsis* and other land plants, γTuRCs are present and contain a complete set of proteins important for functional complexes (Murata *et al.*, 2007). The γTuRCs comprise all GCPs as their human counterparts, indicating conservation of similar mechanism of microtubule assembly in centrosomal and acentrosomal cells (Seltzer *et al.*, 2007). GCP2 – GCP6 probably function as scaffold for interaction between microtubule minus end and 13 γ-tubulin subunits (GCP1). Furthermore, GCP2 is involved in a proper organization of cortical microtubule array by positioning of γ-tubulin to preexisting microtubules (Nakamura and Hashimoto, 2009). Another complex member, NEDD1, is probably involved in microtubule nucleation at nuclear envelope at the onset of mitosis (Zeng *et al.*, 2009). A GCP3-interacting protein GIP1 is a recently described structural subunit of γTuRCs in *Arabidopsis*. It is a homolog of human MZT1, involved in proper localization of γ-tubulin complex components and organization of microtubules (Janski *et al.*, 2012; Batzenschlager *et al.*, 2013).

4.3. Plant-specific microtubular arrays

In animal cells, microtubules usually form interphase cytoplasmic array, a spindle during mitosis and mid-body during cytokinesis. In plants, however, four distinct microtubular structures are observed, some of them with no parallels in animal cells. These are interphase cortical array, preprophase band, plant spindle and phragmoplast. These specific microtubule structures are maintained by cooperation with specific MAPs.

4.3.1. Interphase cortical array

Plant cells do not possess discrete MTOCs, but dispersed γ-tubulin foci around whole

cell cortex where the microtubule nucleation occurs (reviewed in Schmit, 2002). Cortical array consists of bundles of microtubules that interact with the cortex, thereby determining the axis of cell-wall expansion and cell growth (Paradez *et al.*, 2006). Cortical microtubules are organized from discrete loci containing γ-tubulin that are present on existing microtubules or microtubule bundles (Murata *et al.*, 2005). Therefore the organization depends on interactions between microtubules and activity of MAPs, such as microtubule organization 1 (MOR1) (Wasteneys *et al.*, 2002) (Figure 7F).

4.3.2. Preprophase band

Position and orientation of cell division plane in plants is determined prior to mitosis through special structure called preprophase band (PPB). PPB is a cortical array of microtubules that forms during G2/prophase at the place of future cell division site. Later, during the formation of prophase spindle, microtubules are randomly nucleated and organized perpendicular to the plane of PPB. Therefore, PPB facilitates bipolar organization of perinuclear microtubules before nuclear envelope breakdown (Chan *et al.*, 2005; Ambrose and Cyr, 2008). Microtubular bridges extending between the nucleus and PPB before establishment of bipolarity, transmit forces that organize perinuclear microtubules from their random distribution to bipolar spindle (Ambrose and Cyr, 2008). Furthermore, RanGAP1 was found to be the positive marker for PPB to cell division site memory throughout the cell cycle as it persists at the future cell division site after PPB disassembly (Xu *et al.*, 2008) (Figure 7A).

4.3.3. Plant mitotic spindle

The main difference between animal and plant mitotic spindle comes from the presence of distinct MTOCs - centrosomes - in animal cells. Therefore, the plant spindle poles are broader and astral microtubules are not present (Figure 7B, C). Instead of being nucleated at distinct points, plant spindle poles are formed in multiple dispersed places at nuclear envelope containing γ-TuRCs (Drykova *et al.*, 2003). The assembly is regulated by microtubule-associated protein TPX2 (Vos *et al.*, 2008). Furthermore, the organization of mitotic spindle in plant cells relies more than in animals on kinetochores as organizing centers.

4.3.4. Phragmoplast

In contrast to animal cytokinesis, which occurs through contractile ring of actin filaments forming on the surface of the cell, plant cells separation is ensured through

phragmoplast (Figure 7D). The phragmoplast forms during late cytokinesis from the structures of mitotic spindle and serves to track vesicles with cell wall material to the growing cell plate as well as a scaffold for cell plate assembly (Bajer and Allen, 1996). It is formed from microtubules, microfilaments and endoplasmic reticulum that assemble in two sets perpendicular to the future cell plate. As the cell plate grows upwards to the cell surface, phragmoplast microtubules appear at the edges of growing plate.

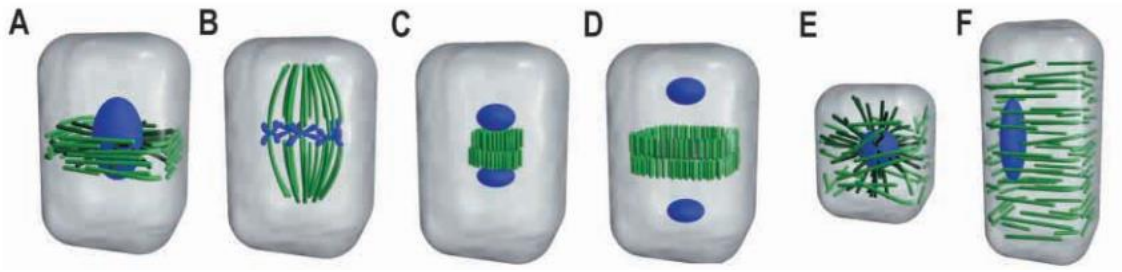

Figure 7: **Plant microtubular structures.** A – preprophase band, B – metaphase spindle, C – the plant spindle in anaphase, D – phragmoplast, E – perinuclear microtubule array after completion of mitosis, F – cortical microtubules during interphase. Adapted from Wasteneys (2002).

4.4. Microtubule populations within the spindle

During cell division, microtubules rapidly reorganize to a mitotic spindle. In cells which possess centrosomes, the centrosome-nucleated microtubules become shorter and more dynamic, leading to disassembly of interphase microtubule network. In addition to centrosome-based microtubule nucleation, *de novo* microtubule assembly occurs around chromosomes after nuclear envelope breakdown. Local stabilization of centrosomal and non-centrosomal microtubules leads to formation of bipolar mitotic spindle with microtubule minus-ends tethered around the spindle pole in case of centrosomal MTs, or scattered at spindle pole in case of acentrosomal MTs (Walczak and Heald, 2008). There are different types of microtubules that define the basic feature of metaphase mitotic spindle – kinetochore MTs, polar MTs and astral MTs.

The most important population of microtubules is kinetochore MTs or k-fibers. K-fibers connect duplicated chromosomes to spindle poles and ensure segregation of sister chromatids to two daughter cells. In some fungi such as *Saccharomyces cerevisiae* with a small centromere, only one microtubule connects to each chromatid (Winey *et al.*, 1995), whereas in most species, kinetochore fibers are formed by a bundle of 20 – 30 microtubules (McEwen

et al., 1997). Connection between chromosome and microtubules occurs at kinetochore, macromolecular structure formed at centromere of each sister chromatid in a process called k-fibre maturation, which occurs between prometaphase and anaphase (McEwen *et al*., 1997). In animals, the k-fibre plus ends are attached to kinetochore through macromolecular protein complex called KMN (KNL1-MIS12-complex-NDC80) network. Erroneous attachments are released for error correction through a regulation mechanism involving Aurora kinase B (Kelly and Funabiki, 2009). Dramatic shortening of k-fibers occurs at anaphase leading to chromosome segregation (Rogers *et al*., 2004). K-fibers are highly resistant to depolymerization by various microtubule-destabilizing agents that destabilize other types of spindle microtubules. High stability of k-fibers is a prerequisite for rigid connection and efficient transmission of forces to chromosomes. In contrast to other types of microtubules in the spindle, kinetochore microtubules are absolutely essential for spindle function (Rieder *et al.*, 1998).

Homologous components of KMN regulatory network were described also in plants. *At*Aurora3, homolog of Aurora B in *Arabidopsis*, is localized at kinetochore region of mitotic chromosomes (Demidov *et al*., 2005). Homologs of NDC80 and Mis12 were identified in maize and localize to kinetochore (Du and Dawe, 2007; Li and Dawe, 2009). *At*Mis12 localizes to centromeric regions of chromosomes throughout the cell cycle and colocalizes with CENH3, centromeric histone H3 variant. A complete loss of *At*Mis12 causes embryo lethality, pointing to an important role in cell cycle regulation, similarly to its human homologue (Sato *et al*., 2005). Other components of NDC80 complex were identified by sequence similarity (Meraldi *et al*., 2006).

Polar microtubules span through the spindle midzone and interact with oppositely oriented microtubules (Cai *et al.*, 2009). They ensure proper microtubule dynamics as well as maintaining spindle size (Houghtaling *et al.*, 2009). In addition, they provide one of the forces for chromosome congression to the metaphase plate through the interaction with chromokinesins (Vaneste *et al*., 2011). Polar microtubules are rearranged to central spindle during anaphase (Glotzer, 2009), and are organized in the same way as the animal ones, with microtubule minus-ends in MTOCs and plus ends at the spindle midzone. Spindle poles are not as tightly focused as in animal cells and multiple minipoles might be formed (Otegui and Staehelin, 2000). They usually co-join k-fibers to form thick bundles (Jensen and Bajer, 1973). Polar microtubules of each half-spindle interact at spindle midzone, and give rise to phragmoplast at anaphase onset (Samuels *et al*., 1995).

Astral microtubules diverge from centrosomes or spindle pole bodies to cell cortex.

Astral microtubules play a major role in centrosome separation during mitotic prophase (Rosenblatt, 2005). Furthermore, they are required for proper positioning of growing contractile ring, analog of plant phragmoplast in animal and yeast cells, that divides the two forming nuclei (Tseng *et al.*, 2012). Chan *et al*. (2005) observed astral-like microtubules also in *Arabidopsis* cells. They appeared after phragmoplast formation around the daughter nuclei and contacted the cell cortex.

4.5. Chromatin induced spindle assembly

Mitotic spindle assembly occurs through dramatic reorganization of interphase microtubular arrays. At the onset of mitosis, peripheral microtubules are destabilized and spindle microtubules are preferentially stabilized. Two models for microtubule nucleation have been proposed: centrosome-mediated "search and capture model" (Kirschner and Mitchison, 1997) and chromatin-mediated "spindle self-organization" (Dasso, 2002). The "search and capture model" suggests that microtubule nucleation occurs at centrosomes, microtubules explore the cytoplasm until they are captured by kinetochores. Captured microtubules are selectively stabilized and favor the formation of bundled k-fibers (Hayden *et al*., 1990). In this model, chromosomes have a passive role and wait for incorporation into the mature spindle (O'Connell and Khodjakov, 2007). In chromatin-mediated "spindle self-organization model", microtubule nucleation occurs at the vicinity of chromosomes with the Ran GTP, independently of centrosomes (Dasso, 2002; Gruss and Vernos, 2004). Although both mechanisms involve to some extent the same protein regulators, chromatin-mediated microtubule nucleation is the main pathway employing the activated RanGTP directly for the assembly of spindle. Furthermore, many organisms do not contain extrachromosomal microtubule nucleating centers such as centrosomes and thus the search and capture model does not seem realistic.

The first observations of mitotic spindle assembly in organisms lacking centrosomes were done on plant cells already in 1982 (De Mey *et al*., 1982). However, mitotic spindle assembly in acentrosomal cells was deeply characterized in *Xenopus laevis* egg extracts. The extracts are suitable systems as they contain both nuclear and cytoplasmic proteins required for the first cell cycles during embryo development. The observation that DNA-coated beads incubated in *Xenopus* egg extracts could stimulate assembly of bipolar mitotic spindle supported the idea that chromatin can stimulate spindle formation (Heald *et al*., 1996; Walczak *et al*., 1998). Recent studies provided evidenced for the existence of two independent non-centrosomal microtubule assembly pathways, one triggered by RanGTP (Carazo-Salas *et*

al., 1999) and the other controlled by chromosome passenger complex (CPC) (Sampath *et al*., 2004).

Xenopus egg extracts were the first system where the role of RanGTP in the assembly of mitotic spindle was described (Carazo-Salas *et al.*, 1999). Spatially, the system is characterized by localization of guanosine nucleotide exchange factor RCC1 on chromatin, thereby increasing the RanGDP to RanGTP conversion in cell nucleus and around chromosomes. On the other hand, GTPase activating protein RanGAP and its cofactors are localized distantly from chromatin. Two mechanisms for spatial organization and timing of mitotic structures organization have been proposed in animals: "Ran gradient model" (Caudron *et al*., 2005) and "Protein localization model" (Arnaoutov *et al*., 2005). According to the Ran gradient model, particular reactions require different concentrations of RanGTP. Thus, high RanGTP concentrations induce microtubule nucleation in the vicinity of chromosomes, whereas the longer-range effects on MTs stabilization require much lower RanGTP concentration (Caudron *et al*., 2005). The protein localization model proposes that RanGTP controls protein complex assembly and disassembly, but the gradient of RanGTP would not provide a spatial signal. The example could be transporter CRM1, which regulates microtubule assembly by releasing spindle assembly factors (Arnaoutov *et al*., 2005).

4.6. Spindle assembly factors released by RanGTP

The mechanism by which RanGTP regulates mitotic spindle assembly is closely related to the regulation of nucleocytoplasmic transport. The role of RanGTP is mediated through importin αβ dimers, which bind NLS of spindle assembly factors and therefore inhibit their function (Gruss *et al*., 2001). The activity of spindle assembly factors (SAFs) is inhibited until RanGTP releases them from importin αβ binding, similarly as in the nuclear import (Nachury *et al*., 2001; Wiese *et al*., 2001). SAFs tend to be transported to nucleus during interphase, but the main pool of them is localized in cytoplasm and the function is performed after nuclear envelope breakdown. To date, a number of RanGTP regulated SAFs has been identified, and most of them are involved in microtubule nucleation, stabilization and spindle organization. Other proteins, which are not directly regulated by RanGTP, are also required for spindle assembly performed by this pathway. γ-Tubulin and ch-TOG can be mentioned as examples (Meunier and Vernos, 2012). RanGTP-regulated SAFs promote function toward microtubules. They have various functions in the spindle assembly and often act cooperatively. For example, TPX2 nucleates microtubules and therefore produces substrates for other associated factors that stabilize microtubules, including TACC/maskin, NuSAP, HURP and XMAP 215. Later

on, some of these interactions promote spindle pole localization of AuroraA which phosphorylates other SAFs (Kalab and Heald, 2008).

4.6.1. TPX2

The first SAF identified in animals to be regulated in this way was microtubule-associated protein TPX2 (Targeting Protein for Xklp2) (Gruss *et al*., 2001). TPX2 localizes to nucleus during interphase, and after nuclear envelope breaks down, it is released from inhibitory binding to importin αβ by RanGTP. Free TPX2 then stimulates microtubule nucleation at kinetochores and around chromosomes (Gruss and Vernos, 2004). TPX2 localization within mitotic spindle especially at the plus end of microtubules in proximity to chromatin was observed in *Xenopus* (Witmann *et al*., 2000). Depletion of TPX2 from human cells abolishes microtubule nucleation around chromatin as well as bipolar spindle formation. The proper mechanism of TPX2 regulation remains unknown. However, it is possible that it catalyzes MT nucleation from γ-TURCs around chromatin in RanGTP dependent manner and also stabilizes kinetochore fibers (Aguirre-Portolés *et al*., 2012).

In human cells, TPX2 is phosphorylated by Aurora A kinase, a member of the serine/threonine family of kinases (Kufer *et al*., 2002). TPX2 was also shown to be Aurora kinase A activator. Activation of the kinase is required for its proper localization to mitotic spindle in human and *Caenorhabditis elegans* (Kufer *et al*., 2002; Ozlu *et al*., 2005). Aurora A is one of the possible components that regulate microtubule growth around chromosomes. It was shown to nucleate microtubules in *Xenopus laevis* egg extracts (Tsai and Zheng, 2005). Furthermore, Bird and Hyman (2008) revealed that interaction of TPX2 and Aurora A is required for correct spindle length. Because TPX2 is released from importins by RanGTP, which is formed by guanine nucleotide exchange factor RCC1 in the vicinity of chromosomes, chromosomes may therefore activate Aurora A through TPX2 (Tsai *et al*., 2003). TPX2 perform also other microtubular roles unrelated to Aurora kinase A. Eckerdt *et al*. (2008) revealed that C-terminal domain of TPX2 interacts with Eg5, a kinesin motor protein in *Xenopus* (Eg5 that bundles microtubules and pulls them past each other). TPX2 helps to settle Eg5 on microtubules and also regulates the motor ability of the protein to slide on microtubules, thereby regulating the proper spindle length (Ma *et al*., 2011). The accurate control of the spindle assembly is regulated at mitotic exit, when TPX2 is degraded by anaphase-promoting complex/cyclosome (APC/C^{cdh1}) (Stewart and Fang, 2005).

*At*TPX2, *Arabidopsis* homologue of TPX2 was identified by Vos *et al*., (2008). *At*TPX2 localizes to cell nucleus during interphase and with microtubular arrays during mitosis and is

essential for nuclear envelope breakdown and prospindle assembly, which might indicate putative interaction of TPX2 with newly assembled microtubular arrays. After nuclear envelope breakdown, the protein is localized on kinetochore microtubule bundles and is degraded during telophase (Vos *et al*., 2008). The presence of TPX2 and TPX2-related proteins in other plant species has been proposed (Evrard *et al*., 2009) and, indeed, Panteris *et al*., (2013) recently identified TPX2 homologue in a fern *Asplenium nidus*. The protein colocalized with cortical microtubules in post-cytokinetic cells of leaves, pointing to possible involvement of fern TPX2 in MTOC activity (Panteris *et al*., 2013). Less is known about the plant TPX2 regulation. Petrovská *et al*., (2011) observed colocalization and physical interaction of *At*TPX2 with *At*Aurora1on MTs in cell cycle specific manner.

4.6.2. NuMA1

Nuclear mitotic apparatus protein 1 (NuMA1) is a highly abundant component of nuclear matrix with structural role in nuclear maintenance during interphase (Cleveland *et al*., 1995). In mitosis, it is involved in establishment of focused spindle poles (Silk *et al*., 2009), and together with cytoplasmic dynein, in tethering spindle microtubules to the mammalian centrosomes (Merdes *et al*., 1996).

NuMA homologues of 210, 220 and 230 kDa were identified in onion nuclear matrix by their cross-reactivity with *Xenopus* NuMA antibodies. They showed the same distribution in interphase nucleus and spindle matrix during mitosis as its animal counterparts (Yu and Moreno-Diaz de la Espina, 1999).

4.6.3. Rae1

RNA export factor 1 (Rae1) is nucleoporin and microtubule associated protein involved in export of messenger RNA and bipolar spindle assembly. Interestingly, Rae1 is ribonucleoprotein and requires RNA-binding for promoting its spindle assembling activity (Blower *et al*., 2005). During mitosis, Rae1 specifically interacts with NuMA and through the interaction increases microtubule-crosslinking capacity of NuMA and stabilizes microtubule minus ends, which is critical for normal bipolar spindle assembly (Wong *et al*., 2006). Mammalian Rae1 also cooperates with checkpoint kinase Bub3 to ensure correct passage through mitotic spindle checkpoint and chromosome segregation (Babu *et al*., 2003). Rae1 and Nup98 form a complex with APC/C and prevent the degradation of securin, anaphase inhibitor that regulates sister chromatid separation before amphitelic attachment of all chromosomes to mitotic spindle (Jeganathan *et al*., 2005).

Homologue of Rae1 (*Nb*Rae1) was identified in *Nicotiana benthamiana* and was found to localize along the entire length of mitotic microtubules throughout the whole mitosis and in cell plate midzone. In contrast to animal Rae1, no *Nb*Rae1 signal was observed around spindle poles. Inhibition of protein expression using RNAi resulted in defects in spindle organization and chromosome segregation, a role which seems to be conserved among animals and plants (Lee *et al*., 2009).

4.6.4. NuSAP1

Nucleolar and spindle-associated protein (NuSAP1) was identified as microtubule- and chromatin-binding protein in animals. During interphase, the protein is actively transported to nucleus, whereas in mitosis it associates with mitotic spindle (Raemaekers *et al*., 2003). NuSAP is involved in microtubule bundling in *Xenopus* egg extracts (Ribbeck *et al*., 2006) and in microtubule stabilization around chromatin (Ribbeck *et al*., 2007). At anaphase onset, NuSAP is degraded by APC/C (Li *et al*., 2007). No NuSAP homologue has been identified in plants.

4.6.5. HURP

Human hepatoma upregulated protein (HURP) was identified by Tsou and colleagues in human hepatoma and is another substrate of Aurora A (Tsou *et al*., 2003; Yu *et al*., 2005). It binds importin β and promotes RanGTP dependent nucleation of microtubules in the vicinity of chromosomes. Furthermore, HURP causes bundling of microtubules and probably through this mechanism stabilize kinetochore microtubules (Silljé *et al*., 2006). Similarly to NuSAP, no HURP homologue has been identified in plants to date.

4.7. Spindle assembly checkpoint

Spindle assembly checkpoint (SAC) is a control mechanism for proper chromosome-to-microtubule attachment at kinetochores. SAC is activated as long as some of the chromosomes fail to connect to kinetochore fibers and blocks mitotic progression in metaphase (Musacchio *et al*., 2002). Spindle checkpoint proteins were first identified through genetic screen in budding yeast. They include Mitotic arrest deficient genes 1-3 (Mad 1 – 3) (Li and Murray, 1991) and Budding uninhibited by benzimidazole genes 1 and 3 (Bub1 and Bub3) (Hoyt *et al*., 1991) that are conserved in all eukaryotes. In budding yeast, SAC is a non-essential pathway important only in response to disruption in the kinetochore microtubule attachment (Li and Murray, 1991; Hoyt *et al*., 1991). In metazoans, it is an essential pathway,

which prevents chromosome missegregation (Musacchio and Salmon, 2007). The signal for SAC activation is generated by lack of tension when some of the chromosomes are not bi-oriented. Aurora kinase B is the regulator of SAC sustainability (Ditchfield *et al*., 2004).

Once all chromosomes are bi-oriented in the spindle midzone (amphitelic attachment), all checkpoint components are released, which is the signal that all chromosomes are under bipolar tension and segregation may start. At the same time, Mad1, Mad2 and BubR1 (Bub 1-related, in higher eukaryotes) release the regulatory component Cdc20 that activates anaphase promoting complex/cyclosome (APC/C^{Cdc20}) (Fang *et al*., 1998; Sudakin *et al*., 2001). APC/C is responsible for ubiquitination and degradation of securin. Therefore, separase is free to cleave cohesins that hold sister chromatids together at centromeres (reviewed in Yu, 2002). Furthermore, it is involved in degradation of other spindle assembly factors, which are protected by its interaction with microtubules until spindle disassembly (Song *et al*., 2014).

Plant homologues of spindle assembly checkpoint components were identified in *Arabidopsis* (Lermontova *et al*., 2008; Caillaud *et al*., 2009), maize (Yu *et al*., 1999) and wheat (Kimbara *et al*., 2004). Plant BUBR1, BUB3.1 and MAD2 are localized at unattached kinetochores similarly to animal counterparts, thereby referring the conserved function of SAC proteins. In contrast to animal cells, the checkpoint proteins are not recruited to kinetochores during normal mitosis, which might be related to differences in spindle formation in animals and plants (Caillaud *et al*., 2009).

5. Aims

The principal goal of this thesis was to obtain a deeper insight into the regulatory pathways involved in chromosome segregation, the key event in cell cycle. The thesis focused mainly on the investigation of uncharacterized and/or poorly characterized proteins involved in RanGTPase pathway in *Arabidopsis thaliana*. Particular aims were as follows:

- To find homologues of human RanBPM protein in the genome of *Arabidopsis thaliana* and use a broad spectrum of methods, such as molecular biology, cell biology and biochemistry to characterize its function in the model plant,
- To characterize *At*TPX2 protein, the important player in mitotic spindle assembly with regards to different organization of microtubule-nucleating machinery in plants,
- To analyze the relationship between *At*TPX2 and *At*Aurora kinase 1 and 3.

6. Conclusions

In this thesis, I have explored proteins involved in nucleocytoplasmic transport and chromosome segregation regulated by RanGTPase pathway in the model plant *Arabidopsis thaliana*. Firstly, *Arabidopsis* homologue of human RanBPM protein was identified and characterized. *At*RanBPM is present in large molecular weight complexes and probably involved in protein degradation. Next, microtubule-associated protein TPX2 was described and nuclear function of this protein was proposed. *At*TPX2 is involved in chromatin-induced mitotic spindle assembly most likely regulated by RanGTPase pathway. Furthermore, it was characterized as regulator of *At*Aurora kinase1, important mitotic kinase involved among others in phosphorylation of histone H3. Despite some intriguing differences in microtubule organization between animals and plants, such as the absence of distinct microtubule-organizing centers in plants and presence of plant-specific microtubular organelles, there are similarities in chromosome segregation machinery in plants in terms of its regulation.

6.1. Characterization of *Arabidopsis thaliana* RanBPM

Based on sequence similarity I have identified homologue of the human RanBPM in *Arabidopsis thaliana*. Despite the differences in size, *At*RanBPM has the same domain composition as its human counterpart. *At*RanBPM neither colocalized with γ-tubulin, the main microtubule nucleating center in plants, nor with microtubules. Consistent with the microscopic observation, *At*RanBPM was not enriched with microtubules in dividing cells. *At*RanBPM-GFP was mainly localized in cytoplasm, and only a small fraction of protein was observed in nuclei. Furthermore, *At*RanBPM was found to be part of a high molecular weight protein complex. After LC-MS analyses of the complex members, we found a similarity in composition with human and budding yeast CTLH-complexes. Although the particular biological role of the complexes is not yet known, it seems that they are involved in degradation processes mediated by RING E3 ubiquitin ligases (Francis *et al.*, 2013) (Publication I).

6.2. Overexpressed *At*TPX2 cause formation of microtubule bundles in nucleus and around nuclear surface

To determine more precisely the mechanism of microtubule nucleation and spindle assembly regulated by *At*TPX2, I have characterized *Arabidopsis* cells overexpressing *At*TPX2. Early after transformation of *At*TPX2-GFP construct under control of 35S promoter

to *Arabidopsis* cells, TPX2 induced at first formation of nuclear dots and patches. Later fibrilar structures and thick bundles of microtubules were formed in nuclei and perinuclear area. The microtubule structures were heavily decorated by *At*TPX2. Microtubule bundles were resistant to treatment with microtubule-depolymerizing drugs and were not related to apoptotic fibers described by Moss *et al*. (2009). Immunofluorescence analysis confirmed colocalization of *At*TPX2 presented in dots with importin and Ran, however, the amount of importin and Ran decreased after assembly of microtubule bundles. Both proteins were also identified to interact with *At*TPX2 *in vitro*. Furthermore, colocalization with γ-tubulin, the main component of plant MTOCs was confirmed.

Taken together, the accumulation of importin and Ran in cell nuclei with overexpressed *At*TPX2 indicates import of *At*TPX2 to cell nuclei. Reduction of nuclear signal of importin after formation of microtubular arrays indicates that microtubule formation was triggered by *At*TPX2 released from importin inhibition, possibly in RanGTPase dependent manner (Publication II).

6.3. *At*TPX2 activates *At*Aurora1 *in vitro* resulting in increased histone H3 phosphorylation

Based on the sequence similarity between human and plants' homologs I have tested whether *At*TPX2 is phosphorylated by *At*Aturora1 and *At*Aurora3, homolog of Aurora B in *Arabidopsis*. Recombinant proteins were prepared in *Escherichia coli* and phosphorylation of *At*TPX2 by both kinases was tested *in vitro*. Only *At*Aurora1 and not *At*Aurora3 were able to reproducibly phosphorylate *At*TPX2. Next, the possible increase in autophosphorylation activity of *At*Aurora1 after *At*TPX2 activation was checked. Indeed, *At*Aurora1 autophosphorylation activity increased dramatically. Furthermore, the lack of activation in case of *At*TPX2ΔN, truncated version without Aurora binding domain, suggests that it is probably Aurora binding domain in *At*TPX2 molecule which is responsible for kinase activation. Due to the activation of kinase with a very low amount of *At*TPX2 we can assume *At*TPX2 to be a potent activator of *At*Aurora1. Therefore, the mechanism of Aurora kinase activation among plants and animals seems to be conserved. Furthermore, the increase in autophosphorylation resulted in increased phosphorylation of histone H3, one of the most important downstream targets of the kinase (Publication III).

Altogether, *At*TPX2 appears as important regulator of *At*Aurora kinase 1 activity not only in targeting the kinase to mitotic spindle, but also in increasing its autophosphorylation activity towards histone H3. Activation of *At*Aurora1 by *At*TPX2 could be a mechanism for

translation of RanGTP signaling to phosphorylation cascade performed by Aurora kinases at kinetochores. Increased *At*Aurora1 activity might also activate various downstream targets such as histones or spindle assembly factors (Figure 8). Therefore the involvement of *At*TPX2 not only in microtubule nucleation, but also in regulation of chromosome segregation is plausible.

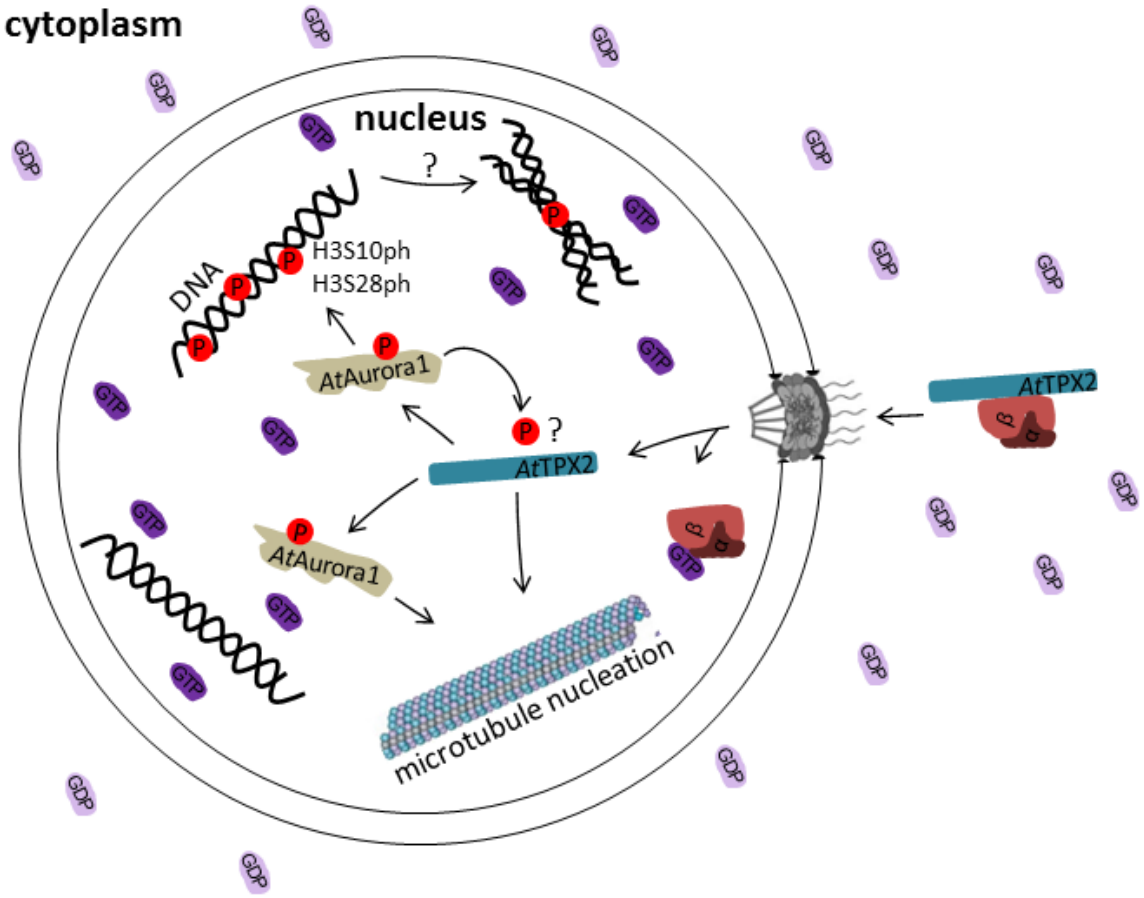

Figure 9: Model for *At*TPX2 – *At*Aurora1 cooperation in mitotic spindle assembly and chromation modification. In interphase cytoplasm where there is a high concentration of RanGDP, *At*TPX2 is bound to importin αβ. After transport to cell nucleus, *At*TPX2 is released from the complex in RanGTP dependent manner. Free *At*TPX2 then stimulates microtubule nucleation and probably also mitotic spindle assembly. *At*Aurora1 might be involved in this process. Furthermore, *At*TPX2 acts as co-activator of *At*Aurora1, which in turn stimulates further microtubule nucleation. *At*Aurora1 also phosphorylates *At*TPX2. Whether *At*TPX2 needs to be phosphorylated for sufficient microtubule nucleation remains to be determined. Moreover, activated and autophosphorylated *At*Aurora1 phosphorylates histone H3 at serine 10 and serine 28 residues. If this *At*TPX2-dependent phosphorylation is connected to chromatin condensation is not clear. It is tempting to speculate that *At*TPX2 and *At*Aurora1 act as a positive feedback loop through series of activation and phosphorylation events for fast and accurate coordination of mitotic events such as mitotic spindle formation and chromosome segregation. Such hyperactivation cascade could stimulate fast and precise reconstruction of mitotic chromatin and/or mitotic spindle formation. E. Tomaštíková (this work).

7. Publications (ISI Web of Knowledge)

7.1. Scientific papers

I. Tomaštíková E, Cenklová V, Kohoutová L, Petrovská B, Váchová L, Halada P, Kočárová G, Binarová P. Interactions of an Arabidopsis RanBPM homologue with LisH-CTLH domain proteins revealed high conservation of CTLH complexes in eukaryotes. BMC Plant Biol. 2012;12:83. IF 4.354 (Appendix I).

II. Petrovská B, Jeřábková H, Kohoutová L, Cenklová V, Pochylová Ž, Gelová Z, Kočárová G, Váchová L, Kurejová M, Tomaštíková E, Binarová P. Overexpressed TPX2 causes ectopic formation of microtubular arrays in the nuclei of acentrosomal plant cells. J Exp Bot. 2013;64(14):4575-4587. IF 5.242 (Appendix II).

III. Tomaštíková E, Demidov D, Jeřábková H, Binarová P, Houben A, Doležel J, Petrovská B. TPX2 protein of *Arabidopsis* activates Aurora kinase 1, but not Aurora kinase 3 *in vitro* . Ready for submission (Appendix III).

7.2. Popular papers

I. Tomaštíková E: Význam aurorakináz u rostlin. Vesmír, 2012, 91 (142):327 – 328 (Appendix IV).

8. Abstracts

I. Tomaštíková E, Binarová L, Cenklová V, Petrovská B, Gelová, Z, Pochylová Ž, Tyčová M, Binarová P. Characterization of *Arabidopsis* At RanSPRY protein, a new member of RanGTPase pathway. 19. Cytoskeletal Club 2011, Vranovská Ves, Czech Republic (Appendix V).

II. Cenklová V, Petrovská B, Binarová L, Gelová Z, Tomaštíková E, Tyčová M, Volc J, Binarová P. At RanSPRY is a new member of proteins od Ran GTPase regulatory pathway. Plant Biotechnology: Green for Good 2011, Olomouc, Czech Republic (Appendix VI).

III. Petrovská B, Cenklová V, Pochylová Ž, Kourová H, Tyčová M, Tomaštíková E, Binarová L, Binarová P. *Arabidopsis* Aurora1 and its activator TPX2 associate with microtubules and form a polar gradient during rearrangement of mitotic microtubules. Plant Biotechnology: Green for Good 2011, Olomouc, Czech Republic (Appendix VII).

IV. Tomaštíková E, Binarová L, Cenklová V, Petrovská B, Gelová Z, Pochylová Ž, Tyčová M, Volc J, Binarová P. Characterization of SPRY-domain containing protein At RanSPRY in *Arabidopsis thaliana*. IX International Conference of Ph.D. Students of Experimental Plant Biology „Fresh Insights in Plant Affairs“ 2011, Prague, Czech Republic (Appendix VIII).

V. Tomaštíková E, Petrovská B, Kohoutová L, Cenklová V, Halada P, Binarová P. Protein interaction of *Arabidopsis* RanBPM homologue reveal conservation of CTLH complexes in eukaryotes. X International Conference of Ph.D. Students of Experimental Plant Biology 2012, Brno, Czech Republic (Appendix IX).

VI. Tomaštíková E, Doležel J, Houben A, Demidov D. Differential activity of *Arabidopsis* Aurora kinase members towards histone H3. 9th Plant Science Student Conference 2013, Halle, Germany (Appendix X).

VII. Tomaštíková E, Doležel J, Houben A, Demidov D. Differential activity of *Arabidopsis* Aurora kinase members towards histone H3. Plant Biotechnology: Green for Good 2013, Olomouc, Czech Republic (Appendix XI).

VIII. Jeřábková H, Petrovská B, Cenklová V, Pochylová Ž, Gelová Z, Váchová L, Kurejová M, Tomaštíková E, Binarová P. Formation of ectopic microtubular fibres within nuclei and with nuclear envelope requires overproduction of *Arabidopsis* TPX2 protein. Plant Biotechnology: Green for Good 2013, Olomouc, Czech Republic (Appendix XII).

IX. Tomaštíková E, Doležel J, Houben A, Demidov D. Differential *Arabidopsis* Aurora

kinase family members activities on histone H3. 3rd European Workshop on Plant Chromatin 2013, Madrid, Spain (Appendix XIII).

X. Tomaštíková E, Demidov D, Jeřábková H, Houben A, Doležel J, Petrovská B. *Arabidopsis* TPX2 activates Aurora kinase 1 in vitro. SEB Annual Meeting 2014, Manchester, United Kingdom (Appendix XIV).

XI. Jeřábková H, Petrovská B, Cenklová V, Pochylová Ž, Gelová Z, Váchová L, Kurejová M, Tomaštíková E, Binarová P. Overexpressed AtTPX2 reinforces microtubule formation in the nuclei of acentrosomal plant cells. SEB Annual Meeting 2014, Manchester, United Kingdom (Appendix XV).

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10.Abbreviations

APC/C^{cdh1} – anaphase-promoting complex/cyclosome

ATPase – adenosine triphosphatase

Bub1, 3 – budding uninhibited by benzimidazole 1, 3

BY-2 – bright yellow 2, tobacco

CAS – cellular apoptosis ausceptibility

CPC - chromosome passenger complex

CRA - CT11-RanBPM

CTLH – C terminal to LisH motif

EB1 – end-binding protein 1

FG-repeat Nups – phenylalanine-glycine repeat domains nucleoporins

GCP - γ-tubulin complex protein

GDP –guanosine diphosphate

GIP – GCP3-interacting protein

Gp210 – Glycoprotein of 210 kDa

GTP – guanosine triphosphate

HEAT repeats – Huntingtin, elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and the yeast kinase TOR1

HURP – human hepatoma upregulated protein

IBB – importin β binding domain

INM – inner nuclear membrane

KASH-domain - Klarsicht/ANC-1/SYNE1 homology

KMN – KNL1-MIS12-complex-NDC80 network

LINC1 – 4 – little nuclei 1 – 4

LINC complexes – linker of nucleoskeleton to cytoskeleton complexes

LisH – Lis homology domain

Mad 1 – 3 – mitotic arrest deficient 1-3

MAF1 – MFP1 attachment factor 1

MAP – microtubule associated protein

MFP1 – MAR-binding filament-like protein 1

MOR1 – microtubule organization 1

MT – microtubule

MTOC – microtubule organizing centre

NDC1 – nuclear Division Cycle 1NLS – nuclear localization signal

NES – nuclear export signal

NMCP 1 – nuclear matrix constituent protein 1

NPC – nuclear pore complex

NTF2 – nuclear transport factor 2

NTR – nuclear transport receptor

NUMA1 – nuclear mitotic apparatus protein 1

Nups – nucleoporins

NuSAP1 – nucleolar and spindle-associated protein

ONM – outer nuclear membrane

PPB – preprophase band

RAE1 – mRNA export factor 1

Ran – Ras-related nuclear protein

RanBP1 – Ran binding protein 1

RanBP2 – Ran binding protein 2

RanBPM – Ran binding protein in microtubule organizing centre

RanGAP – RanGTPase activating protein

RanGDP – Ran guanosine diphosphate

RanGEF – Ran guanine nucleotide exchange factor

RanGTP – Ran guanosine triphosphate

RCC1 – regulator of chromosome condensation 1

SAF – spindle assembly factors

Scorpin – SPRY domain containing Ran binding protein

SPR1 – spiral 1

SPRY – SPIa and ryanodine receptor domain

SUN - Sad1p/Unc-84

TPX2 – targeting protein for Xklp2

WIPs – WPP-domain interacting proteins

WITs – WPP-domain interacting tail-anchored proteins

WPP domain - tryptophan/proline/proline motif domain

γ-TuCs - γ-tubulin complex

γTuRC – γ-tubulin ring complexes

+TIP - plus-end tracking protein

APPENDIX I

Interactions of an *Arabidopsis* RanBPM homologue with LisH-CTLH domain proteins revealed high conservation of CTLH complexes in eukaryotes

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Interactions of an *Arabidopsis* RanBPM homologue with LisH-CTLH domain proteins revealed high conservation of CTLH complexes in eukaryotes

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Abstract

Background: RanBPM (Ran-binding protein in the microtubule-organizing centre) was originally reported as a centrosome-associated protein in human cells. However, RanBPM protein containing highly conserved SPRY, LisH, CTLH and CRA domains is currently considered as a scaffolding protein with multiple cellular functions. A plant homologue of RanBPM has not yet been characterized.

Results: Based on sequence similarity, we identified a homologue of the human RanBPM in *Arabidopsis thaliana*. AtRanBPM protein has highly conserved SPRY, LisH, CTLH and CRA domains. Cell fractionation showed that endogenous AtRanBPM or expressed GFP-AtRanBPM are mainly cytoplasmic proteins with only a minor portion detectable in microsomal fractions. AtRanBPM was identified predominantly in the form of soluble cytoplasmic complexes ~230 – 500 kDa in size. Immunopurification of AtRanBPM followed by mass spectrometric analysis identified proteins containing LisH and CRA domains; LisH, CRA, RING-U-box domains and a transducin/WD40 repeats in a complex with AtRanBPM. Homologues of identified proteins are known to be components of the C-terminal to the LisH motif (CTLH) complexes in humans and budding yeast. Microscopic analysis of GFP-AtRanBPM *in vivo* and immunofluorescence localization of endogenous AtRanBPM protein in cultured cells and seedlings of *Arabidopsis* showed mainly cytoplasmic and nuclear localization. Absence of colocalization with γ-tubulin was consistent with the biochemical data and suggests another than a centrosomal role of the AtRanBPM protein.

Conclusion: We showed that as yet uncharacterized *Arabidopsis* RanBPM protein physically interacts with LisH-CTLH domain-containing proteins. The newly identified high molecular weight cytoplasmic protein complexes of AtRanBPM showed homology with CTLH types of complexes described in mammals and budding yeast. Although the exact functions of the CTLH complexes in scaffolding of protein degradation, in protein interactions and in signalling from the periphery to the cell centre are not yet fully understood, structural conservation of the complexes across eukaryotes suggests their important biological role.

Keywords: Arabidopsis homologue of RanBPM, CTLH-complex, LisH-CTLH domain proteins

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Background

Ras-related nuclear proteins (Rans) are abundant small GTPases, associated with Ran-specific nuclear GEFs (guanine nucleotide exchange factors), cytoplasmic GAPs (GTPase activating proteins), and with RanBP1 (Ran binding protein 1) that stimulate RanGTP hydrolysis in the cytoplasm [1,2]. The *Arabidopsis* genome contains three genes encoding AtRan [3], two genes encoding AtRanGAP related proteins [4] and three genes for RanBP1 isoforms – RanBP1a, RanBP1b and RanBP1c [3,5]. Plant Ran binding proteins (RanBPs) display significant homology with yeast and mammalian RanBPs, but there is little evidence for their biological function [6,7].

One RanBP in animal cells, RanBPM (RanBP9), was identified in a yeast two-hybrid screen with Ran as a bait. RanBPM comprises four domains – SPRY, LisH, CTLH and CRA and is homologous to the human RanBP10 protein [8]. Although RanBPM and RanBP10 have been shown to bind the Ran protein, they do not contain a consensus Ran-binding sequence [9]. RanBPM was defined as a member of the Scorpin family of proteins (SPRY-containing Ran binding protein) with a unique domain organization [10]. As reviewed in Suresh et al. [11], numerous protein interactions described for the RanBPM protein suggest its multiple roles in the regulation of protein stability, cell cycle regulation and other as yet undefined cellular processes.

RanBPM was reported to be a part of the large CTLH (C-terminal to the LisH motif) complexes [12-14]. CTLH complexes composed of LisH, CTLH and CRA domain containing proteins, transducin/WD40 repeat proteins, and armadillo repeat proteins have been found in mammals and yeast [15,16]. Mammalian and yeast CTLH complexes are structurally conserved but their biological function is still not fully understood. In yeast, the CTLH complex of Gid/Vid proteins plays a role in vacuole and proteasome-dependent fructose-1,6-bisphosphatase degradation [16]. Similarly, it has been suggested that CTLH complexes partake in lysosome and proteasome-dependent proteolysis in mammalian cells [17]. Data on proteins with SPRY, LisH, CTLH or CRA domain-containing proteins in plants are limited. In *Pinus radiata*, the SPRY domain containing protein, SEPR11, is the homologue of a Trithorax-group member and is involved in plant reproduction and development [18]. In *Oryza sativa,* the LisH domain-containing protein, OsLIS-L1 is required for male gametophyte formation and the first internode elongation [19].

Here we provide data on an *Arabidopsis* homologue of RanBPM that belongs to the uncharacterized family of plant SPRY, LisH, CTLH and CRA domain-containing proteins. We used *in silico* analysis, biochemical, proteomic and microscopic analyses *in vivo* and *in situ* to characterize AtRanBPM. We found that the AtRanBPM protein is present predominantly in the form of large cytoplasmic protein complexes that are structurally homologous to the CTLH type of complexes described in mammals and budding yeast.

Results

The Arabidopsis homologue of RanBPM is a SPRY-domain containing protein

By homology search of the *Arabidopsis thaliana* genome, we found a SPRY-domain containing protein AtRanBPM (At1g35470), which is a homologue of the human RanBPM (RanBP9) protein. The *AtRanBPM cDNA* contains a single open reading frame and consists of 467 amino acids. *AtRanBPM* is a member of the HOM002780 gene family that comprises 44 genes in 21 plant species, particularly from the ORTHO002658 subfamily. In *Arabidopsis* there are three paralogues of AtRanBPM (At4g09310, At4g09200, At4g09250**)** and one gene originating from the segmental duplication of chromosome 1 (At4g09340) (Plaza 2.0; A resource for plant comparative genomics, [20]). The products of these genes are described as SPRY domain-containing proteins but their biological roles in plants have not yet been identified.

The AtRanBPM protein is composed of four domains – SPRY, LisH, CTLH and CRA (Figure 1A). SPRY, LisH, CTLH and CRA domain-containing proteins are widely spread across eukaryotes. The protein encoded by *AtRanBPM* is annotated as a protein of unknown function (TAIR; The Arabidopsis Information Resource, [21]). As shown in Figure 1B, AtRanBPM has close homologues in other plant species such as *Ricinus communis* (67% identities, 79% positives), *Vitis vinifera* (66%, 78%), *Populus trichocarpa* (67%, 80%), *Sorghum bicolor* (57%, 74%), *Zea mays* (57%, 74%) and *Oryza sativa Japonica group* (57%, 73%) (Figure 1B, Additional file 1). The *Arabidopsis* RanBPM shows a close identity and similarity to its SPRY, LisH, CTLH and CRA domains with mammalian RanBPM and RanBP10 (Figure 1C, Additional file 2) (WU-BLAST, Basic Local Alignment Search Tool, [22]). However, there is an insertion of about 150 amino acids between the CTLH and CRA domains in human proteins (Figure 1C). A phylogenetic analysis performed using MEGA5 software (MEGA 5.02, Molecular Evolutionary Genetics Analysis, [23]) revealed that *Arabidopsis* RanBPM grouped with homologues from other plant species. The metazoan RanBPM homologues grouped in a separated clade. The *Saccharomyces* homologue, Gid1/Vid30, appears to be ancestral to the plant and metazoan clades (Additional file 3).

SPRY, LisH, CTLH and CRA conserved domains are mainly involved in protein-protein interactions. The SPRY domain (SPla and the RYanodine Receptor) was originally identified as a structural motif in ryanodine receptors [24]. Proteins with SPRY domains are involved in intracellular calcium release, as is the case for ryanodine

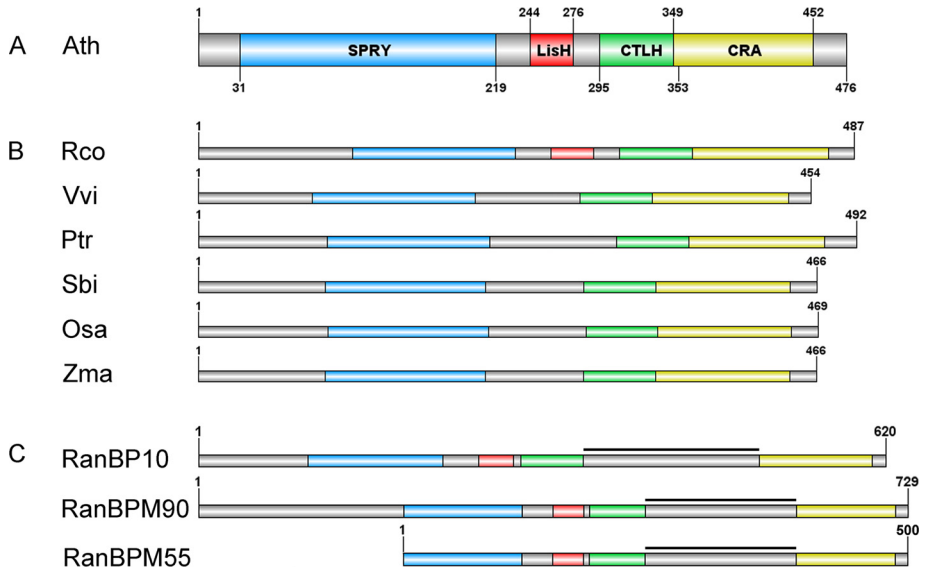

Figure 1 Domain organization of *Arabidopsis* RanBPM protein and its plant and human homologues. A- AtRanBPM protein consists of highly conserved SPRY, LisH, CTLH, and CRA domains. **B**- Comparison of AtRanBPM protein with its closest plant homologues. *Rco – Ricinus communis*, *Vvi – Vitis vinifera*, *Ptr – Populus trichocarpa*, *Sbi – Sorghum bicolor*, *Osa – Oryza sativa*, *Zma – Zea mays*. **C**- Comparison of AtRanBPM protein with its closest human homologues. Bold black lines indicate the 150 amino acids insertion between CTLH and CRA domains present only in human but not in plant RanBPM proteins. RanBPM90 – RanBPM of 90 kDa, RanBPM55 – RanBPM of 55 kDa.

receptors, in RNA metabolism and in regulatory and developmental processes [25]. The LisH domain (Lissencephaly type-1-like homology motif) functions as a stable homodimerization and dimerization motif and its contribution to the dynamics of microtubules has been suggested [26]. Located adjacent to the C-terminus of the LisH domain is a C-terminal to the LisH motif (CTLH) that has a putative α-helical structure with an as yet undescribed function. The CRA domain (CT11-RanBPM) was found to be a motif in the C-terminal part of RanBPM and RanBP10 and represents a protein-protein interaction domain often found in Ran-binding proteins [27].

Putative interaction sites of AtRanBPM were analysed using ELM (The Eukaryotic Linear Motif for functional sites in proteins, [28]) and SUMOsp 2.0 databases (SUMOylation Sites Prediction, [29]) (Figure 2). There are common motifs in the AtRanBPM amino acid sequence, such as phosphorylation consensus sites for phosphoinositide-3-OH kinase related kinases, a protein kinase A phosphorylation site, a mitogen-activated protein kinase phosphorylation site and docking motif, and a putative cyclin recognition site. Further, the AtRanBPM amino acid sequence possesses forkhead-associated phosphopeptide binding domains and a Y-based sorting signal which might act in endosomal and secretory pathways.

Protein expression levels, cellular distribution and molecular forms of AtRanBPM

The AtRanBPM coding sequence [EMBL:AEE31799] was cloned into Gateway-compatible plant binary vectors for N-terminal GFP and C-terminal GFP fusion. *Arabidopsis* cell suspension cultures were transformed according to Mathur et al. [30]. Transformed *Arabidopsis* plants were obtained through the floral-dip method [31].

An antibody against a peptide from the C-terminal part of the AtRanBPM molecule was produced in rabbits and affinity purified using the immunogenic peptide. When cell lines expressing GFP-AtRanBPM were analysed by Western blotting of separated *Arabidopsis* extracts, the antibody recognized a band of 52 kDa, the predicted molecular mass (MW) of AtRanBPM, and a band corresponding to the MW of GFP-AtRanBPM (Figure 3A). Western blot further showed that the protein levels of AtRanBPM were much lower in *Arabidopsis* seedlings than cell cultures. In cultured *Arabidopsis* cells, there was no significant difference in AtRanBPM protein levels between dividing cells and stationary growing non-dividing cells (Figure 3B). Similarly, there was no difference in AtRanBPM protein levels between four, eight and 15 day old *Arabidopsis* seedlings. Our data correspond to those on AtRanBPM expression found in the publically available Affymetrix expression databases, the Arabidopsis eFP browser [32], and the Genevestigator database [33] where AtRanBPM has a constant level of expression in analysed tissues as well as during seedling development.

The cellular distribution of AtRanBPM was analysed using differential centrifugation. As shown in Figure 3C, the majority of the protein was cytoplasmic and only small amounts sedimented either in low speed pellets

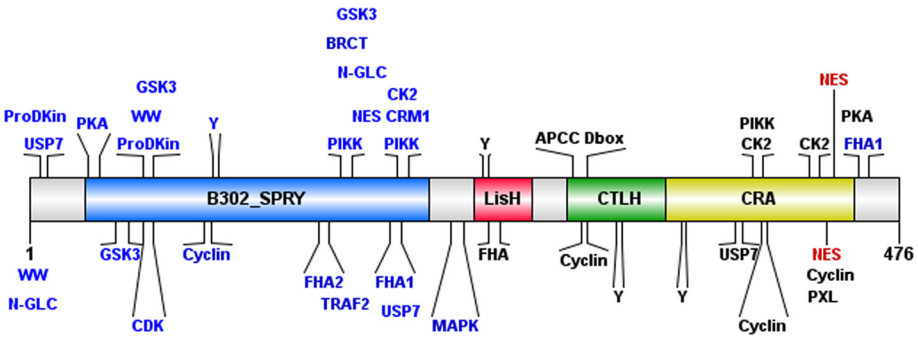

Figure 2 Putative interaction sites and binding motifs in AtRanBPM amino acid sequence. To predict putative interaction sites and binding motifs ELM [28] and SUMOsp 2.0 [29] databases were used.

or in the microsomal pellet (P100). The fact that AtRanBPM was also sedimented from a high-speed cytoplasmic S100 extract to pellet P200 indicated that the protein might be present in the form of higher MW complexes. The separation of S100 high speed extract by native polyacrylamide gel electrophoresis (native PAGE) confirmed cell fractionation data and suggested that AtRanBPM is a component of protein complexes with a molecular mass of around ~230 – 500 kDa (Figure 3D). Further, we immunopurified GFP-AtRanBPM using the GFP trap. Separation of purified GFP-AtRanBPM by native PAGE followed by Western blotting showed the

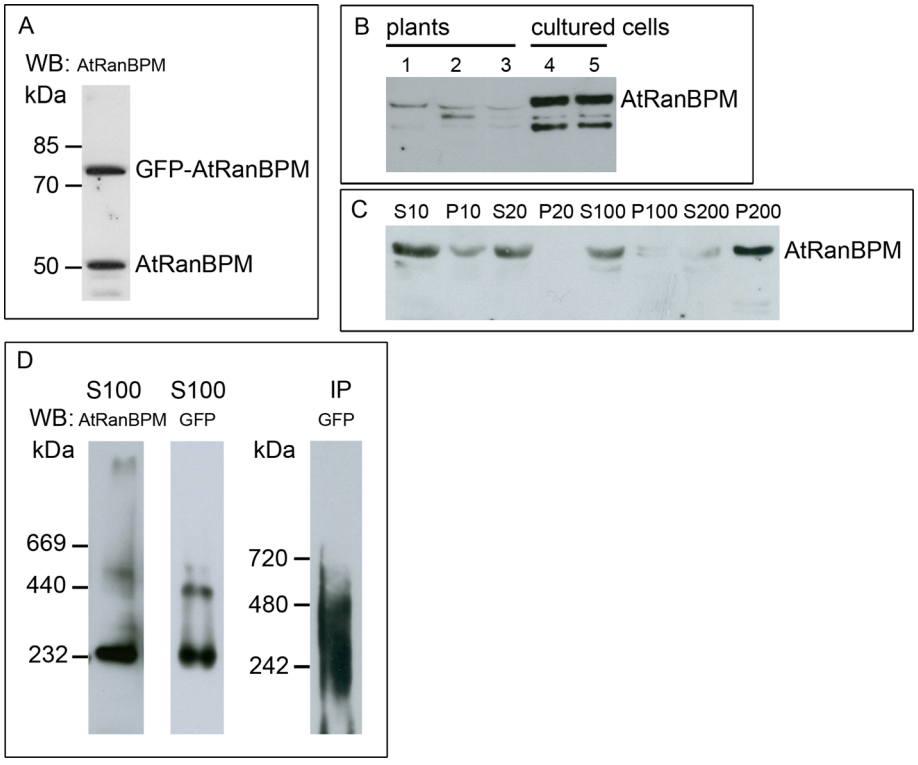

Figure 3 Cellular distribution of AtRanBPM and AtRanBPM complexes. A- The antibody against AtRanBPM recognizes a band of 52 kDa, which corresponds to endogenous AtRanBPM protein, and a band of 80 kDa, which corresponds to expressed GFP-AtRanBPM from cultured cells expressing GFP-AtRanBPM. **B**- Comparison of protein levels of AtRanBPM in *Arabidopsis* seedlings and cultured cells. Lines 1, 2, and 3 – fractions S10 from four-, eight-, and 15-d-old seedlings; lines 4 and 5 – fractions S10 from exponentially growing cultured cells (3 days after subculture) and stationary phase cultured cells (6 days after subculture). Per line, 25 μg of protein content of S10 fraction was loaded. **C**- Distribution of AtRanBPM in the cellular fractions of *Arabidopsis* cultured cells. The majority of the protein was present in cytoplasm (fractions S10, S20, S100), a small amount of the protein was found in pellet P10. AtRanBPM was spun down to high-speed pellet P200 probably in the form of complexes. **D**- GFP-AtRanBPM complexes from high-speed supernatant S100 and from immunoprecipitate (IP) were resolved by non-denaturing PAGE and detected by anti-AtRanBPM antibody or anti-GFP antibody as indicated. For S100 fractions, 15 and 10 μg of protein content was loaded.

presence of complexes of similar size as observed when high-speed supernatant S100 was analysed (Figure 3D). Altogether these data showed that AtRanBPM is present predominantly in the form of large cytoplasmic complexes.

Analysis of AtRanBPM protein complexes by mass spectrometry

To analyse putative interactors of AtRanBPM in the complexes, GFP-AtRanBPM and copurified proteins were separated by SDS-PAGE followed by silver staining, and specific bands were excised and analysed by MALDI-MS (Figure 4A). GFP immunopurification performed in extracts from wild type cell was used as a negative control. Neither AtRanBPM protein nor bands corresponding to the MW of proteins copurified with GFP-AtRanBPM were observed in the negative controls and only background contamination such as HSPs and cytoskeletal proteins were detected by MALDI-MS (Additional file 4). We found that proteins reproducibly copurifying with AtRanBPM belong to CTLH-domain containing proteins including LisH (At1g61150), CRA and U-box (At3g55070, At4g37880), and to the transducin/WD-40 domain-containing proteins At5g08560, and At5g43920 (Table 1, Figure 4B). Alternatively to MALDI-MS, we used LC-MALDI-MS/MS analysis of eluates from the GFP trap to confirm the interaction of GFP-AtRanBPM with the proteins mentioned above.

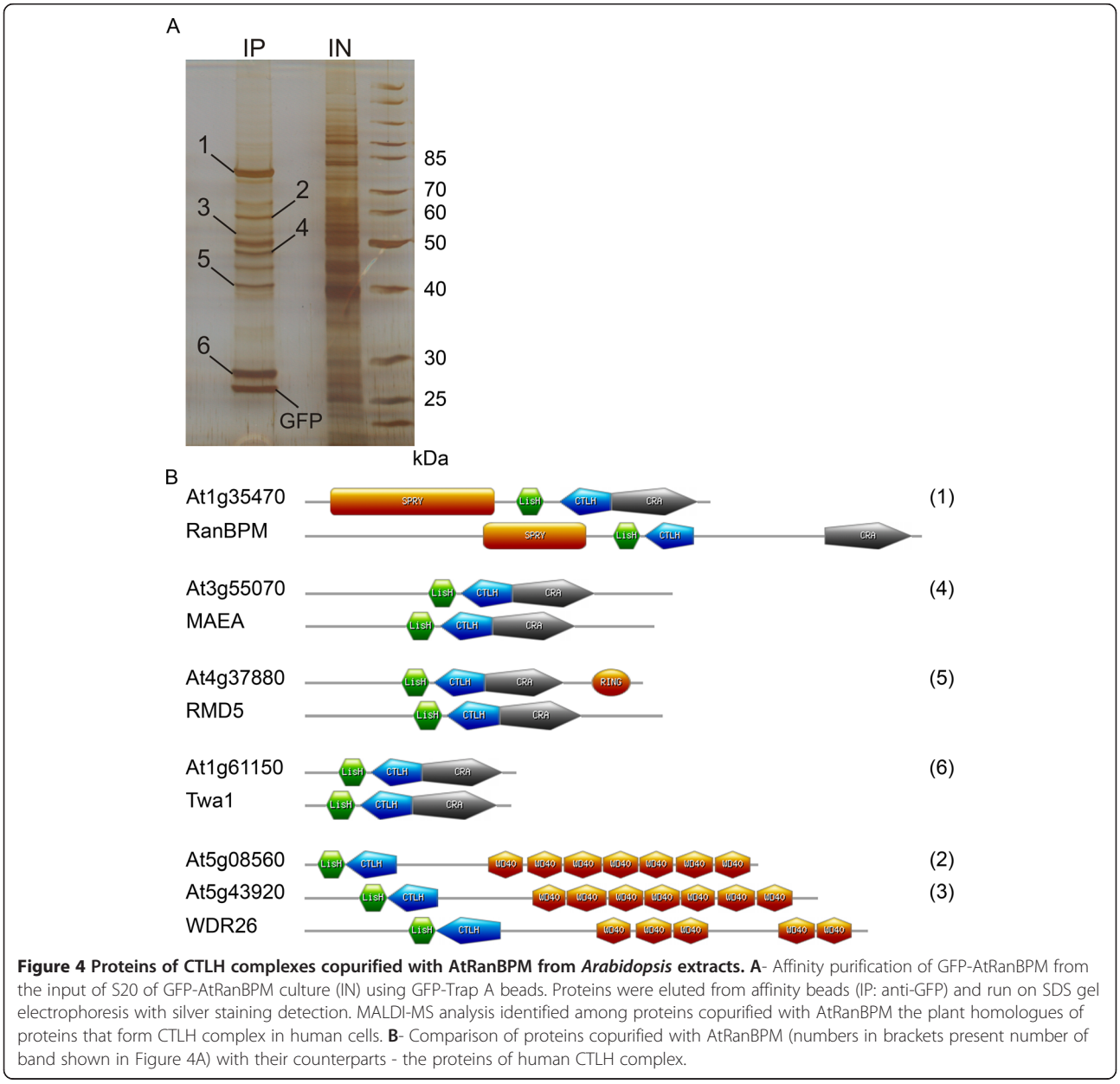

Figure 4 Proteins of CTLH complexes copurified with AtRanBPM from *Arabidopsis* extracts. A- Affinity purification of GFP-AtRanBPM from the input of S20 of GFP-AtRanBPM culture (IN) using GFP-Trap A beads. Proteins were eluted from affinity beads (IP: anti-GFP) and run on SDS gel electrophoresis with silver staining detection. MALDI-MS analysis identified among proteins copurified with AtRanBPM the plant homologues of proteins that form CTLH complex in human cells. **B**- Comparison of proteins copurified with AtRanBPM (numbers in brackets present number of band shown in Figure 4A) with their counterparts - the proteins of human CTLH complex.

Table 1 Proteins copurified with AtRanBPM are members of CTLH complexes

Protein name	AGI number	Band No.	MW [kDa]	No. peptides	Sequence coverage [%]	Aranet/ ATTED-II	Members of CTLH complex in other organisms
AtRanBPM	At1g35470	1	52	17/41	41/60		RanBPM [Q96S59] Gid1/Vid30 [P53076]***
LisH and RanBPM domain-containing protein	At1g61150	6	25	8/26	41/82	-/+	Twa1 [Q9NWU2]**Gid8 [P40208]***
LisH/CRA/RING-U-box domain-containing protein	At3g55070	4	48	10/17	28/31	+/-	MAEA [Q7L5Y9]*Gid9p [P40492]***
LisH/CRA/RING-U-box domain-containing protein	At4g37880	5	44	9/8	32/22	+/-	RMD5 [Q9H871]*Gid2 [Q12508]***
Transducin/WD40 domain-containing protein	At5g08560	2	66	17/14	38/31	+/-	WDR26 [Q9H7D7]
Transducin/WD40 domain-containing protein	At5g43920	3	60	6/4	13/7	+/-	WDR26 [Q9H7D7]

Proteins (for band numbering see Figure 4A) copurified with AtRanBPM protein were identified by both MALDI-MS and LC-MALDI-MS/MS analysis. The values before and after the slash in the columns "No. peptides" and "Sequence coverage" refer to MALDI-MS and LC-MALDI-MS/MS data, respectively. Homologues of human CTLH-complex members reported by Kobayashi et al., [15] (*), proteins in the complex described by Umeda et al. [12] (**), yeast homologues in the Gid complex published by Regelmann et al. [16] (***).

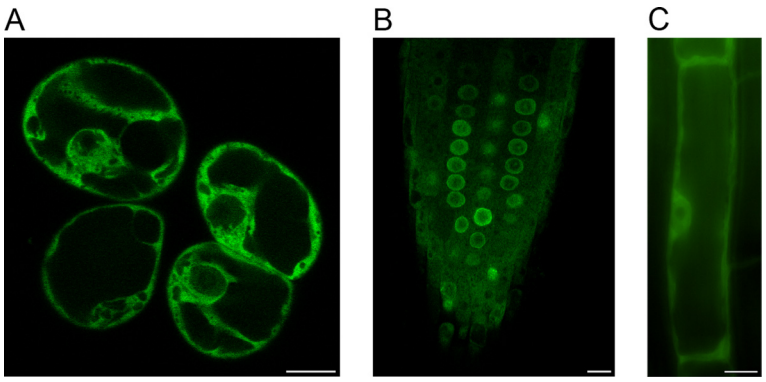

Figure 5 *In vivo* localization of GFP-AtRanBPM in *Arabidopsis* cells and seedlings. A- Localization of GFP-AtRanBPM in *Arabidopsis* suspension culture. The protein is localized in the cytoplasm and in the perinuclear area. Weaker signal is present in nuclei. **B**- Root tip of 8 days old *Arabidopsis* plant. Weak cytoplasmic signal for GFP-AtRanBPM is enriched in the perinuclear area. **C**- Cytoplasmic and perinuclear localization of GFP-AtRanBPM in differentiated cells of root. Bars = 10 μm.

Database search indicated that the proteins copurified with AtRanBPM were homologous to protein components of the human CTLH complex [15]. The CTLH complex is annotated in CORUM (The comprehensive resource for mammalian complexes, [34]) as a complex with a putative function in regulating cell migration, nucleokinesis, chromosome segregation and microtubule dynamics. Components of the CTLH complex are conserved amongst animal species and their presence is predicted for other eukaryotes such as plants and fungi. A relatively lower degree of homology with individual mammalian and plant members of CTLH complexes (Figure 4B, Additional file 5) is due to the fact that part of the molecule separating highly conserved domains contains insertions and deletions of various lengths, differing among plant, mammalian and/or yeast homologues.

The LC-MALDI-MS/MS analysis also identified among proteins copurified with AtRanBPM Armadillo-repeat-containing protein (At3g08947) (Additional file 6). However, the protein was not proven to be a homologue of the human armadillo repeat ARMC8, a member of the CTLH complex [34,35]. Further, Yippee family proteins (At5g53940, At2g40110) and Yippee-like protein (At3g08890) were copurified with AtRanBPM and identified by LC-MALDI-MS/MS (Additional file 6). Yippee-like proteins belong to the YPEL family of proteins whose members have been described as interactors of the human RanBPM [10]. Proteins At5g08560, At5g43920, At3g55070, and At3g37880, copurified with AtRanBPM were predicted to interact within a functional network in the Aranet database (Probabilistic Functional Gene Network for Arabidopsis thaliana, [36]) (Table 1).

Together these data suggest that plant AtRanBPM protein, like its yeast and mammalian homologues, is a component of a cytoplasmic multiprotein complex where it interacts with LisH-CTLH domain-containing proteins and armadillo-repeat-containing proteins.

In vivo localization of GFP-AtRanBPM and AtRanBPM immunofluorescence labelling in Arabidopsis cultured cells and seedlings

In cultured cells of *Arabidopsis,* the GFP-AtRanBPM signal was localized in the cytoplasm and in the perinuclear area of interphase cells, with a weaker signal detected in nuclei (Figure 5A). We observed no gain-of-function phenotype of *Arabidopsis* seedlings expressing GFP-AtRanBPM from Gateway vectors. Similarly to cultured cells, the GFP-AtRanBPM signal was localized in the cytoplasm, in nuclei and accumulated in the vicinity of nuclei in dividing zone of roots (Figure 5B). In differentiated root cells, GFP-AtRanBPM was cytoplasmic and in the perinuclear area (Figure 5C, Additional file 7). C-terminal GFP fusions showed a similar localization pattern when expressed transiently in cultured cells of *Arabidopsis* (Additional file 8).

Immunofluorescence analyses showed that the AtRanBPM protein was distributed patchily in the cytoplasm and nuclei; in non-dividing cells it accumulated in the perinuclear region and in dividing cells, the signal was slightly enriched in the area of the mitotic spindle and the phragmoplast (Figure 6A). Further we compared localization of the endogenous protein and GFP-AtRanBPM by double labelling with anti-AtRanBPM and anti-GFP antibodies. We confirmed a similar localization pattern for AtRanBPM and expressed AtRanBPM with a cytoplasmic signal slightly enriched in the perinuclear area and a weaker nuclear signal (Figure 6B).

To determine whether the AtRanBPM protein plays a role in microtubule organization like the truncated version of human RanBPM [37], we studied the colocalization of AtRanBPM with the centrosomal protein, γ-tubulin. In acentrosomal plant cells, γ-tubulin is present with dispersed sites of microtubule nucleation in the cytoplasm, with nuclei, and on membranes and microtubules [38]. We found that the *Arabidopsis* homologue

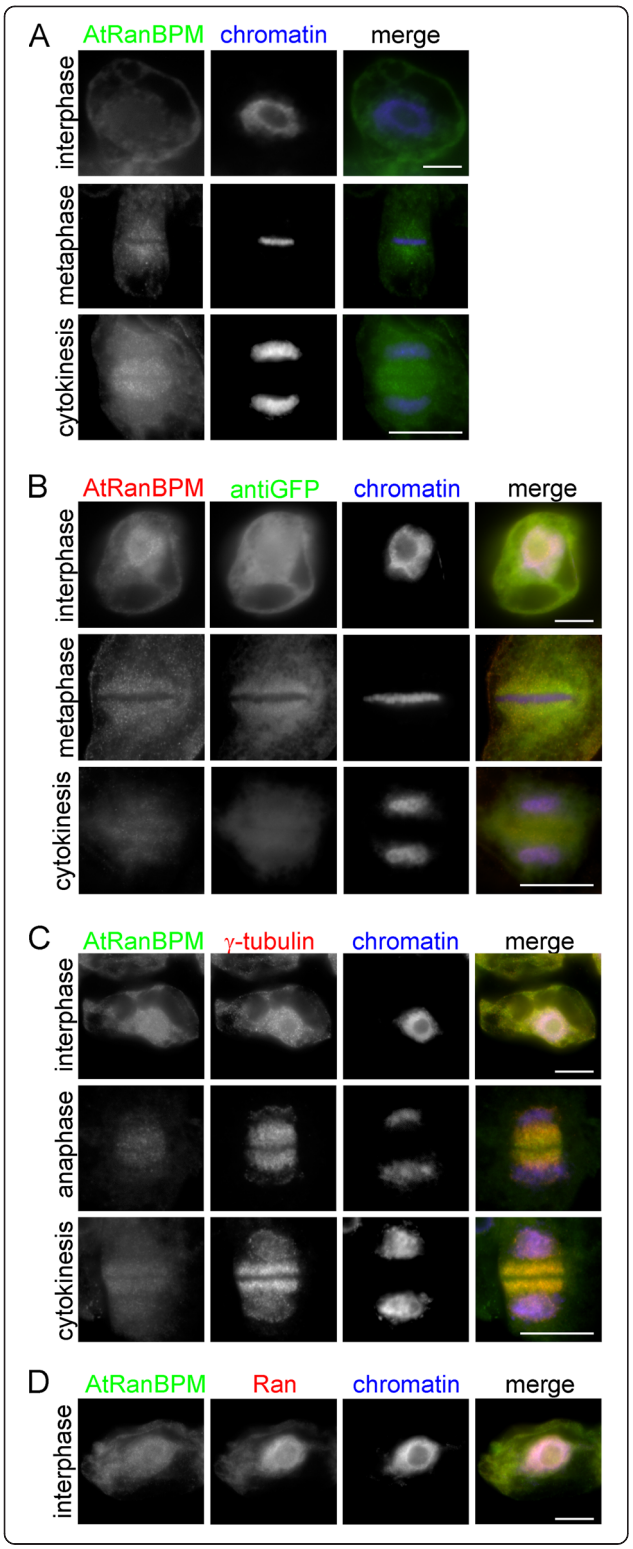

Figure 6 Immunolocalization of AtRanBPM in *Arabidopsis* cultured cells. A- Immunolocalization of endogenous AtRanBPM with affinity purified anti-AtRanBPM antibody. Signal for AtRanBPM was cytoplasmic with slight accumulation in the vicinity of nuclei and in dividing cells with slight enrichment in spindle and phragmoplast area. (AtRanBPM – green, chromatin – blue). **B**- Double immunofluorescence analysis of cells expressing GFP-AtRanBPM confirmed similar localization pattern for expressed GFP protein and for endogenous AtRanBPM protein (Figure 6A) (GFP-AtRanBPM – green, endogenous AtRanBPM – red, chromatin – blue). **C**- Double immunofluorescence analysis of AtRanBPM (green) and γ-tubulin (red) did not confirm colocalization of both proteins in interphase and in dividing cells. **D**- Double immunofluorescence of AtRanBPM (green) and Ran (red). Only a small portion of AtRanBPM is localized in nuclei compared to intensive nuclear signal for Ran protein. Bars = 10 μm.

AtRanBPM did not colocalize with γ-tubulin-positive sites either present with the nuclear envelope or on microtubular arrays (Figure 6C).

As a portion of RanBPM was reported to be associated with the Ran protein in mammalian cells [14] we performed double immunofluorescence labelling of AtRanBPM and Ran protein. In cultured cells of *Arabidopsis*, Ran was localized in the cytoplasm and in nuclei but only a small fraction of AtRanBPM colocalized with nuclear Ran.

Discussion

RanBPM protein was first characterized in human cells as a centrosomal protein involved in microtubule nucleation which colocalized with γ-tubulin at centrosomes and at ectopic nucleation sites [37]. The data on RanBPM initiated an investigation of the role of the RanGTPase pathway and its role in chromatin-mediated microtubule nucleation and spindle assembly [39]. However, later it was found that antibody against the 55 kDa form of RanBPM that was characterized by Nakamura et al. [37] did not recognize the full length 90 kDa RanBPM protein. The truncated form of RanBPM (55 kDa) was shown to be an incorrectly translated product of the *RanBPM* gene and moreover, only the truncated version but not the whole RanBPM molecule was active in microtubule nucleation [14]. The complete RanBPM molecule thus does not show the same properties as the truncated version and it was depicted as a scaffold protein that links and modulates interactions between cell surface receptors and their intracellular signalling pathways [11,40]. The molecular mass of plant AtRanBPM (52 kDa) corresponds to that of the truncated RanBPM rather than to the whole human RanBPM molecule. However, the reason for the apparent discrepancy in size of the full length human and plant RanBPM molecules is an insertion of 150 amino acids between CTLH and CRA domains in the plant protein and the presence of a long stretch of proline and glutamine residues on the N-terminal part of human RanBPM molecule.

We found that AtRanBPM maintained the properties of the full length RanBPM of mammals: (i) while the truncated version of human RanBPM is present in

centrosomal and ectopic microtubule nucleation sites [37], AtRanBPM protein in acentrosomal plant cells did not colocalize with microtubule nucleation sites positive for γ-tubulin; (ii) proteins of the γ-tubulin nucleating machinery were not identified by MALDI-MS or LC-MALDI-MS/MS among proteins interacting with AtRanBPM, nor were they found in coexpression databases and a biological role of AtRanBPM other than in processes relating to centrosomes and microtubules was thus suggested; (iii) the presence of highly conserved SPRY, LisH, CTLH and CRA domains in AtRanBPM indicated its function in mediating multiple protein interactions that were described for the whole molecule of human RanBPM [12,14,15].

AtRanBPM protein was not specifically enriched with microtubules in dividing cells or localized in putative microtubule nucleation sites. Subcellular localization of AtRanBPM corresponded to published data on the subcellular localization of the whole RanBPM molecule in mammalian cells [14,41,42]. Since a weak nuclear signal, observed for human RanBPM was suggested to be a consequence of over-expression of its tagged version [40], we analysed by immunofluorescence the nuclear localization for endogenous AtRanBPM protein. A smaller portion of AtRanBPM was present in nuclei and the possibility that the nuclear signal observed for GFP-AtRanBPM or AtRanBPM-GFP resulted from over expression of GFP fusions was thus excluded. We observed only partial colocalization of Ran and AtRanBPM in nuclei. It would be interesting to address the question whether transport of the AtRanBPM complex between nucleus, perinuclear area and cytoplasm might be regulated by a weak interaction between AtRanBPM and Ran as it was suggested for mammalian RanBPM [14]. Heterogeneity in the localization of mammalian RanBPM might depend on interacting proteins [15]. Conserved domains of RanBPM protein are responsible for interactions and complex formations with a physiologically divergent group of proteins. As reviewed by Suresh et al. [11], RanBPM is a modulator/protein stabilizer, a regulator of transcriptional activity, and has cell cycle and neurological functions. RanBPM interacts with a wide range of receptors [43], acts as a scaffolding protein [44,45], and is involved in signal transduction pathways [44,46], and the apoptotic pathways [47]. Plant homologues of *RanBPM* belong to genes with unknown functions. We found that the product of *AtRanBPM* is predominantly a cytoplasmic protein that is a part of protein complexes with a molecular mass of approximately 230 – 500 kDa. A large protein complex of RanBPM was described by Nishitani et al. [14] and Ideguchi et al. [13]. Later the complex was designated as a CTLH complex [15]. Five from eleven CTLH domain-containing proteins that were identified in databases were described as being a part of the CTLH complex and an interaction within the complex via LisH-CTLH domains was suggested [15]. In *Saccharomyces cerevisiae* all four CTLH domain-containing proteins present in the genome are part of the CTLH-like complex Gid. The Gid complex is suggested to be involved in proteasome-dependent glucose-induced degradation of fructose-1,6-bisphosphatase [16,48]. There are twenty one CTLH-domain containing proteins encoded in the *Arabidopsis* genome and of these, six proteins were identified in our experiments to form CTLH-like complexes with AtRanBPM protein. Though we identified the same spectrum of AtRanBPM interacting proteins in several independent experiments it cannot be ruled out that the members of CTLH complexes exist and remain to be identified. We found that the antibody raised against a peptide from the AtRanBPM sequence did not work in immunopurification experiments. The immunopurifications performed with a panel of antibodies against the AtRanBPM protein might help to disclose the presence of other putative members of plant CTLH complexes. Alternatively, immunopurification of the CTLH complex or pull down experiments via proteins that copurified with AtRanBPM might extend our knowledge of the composition and protein interactions of newly identified CTLH complexes.

All the members of the CTLH complexes that we identified belong to yet uncharacterized plant LisH-CTLH domain-containing proteins. Protein At1g61150 is a homologue of the human Twa1 protein found in yeast by two-hybrid analysis to interact with RanBPM [12]. LisH, CRA, and RING/U-box domain-containing proteins of unknown function (At3g55070, At4g37880), copurified with AtRanBPM, are homologous to human MAEA and RMD5, respectively, that are members of the CTLH complex annotated by Kobayashi et al. [15]. Similarly, LisH and CTLH domain-containing proteins such as RanBP10 or the WD repeat domain 26 (WDR26), were suggested to be putative members of the CTLH complex [15]. We found that uncharacterized *Arabidopsis* transducin/WD-40 domain-containing proteins At5g08560 and At5g43920 which copurified with AtRanBPM, are homologues of human WDR26 protein and thus belong to components of the plant CTLH complex. Corresponding to our microscopic data on cytoplasmic and nuclear localizations of AtRanBPM, the members of the human CTLH complex, proteins RanBPM, RMD5, WDR26, Twa1, and MAEA, were shown to have predominantly cytoplasmic and a weaker nuclear localization [12,15,49].

A component of the human CTLH complex, the armadillo repeat-containing protein ARMC8, when exogenously expressed, upregulates the proteolytic degradation of ectopically expressed α-catenin, and thus was

suggested to play an important role in the ubiquitin-independent and proteasome-dependent degradation of α-catenin [35]. α-Catenin is known to function as a link protein between cadherins and actin-containing filaments of the cytoskeleton [50]; thus a complex might regulate molecular adhesion. We identified Armadillo-repeat-containing protein (At3g08947) among proteins copurified with AtRanBPM but its homology with armadillo repeat ARMC8, a member of the human CTLH complex, was not proven. However, we found that expression of copurified protein At4g37880, a homologue of RMD5 protein, correlated strongly with expression of the armadillo repeat-containing protein At3g01400, one of the homologues of ARMC8, and with expression of the plant adhesion molecule RabGAP/TBC domain-containing protein At3g02460 (ATTED-II, [51]). It is tempting to speculate whether similar function in connecting external signals in adhesion with the cell centre analogous to the cadherin and catenin pathway in animals might exist for plant CTLH complexes. However, further experimental data are needed to prove this hypothetical function of the CTLH complexes in plants.

Besides copurification with CTLH complex proteins, we found that plant AtRanBPM interacts with Yippee proteins (At5g53940, At2g40110) and Yippee-like protein (At3g08890). It was shown in human cells that RanBPM binds members of YPEL (Yippee like) family of proteins involved in a cell division-related functions [10]. Our data showed that binding with Yippee proteins might present one of the AtRanBPM multiple protein interactions in plant cells too.

Conclusions

RanBPM protein was shown to have numerous apparently functionally unrelated protein interactors, including membrane receptors, cytoplasmic and nuclear proteins [12,15]. This suggests its role in a variety of cellular processes. Our biochemical and proteomic studies showed that AtRanBPM is a component of plant homologues of CTLH complexes. The fact that CTLH complexes are present in mammals, yeast and in plants suggests their structural conservation in evolution. CTLH complexes thus might represent a module with important but not fully understood biological functions.

Methods

Plant material

Arabidopsis thaliana (*L.*) *Heynh*. plants, ecotype Columbia were used. Sterilized seeds were sown and stratified at 4 °C for 2 days, then grown under 8 h of light, 16 h of darkness at 20 °C and after 4 weeks, under 16 h of light and 8 h of darkness. Seeds were cultivated on half-strength Murashige and Skoog (MS) medium (Duchefa), supplemented with 0.25 mM MES, 1% saccharose and 1% phytoagar. Transformed seedlings were selected as described in Harrison et al. [52]. *Arabidopsis thaliana* suspension cultures were cultured as described in Drykova et al. [38].

Molecular cloning of AtRanBPM

AtRanBPM coding sequence [EMBL:AEE31799] was obtained by PCR amplification using *Arabidopsis thaliana* cDNA as template and Platinum Pfx DNA Polymerase (Invitrogen). The PCR primers with *attB* sites (underlined) were designed according to the manufacturer's intruction, forward 5' – GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAACTCTTCACCACCACCG – 3', reverse 5' – GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAGTCTCCATTCAGTGACCGCCTTTC – 3' for N-terminal GFP fusion and for C-terminal GFP fusion forward 5' – GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAACTCTTCACCACCACCG – 3', reverse 5' – GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAGTCTCCATTCAGTGACCGCCTTTC – 3´. PCR products were isolated using QIAquick gel extraction kit (Qiagen) and afterwards cloned by Gateway technology (Invitrogen). Gateway binary vectors pK7WGF2,0 for N-terminal GFP fusion, pMDC43 for C-terminal GFP fusion [53] were used.

Stable transformation of cell suspension cultures and plants

Arabidopsis suspension cultures of Landsberg erecta ecotype stably expressing GFP-AtRanBPM or transiently expressing AtRanBPM-GFP were prepared according to the protocol of Mathur et al. [30]. Arabidopsis plants Landsberg erecta ecotype were transformed by the floral-dip method [31].

Preparation of protein extracts and its fractionation by differential centrifugation

Cultured suspension cells or seedlings of *Arabidopsis thaliana* were ground in liquid nitrogen and thawed in extraction buffer in ratio 1:1 weight to volume. Extraction buffer was 50 mM Na-Hepes, pH 7.5, 75 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT supplemented with inhibitors of proteases and phosphatases according to Drykova et al. [38] with increased concentration of 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride to 2 mM and β-glycerophosphate to 60 mM. Crude extracts were centrifuged at 10,000 *g* for 10 min at 4 °C; obtained supernatants S10 were subsequently centrifuged at 20,000 *g* for 0.5 h at 4 °C to gain supernatants S20 and pellets P20. Supernatants S20 were then centrifuged at 100,000 *g* for 1 h at 4 °C when S100 were needed. Supernatants S100 were centrifuged at 200,000 *g* for 1.5 h at 4 °C to gain supernatants S200 and pellets P200 when needed. For differential centrifugation, pellets were

resuspended in a volume of extraction buffer equal to the volume of the corresponding supernatants; equal sample volumes were loaded on SDS-PAGE gel.

Immunoprecipitation of GFP-AtRanBPM

Cell extracts S20 (~ 4 mg protein/mL) were used directly or solubilized by 1% NP-40 (Calbiochem) for 1 h at 4 °C. Immunoprecipitations of GFP-AtRanBPM protein were then performed using GFP-Trap_A (ChromoTek) according to the manufactures' instructions. GFP immunopurification from extracts S20 of wild type Ler *Arabidopsis* culture was used as a negative control.

Protein digestion and mass spectrometry

The analysed protein bands were cut out of the Ag-stained SDS-PAGE gels, trypsin-digested and the proteins were identified by peptide mass mapping as described elsewhere [54]. Alternatively, eluates from the GFP trap were denatured in 8 M urea and digested overnight using trypsin. After desalting the resulting peptides were separated using the Ultimate HPLC system (LC Packings) on a Magic C18AQ column. The eluent from the column was spotted directly onto PAC-target using a ProteineerFC spotting device (Bruker Daltonics). Automatic MALDI MS/MS analyses were performed on an Ultraflex III TOF/TOF (Bruker Daltonics) and the proteins were identified by searching MS/MS spectra against *A. thaliana* subset of NCBI database using the in-house Mascot program (Matrixscience). The high resolution MALDI spectra were acquired on an APEX-Qe 9.4 T FTMS instrument (Bruker Daltonics).

Electrophoresis and immunoblotting

Protein samples after addition of appropriate sample buffer were separated either on 10% SDS-PAGE gels [55] or on 3-10% non-denaturing PAGE gels as described previously in Drykova et al. [38]. Proteins were transferred onto 0.45 μm polyvinylidene fluoride membranes (Millipore) by wet electroblotting and immunodetected with appropriate antibody. Anti-AtRanBPM antibody was designed against peptide of AtRanBPM amino acid sequence (CSTNPNKKDVQRS), raised in rabbits and purified with immunogenic peptide coupled to protein A sepharose beads (GeneScript) and used in dilution 1:100 for Western blotting from non-denaturating PAGE, 1:300-1:500 for Western blotting from SDS-PAGE. Anti-GFP ab290 (Abcam) was diluted 1:2,000. Secondary anti-Rabbit IgG ECL Antibody, HRP Conjugated (GE Healthcare) was diluted 1:10,000; Super Signal West Pico Chemiluminiscent Substrate (Thermal Scientific) was used according to manufacturer's instructions. Alternatively, SDS-PAGE gels were stained with silver, and the selected protein bands analysed by MALDI-MS.

Immunofluorescence

Arabidopsis thaliana suspension cultures were fixed for 30 min using 3.7% paraformaldehyde and processed for immunofluorescence labelling according to Binarová et al. [56]. Primary antibodies used: mouse anti-α-tubulin monoclonal antibody DM1A (Sigma) diluted 1:500, mouse monoclonal anti-γ-tubulin TU-32 (kindly provided by Pavel Dráber, IMG, Prague, Czech Republic) diluted 1:10, mouse anti-GFP antibody (Abcam) diluted 1:1,000, and mouse antibody against human Ran protein (BD Transduction Laboratories) diluted 1:1,000. Anti-AtRanBPM antibody was used in a dilution 1:1,000, anti-rabbit or anti-mouse secondary antibodies DyLight 488 or DyLight 549 (Jackson Immuno Research Laboratories) were diluted 1:250. DNA was stained with DAPI.

Microscopy

Microscopical analysis was performed on an IX81 motorized inverted research microscope CellR (Olympus) equipped with DSU (Disk Scanning Unit) and digital monochrome CCD camera CCD-ORCA/ER. To avoid filter crosstalk, fluorescence was detected using HQ 480/40 exciter and HQ 510/560 emitter filter cubes for DyLight 488 and HQ 545/30 exciter and HQ 610/75 emitter filter cubes for DyLight 549 (both AHF AnalysenTechnique). Confocal images were taken on Olympus FluoView FV1000 based on IX81 microscope using PLAPO 100x/1.45 and UPLSAPO 60x/1.35 objectives. GFP was excited by 473 nm solid state laser and its emission was detected from 485 to 545 nm. Images were processed and analysed using CellR and FV10-ASW (Olympus). Adobe Photoshop 7.0 was used for preparation of figures.

Database search

To identify putative domains, SMART (Simple Modular Architecture Research Tool, [57], http://smart.embl-heidelberg.de/) and Pfam ([58], http://pfam.sanger.ac.uk/) databases were used. Multiple sequence alignment was performed by ClustalX2 [59] and evaluation of similarity and identity was done by WU-BLAST (Basic Local Alignment Search Tool, [22], http://www.ebi.ac.uk/Tools/sss/wublast/). Schematic drawings of protein structure were prepared in DOG (Domain Graph, [60]) and MyDomains (Prosite, http://prosite.expasy.org/cgi-bin/prosite/mydomains/). Search for putative interaction sites in AtRanBPM amino acid sequence was done in ELM (The eukaryotic linear motif resource for functional sites in proteins, [28], http://elm.eu.org/) and SumoSP 2.0 (Sumoylation sites prediction [29], http://sumosp.biocuckoo.org/). Phylogenetic relationships were analysed in MEGA 5.02 (Molecular evolutionary genetic analysis, [23]). Genes with similar expression pattern to AtRanBPM were identified in publically available databases ATTED-II ([51], http://atted.jp/) and AraNet ([36], http://www.functionalnet.org/aranet/).

Putative interactors were analysed by Arabidopsis eFP browser ([32], http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) and Genevestigator ([33], https://www.genevestigator.com/gv/plant.jsp). The information and phylogenetic relationships of human CTLH complex with similar complexes from other species were found in CORUM ([34], http://mips.helmholtz-muenchen.de/genre/proj/corum/).

Additional files

Additional file 1: Multiple sequence alignment of AtRanBPM plant homologues. Sequence alignment of *Arabidopsis* RanBPM with *Ricinus communis*, *Vitis vinifera*, *Populus trichocarpa*, *Sorghum bicolor*, *Oryza sativa* and *Zea mays* homologues. Alignment was done using ClustalX2 software [59]. Sequence data of this alignment can be found at accession numbers [Swiss-Prot:F4HYD7] for At1g35470, [Swiss-Prot:B9S762] for *R. communis*, [Swiss-Prot:F6HWC3] for *V. vinifera*, [Swiss-Prot:B9MWC1] for *P. trichocarpa*, [Swiss-Prot:C5XUT1] for *S. bicolor*, [Swiss-Prot:Q6ZI83] for *O. sativa*, [Swiss-Prot:B6UAR9] for *Z. mays*.

Additional file 2: Identities and similarities of conserved domains between Arabidopsis RanBPM and its human homologues. Sequence alignment was done for conserved domains SPRY (A), LisH (B), CTLH (C) and CRA (D) of full-sized 90 kDa form (RanBPM_90) and 55 kDa form (RanBPM_55) of human RanBPM protein, and RanBP10 with *Arabidopsis* RanBPM using ClustalX2 software [59]. Sequence data of this alignment can be found at accession numbers [Swiss-Prot:Q6VN20] for RanBP10, [Swiss-Prot:Q96S59] for 90 kDa RanBPM and [EMBL:BAA23216] for 55 kDa RanBPM. The sequence of the SPRY domain was encountered from the 99 amino acids in AtRanBPM sequence. E- Levels of identities and similarities in amino acid composition of conserved domains between AtRanBPM and its human homologues RanBPM and RanBP10.

Additional file 3: Phylogenetic analysis of AtRanBPM and its homologues from other eukaryotic species. The tree was constructed by the neighbor-joining method with the MEGA 5.05 software [23]. Branch numbers represent the percentage of bootstrap values in 1000 sampling replicates. The protein accession numbers are [Swiss-prot:F4HYD7] for AtRanBPM At1g35470, [Swiss-prot: Q9SMS1] At4g09340 (segmental genome duplication of chromosome 1), [Swiss-Prot:B9S762] for *R. communis*, [Swiss-Prot:F6HWC3] for *V. vinifera*, [Swiss-Prot:B9MWC1] for *P. trichocarpa*, [Swiss-Prot:C5XUT1] for *S. bicolor*, [Swiss-Prot:Q6ZI83] for *O. sativa*, [Swiss-Prot:B6UAR9] for *Z. mays*, [Swiss-prot: Q6VN20] for human RanBP10, [Swiss-prot: A3KMV8] for RanBP10 from *Bos taurus*, [Swiss-prot: B5LX41] for RanBP10 from *Felis catus*, [Swiss-prot: Q6VN19] for RanBP10 from *Mus musculus*, [Swiss-prot: Q1LUS8] for RanBP10 from *Danio rerio*, [Swiss-prot: Q9PTY5] for RanBP9 from *Xenopus laevis*, [Swiss-prot: Q96S59] for human RanBP9, [Swiss-prot: P69566] for RanBP9 from *Mus musculus*, [Swiss-prot: Q4Z8K6] for RanBP9/10 from *Drosophila melanogaster* and [Swiss-prot: P53076] for Gid1/Vid30 homologue from *Saccharomyces cerevisiae*. Distance bars are given bottom left and bootstrap values are indicated at the nodes.

Additional file 4: Immunopurification of GFP-AtRanBPM protein. Immunopurification of GFP-AtRanBPM from extracts of GFP-AtRanBPM expressing cell cultures (IP GFP-RanBPM). GFP immunopurification from extracts of wild type Ler *Arabidopsis* cells (IP WT) was used as a negative control. A- Proteins were silver stained after separation on SDS-PAGE. Bands corresponding to MW similar of the proteins copurified with GFP-AtRanBPM (IP GFP-RanBPM) were not present in the negative control (IP WT). B- Signal for AtRanBPM was absent in the negative control (IP WT) after detection with anti-AtRanBPM antibody on Western blots. C- Proteins identified by MALDI-MS in negative control (IP WT in A) were background contamination.

Additional file 5: Identities and similarities between proteins copurifying with AtRanBPM and human CTLH complex members. Identities and similarities between *Arabidopsis* and human proteins were analysed in WU-BLAST.

Additional file 6: Additional proteins copurified with AtRanBPM. The proteins were identified by LC-MALDI-MS/MS and the identity of the matched peptides was confirmed by high-resolution MALDI-FTMS with mass accuracy below 1 ppm.

Additional file 7: GFP-AtRanBPM in *Arabidopsis* root cells. AtRanBPM-GFP signal is dynamic and moving with the cytoplasmic stream.

Additional file 8: Cellular localization of C-terminal GFP and N-terminal GFP AtRanBPM fusion proteins. Cells of *Arabidopsis* expressing C-terminal GFP AtRanBPM (AtRanBPM-GFP) showed weak cytoplasmic and nuclear signal and accumulation of perinuclear GFP signal similarly as observed for N-terminal GFP AtRanBPM (GFP-AtRanBPM).

Abbreviations

CRA: CT11-RanBPM; CTLH: C-terminal to LisH; GAP: GTPase activating protein; GEF: Guanine nucleotide exchange factor; GFP: Green fluorescent protein; Gid/Vid proteins: Glucose-induced degradation/vacuole-induced degradation proteins; LC: Liquid chromatography; LisH: Lissencephaly type-1-like homology; RanBP: Ran binding protein; RanBP1: Ran binding protein1; RanBPM: Ran binding protein in microtubule organizing centre; SPRY: SPIa and Ryanodine receptor; Scorpin family: SPRY-containing Ran binding protein family.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ET performed databases search, *in vivo* microscopical analyses and wrote the manuscript. VC carried out immunolocalization and microscopy, and participated in writing the manuscript. LK performed biochemical analysis, isolation of protein complexes and confocal microscopy, BP performed cloning and together with LV, created stably transformed cell lines and plants of *Arabidopsis thaliana*, and performed *in vivo* microscopical analyses. PH performed proteomic analyses. GK undertook immunopurification of protein complexes. PB designed and coordinated experimental plans, and wrote the manuscript. All authors read and approved the final manuscript.

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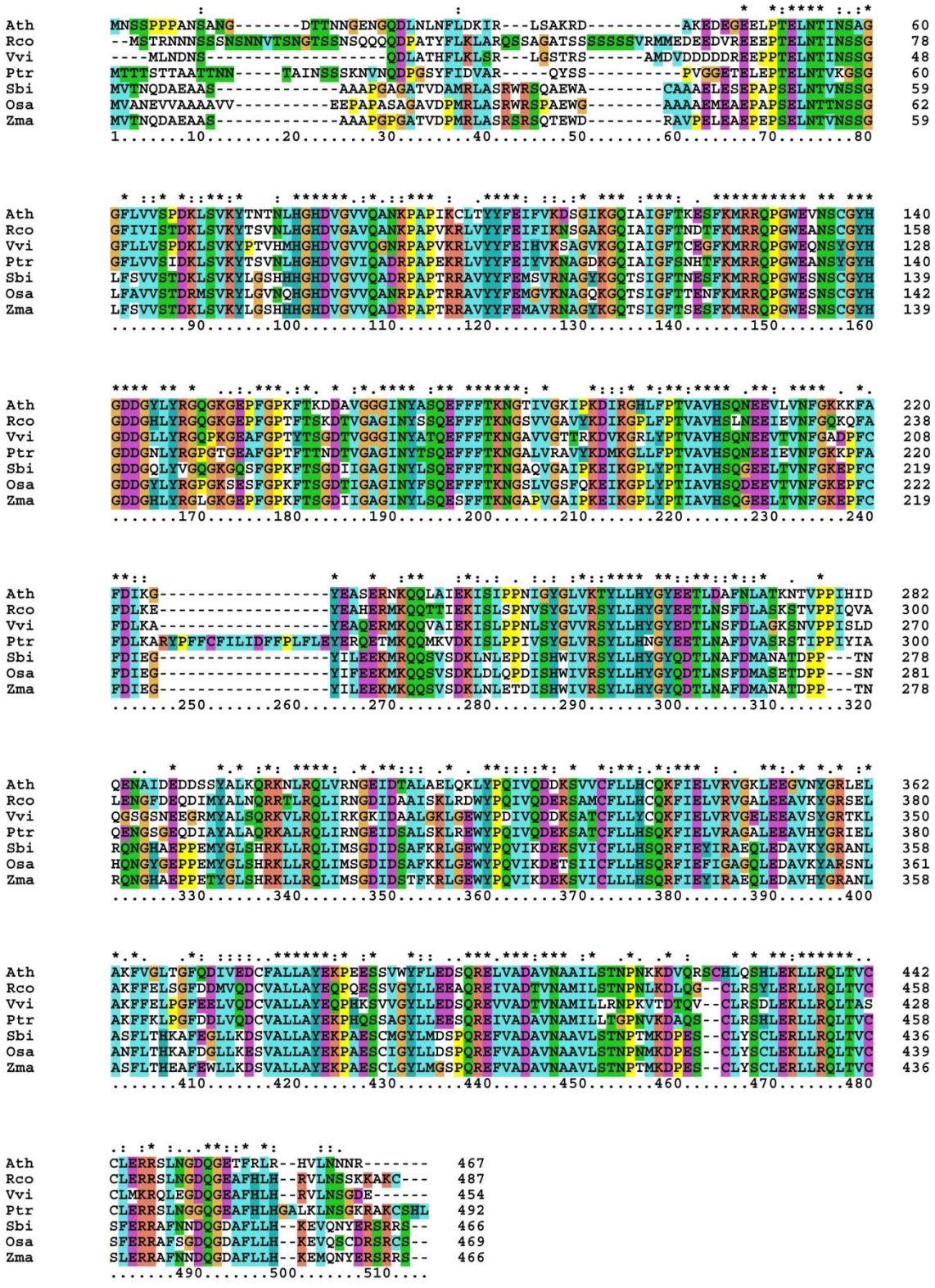
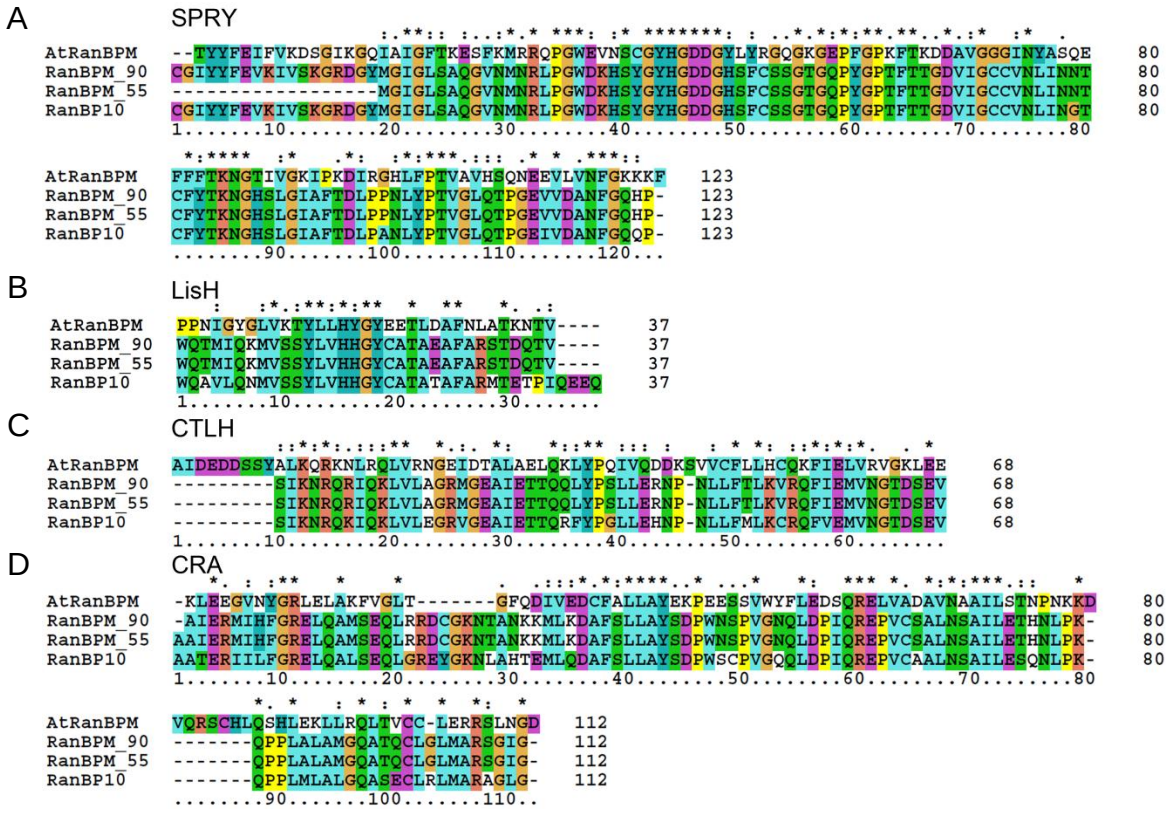


Additional file 1: **Multiple sequence alignment of AtRanBPM plant homologues**. Sequence alignment of *Arabidopsis* RanBPM with *Ricinus communis*, *Vitis vinifera*, *Populus trichocarpa*, *Sorghum bicolor*, *Oryza sativa* and *Zea mays* homologues. Alignment was done using ClustalX2 software (Larkin et al., 2007). Sequence data of this alignment can be found at accession numbers [Swiss-Prot:F4HYD7] for At1g35470, [Swiss-Prot:B9S762] for *R.*

communis, [Swiss-Prot:F6HWC3] for *V. vinifera*, [Swiss-Prot:B9MWC1] for *P. trichocarpa*, [Swiss-Prot:C5XUT1] for *S. bicolor*, [Swiss-Prot:Q6ZI83] for *O. sativa*, [Swiss-prot:B6UAR9] for *Z. mays*.

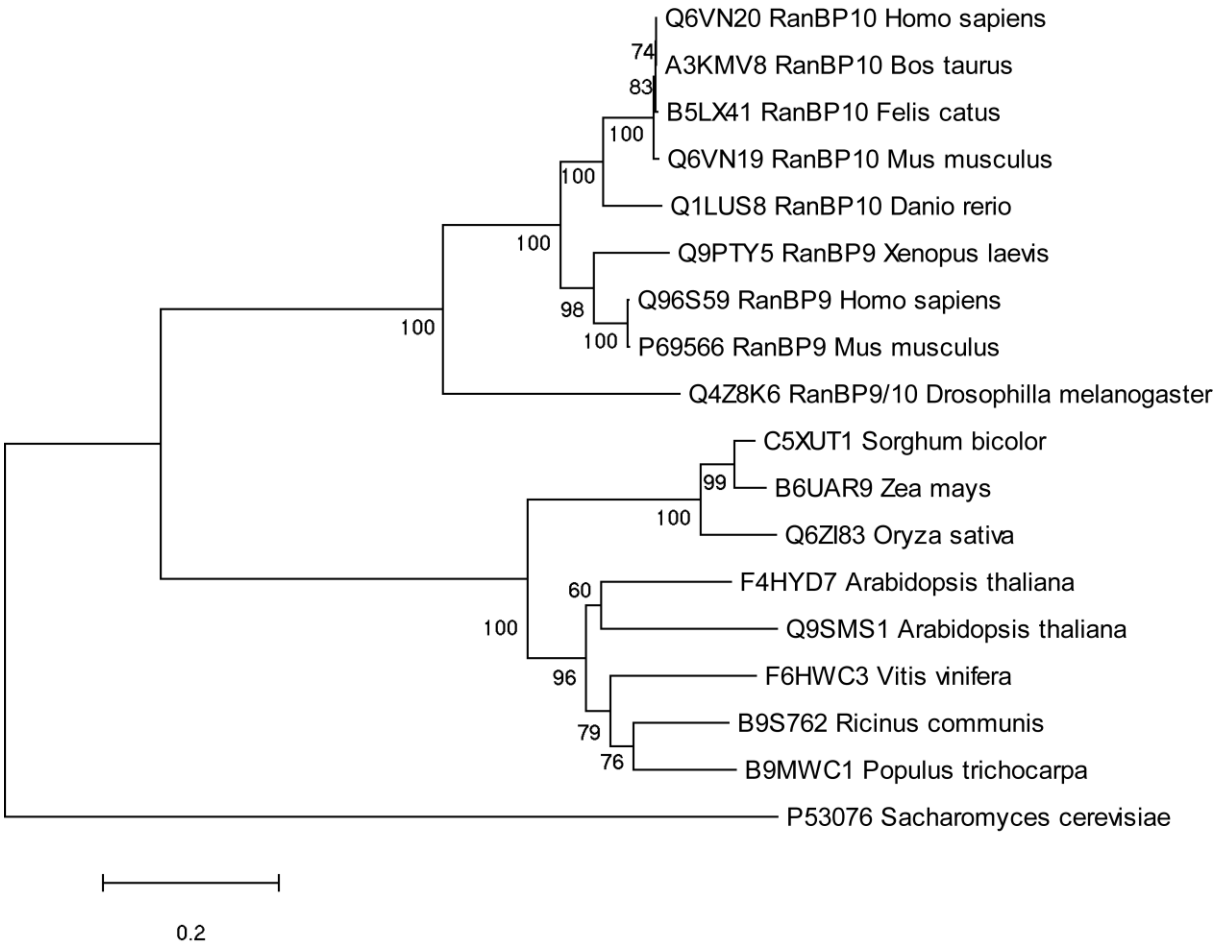


E

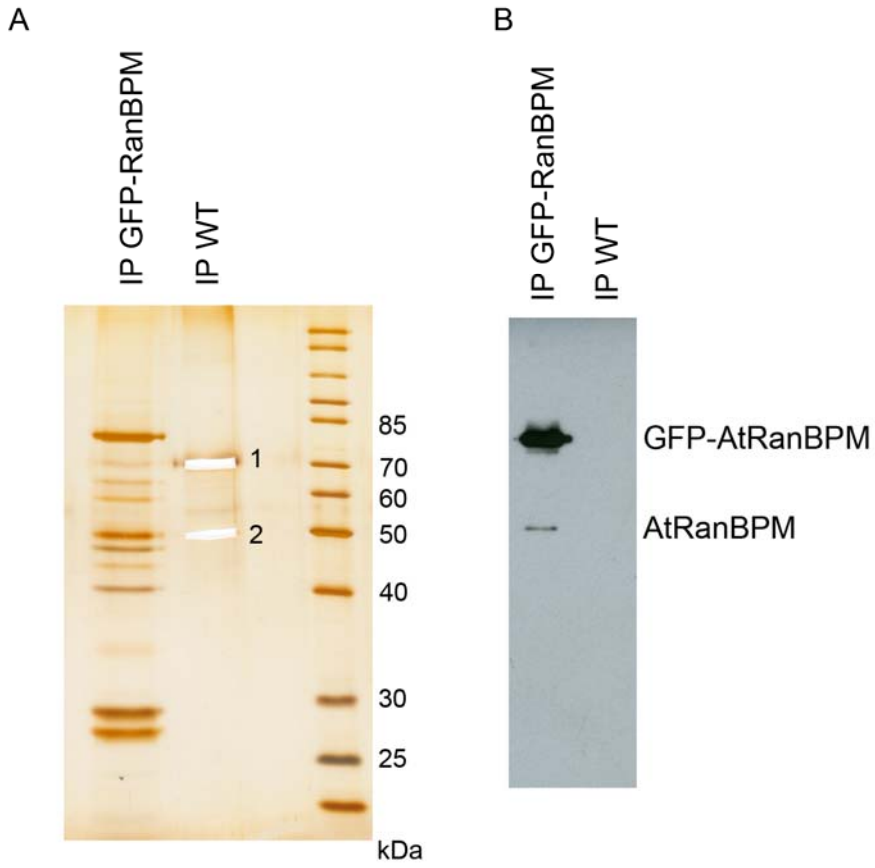
	RanBPM		RanBP10	
	Identities [%]	Similarities [%]	Identities [%]	Similarities [%]
SPRY	40,0	58,3	39,7	58,7
LisH	46,2	69,2	43,5	65,2
CTLH	30,8	67,3	28,8	59,2
CRA	43,8	62,5	41,9	62,8

Additional file 2: **Identities and similarities of conserved domains between *Arabidopsis* RanBPM and its human homologues**. Sequence alignment was done for conserved domains SPRY (A), LisH (B), CTLH (C) and CRA (D) of full-sized 90 kDa form (RanBPM_90) and 55 kDa form (RanBPM_55) of human RanBPM protein, and RanBP10 with *Arabidopsis* RanBPM using ClustalX2 software [58]. Sequence data of this alignment can be found at accession numbers [Swiss-Prot:Q6VN20] for RanBP10, [Swiss-Prot:Q96S59] for 90 kDa RanBPM and [EMBL:BAA23216] for 55 kDa RanBPM. Sequence of SPRY domain was encountered from the 99 amino acid in AtRanBPM sequence. E- Levels of identities and

similarities in amino acid composition of conserved domains between AtRanBPM and its human homologues RanBPM and RanBP10.



Additional file 3: Phylogenetic analysis of AtRanBPM and its homologues from other eukaryotic species. The tree was constructed by the neighbor-joining method with the MEGA 5.05 program. Branch numbers represent the percentage of bootstrap values in 1000 sampling replicates. The protein accession numbers are [Swiss-prot:F4HYD7] for AtRanBPM At1g35470, [Swiss-prot: Q9SMS1] At4g09340 (segmental genome duplication of chromosome 1), [Swiss-Prot:B9S762] for *R. communis*, [Swiss-Prot:F6HWC3] for *V. vinifera*, [Swiss-Prot:B9MWC1] for *P. trichocarpa*, [Swiss-Prot:C5XUT1] for *S. bicolor*, [Swiss-Prot:Q6ZI83] for *O. sativa*, [Swiss-Prot:B6UAR9] for *Z. mays*, [Swiss-prot: Q6VN20] for human RanBP10, [Swiss-prot: A3KMV8] for RanBP10 from *Bos taurus*, [Swiss-prot: B5LX41] for RanBP10 from *Felis catus*, [Swiss-prot: Q6VN19] for RanBP10 from *Mus musculus*, [Swiss-prot: Q1LUS8] for RanBP10 from *Danio rerio*, [Swiss-prot: Q9PTY5] for RanBP9 from *Xenopus laevis*, [Swiss-prot: Q96S59] for human RanBP9, [Swiss-prot: P69566] for RanBP9 from *Mus musculus*, [Swiss-prot: Q4Z8K6] for RanBP9/10 from *Drosophilla melanogaster* and [Swiss-prot: P53076] for Gid1/Vid30 homologue from *Sacharomyces cerevisiae*. Distance bars are given bottom left and bootstrap values are indicated at the nodes.



C

	Protein name	DTB No.	MW [kDa]	No. peptides	Sequence coverage [%]	MSMS confirmation
1	Protein S100-A8	S10A8_HUMAN	11	2	11	LLETECPQYIR
1	Actin, cytoplasmic 1	ACTB_HUMAN	42	4	17	SYELPDGQVITIGNER
1	Keratin, type II cytoskeletal 1	K2C1_HUMAN	66	4	12	No
1	Keratin, type I cytoskeletal 14	K1C14_HUMAN	51	1	1	IRDWYQR

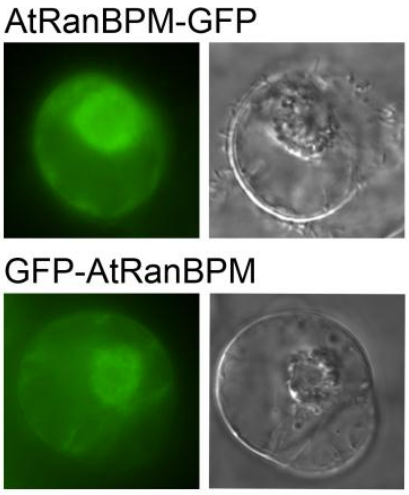
Additional file 4: Immunopurification of GFP-AtRanBPM protein.
Immunopurification of GFP-AtRanBPM from extracts of GFP-AtRanBPM expressing cell cultures (IP GFP-RanBPM). GFP immunopurification from extracts of wild type Ler *Arabidopsis* cells (IP WT) was used as a negative control. A- Proteins were silver stained after separation on SDS-PAGE. Bands corresponding to MW similar of the proteins copurified with GFP-AtRanBPM (IP GFP-RanBPM) were not present in the negative control (IP WT). B- Signal for AtRanBPM was absent in the negative control (IP WT) after detection with anti-AtRanBPM antibody on Western blots. C- Proteins identified by MALDI-MS in negative control (IP WT in A) were background contamination.

Arabidopsis - human	**At1g35470 - RanBPM**	**At3g55070 - MAEA**	**At4g37880 - RMD5**	**At1g61150 - Twa1**	**At5g08560 - WDR26**	**At5g43920 - WDR26**
Identities [%]	31	36	37	42	39	40
Similarities [%]	50	58	59	63	59	58

Additional file 5: Identities and similarities between proteins copurifying with AtRanBPM and human CTLH complex members. Identities and similarities between *Arabidopsis* and human proteins were analyzed in WU-BLAST.

Protein name	AGI number	MW [kDa]	No. peptides	Sequence coverage [%]	Mascot score
LisH/CRA domains containing protein	At1g06060	25	1	4	32
Yippee family protein	At5g53940	15	1	9	71
Yippee-like protein	At3g08890	12	1	17	56
Yippee family protein	At2g40110	15	1	9	47
Armadillo/beta-catenin-like repeat-containing protein	At3g08947	97	4	5	120

Additional file 6: **Additional proteins copurified with AtRanBPM.** The proteins were identified by LC-MALDI-MS/MS and the identity of the matched peptides was confirmed by high-resolution MALDI-FTMS with mass accuracy below 1 ppm.



Additional file 8: Cellular localization of C-terminal GFP and N-terminal GFP AtRanBPM fusion proteins.

Cells of *Arabidopsis* expressing C-terminal GFP AtRanBPM (AtRanBPM-GFP) showed weak cytoplasmic and nuclear signal and accumulation of perinuclear GFP signal similarly as observed for N-terminal GFP AtRanBPM (GFP-AtRanBPM).

APPENDIX II

Overexpressed TPX2 causes ectopic formation of microtubular arrays in the nuclei of acentrosomal plant cells

Petrovská B, Jeřábková H, Kohoutová L, Cenklová V, Pochylová Ž, Gelová Z, Kočárová G, Váchová L, Kurejová M, Tomaštíková E, Binarová P

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RESEARCH PAPER

Overexpressed TPX2 causes ectopic formation of microtubular arrays in the nuclei of acentrosomal plant cells

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Abstract

TPX2 performs multiple roles in microtubule organization. Previously, it was shown that plant AtTPX2 binds AtAurora1 kinase and colocalizes with microtubules in a cell cycle-specific manner. To elucidate the function of TPX2 further, this work analysed *Arabidopsis* cells overexpressing AtTPX2-GFP. Distinct arrays of bundled microtubules, decorated with AtTPX2-GFP, were formed in the vicinity of the nuclear envelope and in the nuclei of overexpressing cells. The microtubular arrays showed reduced sensitivity to anti-microtubular drugs. TPX2-mediated formation of nuclear/perinuclear microtubular arrays was not specific for the transition to mitosis and occurred independently of Aurora kinase. The fibres were not observed in cells with detectable programmed cell death and, in this respect, they differed from TPX2-dependent microtubular assemblies functioning in mammalian apoptosis. Colocalization and co-purification data confirmed the interaction of importin with AtTPX2-GFP. In cells with nuclear foci of overexpressed AtTPX2-GFP, strong nuclear signals for Ran and importin diminished when microtubular arrays were assembled. This observation suggests that TPX2-mediated microtubule formation might be triggered by a Ran cycle. Collectively, the data suggest that in the acentrosomal plant cell, in conjunction with importin, overexpressed AtTPX2 reinforces microtubule formation in the vicinity of chromatin and the nuclear envelope.

Key words: *Arabidopsis thaliana*, AtTPX2, Aurora kinase, fibres, γ-tubulin, importin, microtubules, nuclei, Ran.

Introduction

The targeting protein for Xklp2 (TPX2), is a microtubule-associated protein with multiple functions. Originally, it was identified as a protein required for targeting kinesin-12 (Xklp2) to the spindle pole in *Xenopus* egg extracts (Wittmann *et al.*, 1998). TPX2 and NuMA have been identified as potential downstream effectors of RanGTP in microtubule assembly in *Xenopus* egg extracts and both proteins are the targets of importin blocking activity; they are found in complexes with importin α and β (Gruss *et al.*, 2001; Nachury *et al.*, 2001; Wiese *et al.*, 2001). TPX2 and NuMA proteins colocalize to the interphase nucleus, probably with Ran and importin α and β. This nuclear localization prevents them from acting on microtubules in the cytoplasm until the nuclear envelope breaks down at the beginning of mitosis or meiosis (Kahana and Cleveland, 2001). In the initial phase of mitosis, RanGTP releases TPX2 from its interphase binding partner, importin β, and thus activates TPX2 for bipolar spindle assembly.

TPX2 is also a well-characterized upstream regulator of Aurora A kinase (Kufer *et al.*, 2002; Eyers *et al.*, 2003; Marumoto *et al.*, 2005) and TPX2-activated Aurora A kinase was shown to be essential for Ran-stimulated spindle assembly in the presence/absence of centrosomes (Tsai and Zheng, 2005). TPX2 is an important protein in centrosomal and acentrosomal microtubule nucleation in chromatin and its role with γ-tubulin in chromatin-driven mitotic spindle nucleation in animal cells is well characterized (Wilde and Zheng, 1999; Groen *et al.*, 2004). Recently, TPX2 was identified as a new scaffolding protein and a co-activator of Aurora B in the chromosomal passenger complex (Iyer and Tsai, 2012). Proteomic analysis of human metaphase chromosomes has shown that TPX2 is a nuclear protein belonging to the group of chromosomal fibrous proteins (Uchiyama *et al.*, 2005). However, of the 18 different proteins in this group (e.g. β-actin, vimentin, tubulin), the contribution of TPX2 is unknown (Pederson and Aebi, 2002).

Plant TPX2 contains all of the functional domains of its vertebrate counterpart, but the TPX2 signature motif is present only once in vertebrate sequences compared to twice in plants (Vos *et al.*, 2008; Evrard *et al.*, 2009) where its coiled-coil signature is poorly understood. In *Arabidopsis*, two copies of the TPX2 gene are expressed per genome (Vos *et al.*, 2008), where it is predominantly nuclear during interphase but is actively exported before nuclear envelope breakdown. AtTPX2 is essential for nuclear envelope breakdown and initiation of prospindle assembly (Vos *et al.*, 2008; Evrard *et al.*, 2009). Plant microtubule-associated proteins sharing the same microtubule binding domain as TPX2 play important roles in the organization of microtubular arrays, cell growth, and regulation of cell division (for reviews see Hamada, 2007; Sedbrook and Kaloriti, 2008). Recently, Panteris and Adamakis (2012) speculated about the possible role of fern TPX2 in cortical microtubule assembly.

In 2012, this study group reported that AtAurora1 kinase and AtTPX2 colocalize in plant microtubules in a cell cycle-specific manner, from preprophase to early telophase (Petrovská *et al.*, 2012). In addition, *Arabidopsis* TPX2 protein is intranuclear, and although important mitotic functions for the protein have already been well documented (Vos *et al.*, 2008; Petrovská *et al.*, 2012), any functional role for its accumulation in interphase nuclei is far from being understood.

This study presents data on specific arrays of microtubules decorated with AtTPX2 formed in nuclei and in the vicinity of the nuclear envelope of cells overexpressing AtTPX2-GFP. The formation of nuclear and perinuclear microtubules occurred without participation of Aurora kinase 1 and mitotic signalling. Microtubular arrays heavily decorated with AtTPX2 were not specific to programmed cell death as was described in mammalian cells (Moss *et al.*, 2009). These data on the functions of AtTPX2 in the formation of specific nuclear/perinuclear microtubular arrays and the interaction of importin with AtTPX2 bring further insight to the poorly understood molecular mechanisms of acentrosomal plant microtubule organization.

Materials and methods

Molecular cloning of AtTPX2 *and* AtAurora1

Molecular cloning of *AtTPX2* (At1g03780) and *AtAurora1* (At4g32830) for N- and C-terminal fusions was performed according to Petrovská *et al.* (2012). Gateway binary vectors pK7WGF2,0 for N-terminal GFP fusion, pH7WGR2,0 for N-terminal RFP fusion (Karimi *et al.*, 2002), pMDC43 for C-terminal GFP fusion (Curtis and Grossniklaus, 2003), and pB7RWG2,0 for C-terminal RFP fusion (Karimi *et al.*, 2002) for *AtTPX2* cloning were used, and pGEM T-Easy P2R-P3 (Invitrogen), pGEM T-Easy P4-P1R (Invitrogen), pGEM T-Easy 221 (Invitrogen), and pK7m34GW (purchased from Ghent University, Ghent, Belgium) for *AtAurora1* cloning were used.

Stable transformation of cell suspension cultures and plants

Suspension cultures of *Arabidopsis thaliana* cv. Columbia and cv. Lansberg erecta (Ler) with stable expression of AtTPX2-GFP or/and AtAurora1-RFP were derived as described in Petrovská *et al.* (2012), using the techniques of Mathur *et al.* (1998) and Koroleva *et al.* (2005). *Arabidopsis* Columbia plants were transformed with AtTPX2-GFP using the floral-dip method (Clough and Bent, 1998) as described in Petrovská *et al.* (2012).

Quantitative real-time PCR analysis

Quantitative real-time PCR (qPCR) was performed following MIQE recommendations (Bustin *et al.*, 2009). Total RNA was isolated from *A. thaliana* control and AtTPX2-overexpressed suspension cultures using the Plant RNeasy Extraction Kit (Qiagen). Digestion of DNA during RNA purification was performed using the RNase-Free DNase Set (Qiagen). Purified RNA (100 ng) was reverse transcribed using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) with an anchored-oligo $(dT)_{18}$ primer according to the Roche instructions. *QPDR* using SYBR Green I Dye (Top-Bio, Czech Republic) was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad). Three replicate PCR amplifications were performed for each sample. The PDF2 gene (Czechowski *et al.*, 2005) was used as a reference. Quantification of transcripts of each gene, normalized to the internal reference PDF2 gene (At1g13320), was determined using CFX Manager Software (Bio-Rad). The transcript level of each target gene of control cells or the reference gene in controls or overexpressed AtTPX2 cells, was designated as 1.0. The primers used for real-time PCR were: PDF2For 5′-TAACGTGGCCAAAATGATGC-3′, PDF2Rev 5′-GTTCTCCACAACCGCTTGGT-3′, *AtTPX2*For 5′-AAGCTCGACCTGTGAACAAGA-3′, and *AtTPX2*Rev 5′-CTGGCAGATGTGGTGTACTTCT-3′. To ensure specificity of primers, primer pairs were designed to span across two neighbouring exons and were detected as a single peak in dissociation curve analysis.

Drug treatment

Amiprophos methyl (APM; Duchefa) at a concentration of 5 μM was used for microtubule depolymerization as described Weingartner *et al.* (2001); taxol (Sigma-Aldrich) was used at a concentration 5 μM. Inhibition of Cdk and Aurora kinase activity was done adding 100 μM roscovitine (a gift from Miroslav Strnad, Olomouc, Czech Republic) as described by Planchais *et al.* (1997) and Binarová *et al.* (1998) and 2 μM Aurora kinase inhibitor ZM447439 (Tocris Bioscience) as described by Ditchfield *et al.* (2003).

Co-immunoprecipitation

Co-immunoprecipitations were performed using GFP-Trap A and RFP-Trap A (ChromoTek, Planegg-Martinsried, Germany) according to the manufacturer's instructions using the modified protocol

described in Petrovská *et al*. (2012). The extract from the *A. thaliana* cell culture expressing AtTPX2-GFP or co-expressing AtAurora1-RFP and AtTPX2-GFP (protein concentration 3–4 mg ml^{-1}) after centrifugation at 10,000 *g* for 10 min were used directly or solubilized by 1% NP-40 for 1 h at 4 °C. The extracts were supplemented with double concentration of inhibitors of proteases, with inhibitors of phosphatases and 50 µM MG132 (Sigma) and incubated with GFP-Trap or RFP-Trap beads for 1.5 h at 4 °C. As a negative control, GFP immunoprecipitate from wild-type Ler *Arabidopsis* culture was used. The immunoprecipitated proteins were released by elution with glycine (pH 2.5). Proteins in the eluates were resolved by SDS-PAGE and analysed for importin, γ-tubulin, and AtAurora1 by immunoblotting with rabbit polyclonal anti-importin antibody 1:3000 (Secant Chemicals), affinity-purified rabbit polyclonal antibody AthTU 1:2,500 (Dryková *et al.*, 2003), anti-actin 1:1000 (Affinity BioReagents), anti-GFP and anti-RFP 1:2000 (Abcam and ChromoTek) antibodies, and anti-Ran antibody 1:200 (Transduction Laboratories). Secondary antibodies anti-rabbit and anti-mouse IgG HRP Conjugates (Promega or Amersham-GE Healthcare) were used; Super Signal West Pico Chemiluminiscent Substrate (Thermo Scientific) was used according to the manufacturer's instructions.

Immunofluorescence

Arabidopsis thaliana suspension cultures were fixed for 1 h using 3.7% paraformaldehyde and processed for immunofluorescence as described in Binarová *et al*. (1993). Primary antibodies, anti-α-tubulin monoclonal antibody DM1A (Sigma) at a dilution of 1:500, monoclonal anti-γ-tubulin TU-32 (kindly provided by Pavel Dráber from IMG, Prague, Czech Republic) diluted 1:10, affinity purified rabbit polyclonal antibody AthTU 1:1000 (Dryková *et al.*, 2003), anti-GFP antibody (Abcam) at a dilution 1:1000, anti-actin (Affinity BioReagents) at a dilution 1:1000, anti-phospho-histone H3 (Ser10) antibody (Cell Signaling Technology) at a dilution 1:2000, monoclonal mouse anti-importin antibody (Secant Chemicals, Winchendon, MA) at a a dilution 1:2000, rabbit polyclonal anti-importin antibody (Secant Chemicals) at a dilution 1:3000, and anti-Ran antibody (Transduction Laboratories) at a dilution 1:200 were used with anti-mouse and anti-rabbit conjugated antibodies to FITC, DyLight 488, Cy3, DyLight 550, or Alexa Fluor 647 (Jackson ImmunoResearch Laboratories). DNA was stained with DAPI.

In situ *detection of fragmented DNA*

The *In Situ* Cell Death Detection Kit (Roche) was used for the TUNEL (TdT-mediated dUTP nick-end labelling) test according to the manufacturer's instructions. Besides the TUNEL test, the viability assay (on the basis of its penetration into non-viable cells) was determined by 10 min incubation of cell suspension with 0.1% of Evans blue dye.

Microscopy

Microscopy was performed using an IX81 motorized inverted research microscope CellR (Olympus) equipped with disk scanning unit and digital monochrome CCD camera CCD-ORCA/ER, and using an Olympus IX-81 FV-1000 confocal microscope. To avoid filter crosstalk, fluorescence was detected using HQ 480/40 exciter and HQ 510/560 emitter filter cubes for FITC and HQ 545/30 exciter and HQ 610/75 emitter filter cubes for Cy3 (both AHF Analysen Technique). Images were processed and analysed using CellR Software and Quick Photo Camera Software version 2.3 (Olympus). Images from confocal laser scanning microscopy were taken with PLAPO objective 100×/1.45 using the sequential multitrack mode to avoid bleed-through; excitation and emission wavelengths were 405 and 425–460 nm for DAPI, 473 and 485–545 nm for FITC or DyLight 488, 559 and 575–620 nm for Cy3 or DyLight 550, and 635 and 655–755 nm for Alexa Fluor 647. Green fluorescent protein was excited by 473 nm and emission was detected from 485 to 545 nm. Whenever needed, z-stacks were taken with 0.2 µm z-step. Images were analysed using FV10-ASW (Olympus); 3-D reconstruction and animation from z-stacks, and sectioning of gained 3D objects was performed using Imaris software (Bitplane) in the section and animation mode.

Figures were prepared using Adobe Photoshop 7.0. The quantitative colocalization analyses were performed using ImageJ software with JACoP (Just Another Co-localization Plug-in) plugin (Bolte and Cordeliéres, 2006) based on Pearson's coefficient, overlap coefficient, and Manders' coefficient (colocalization coefficient for channel M1, M2). Costes' approach was expressed with a plot of the distribution of the Pearson's coefficient of randomized images (curve) and of the green channel image (red line) and showed a probability of colocalization. Another development based on Pearson's coefficient used for confirmation of a degree of colocalization was Van Steensel's approach. Li's approach were presented as a set of two graphs, each showing the normalized intensities (from 0 to 1) as a function of the product $(A_i - a)(B_i - b)$ for each channel. Observed positive product $(A_i - a)(B_i - b)$ and dot cloud concentrated on the right side of the $x = 0$ line (although adopting a C-shape) indicated high colocalization.

Results

In silico *analyses suggested nuclear localization as well as nuclear function of AtTPX2.*

The AtTPX2 protein is composed of two domains: TPX2_importin (pfam: PF12214) and TPX2 domain (pfam: PF06886) (Fig. 1A) (Punta *et al.*, 2012). *Arabidopsis* TPX2 has two nuclear localization signals andm unlike other TPX2s, AtTPX2 has a signal for nuclear export (Vos *et al.*, 2008). AtTPX2 also possesses two domains that can mediate its localization to microtubules (Vos *et al.*, 2008; Petrovská *et al.*, 2012). The AtTPX2 protein also contains a short region (amino acids 588–619) that shows significant probability for coiled coil formation, as confirmed using several algorithms: Coils (Lupas *et al.*, 1991), Paircoil (Berger *et al.*, 1995), MultiCoil (Wolf *et al.*, 1997), and ELM (Dinkel *et al.*, 2012). The coiled coil region was found in plant proteins that contain TPX2_importin and TPX2 motifs across various plant species (Fig. 1B); most of them belonged to as-yet uncharacterized or hypothetical plant proteins.

Deeper analysis of *Arabidopsis* TPX2 amino acid sequence (Dinkel *et al.*, 2012) revealed the following: the presence of an HP1 ligand (interacts with chromoshadow domain of heterochromatin-binding protein 1, amino acids 95–99), KEN box (148–152, 275–279, and 667–671), D box (327–645), three cyclin recognition sites (305–309, 332–336, 516–519), several FHA phosphopeptide ligands (predominant in nuclear proteins that are involved in cell cycle checkpoint, DNA repair and transcriptional regulation), mitotic spindle checkpoint protein MAD2 binding motif (361–369), mitogen-activated protein kinase, docking motifs (41–47, 492–502, 538–549, 723–731), sumoylation sites (68–71, 208–211, 529–532, 575–578), and several phosphorylation sites (i.e. PIKK, glycogen synthase kinase 3, PKA; Supplementary Table S1, available at *JXB* online). Localization of proteins with these motifs or interaction sites is typically nuclear. Plant TPX2 is localized in nuclei (Vos *et al.*, 2008; Petrovská *et al.*, 2012). In addition, using Nuc-PLoc (Hong-Bin and Kuo-Chen, 2007) and

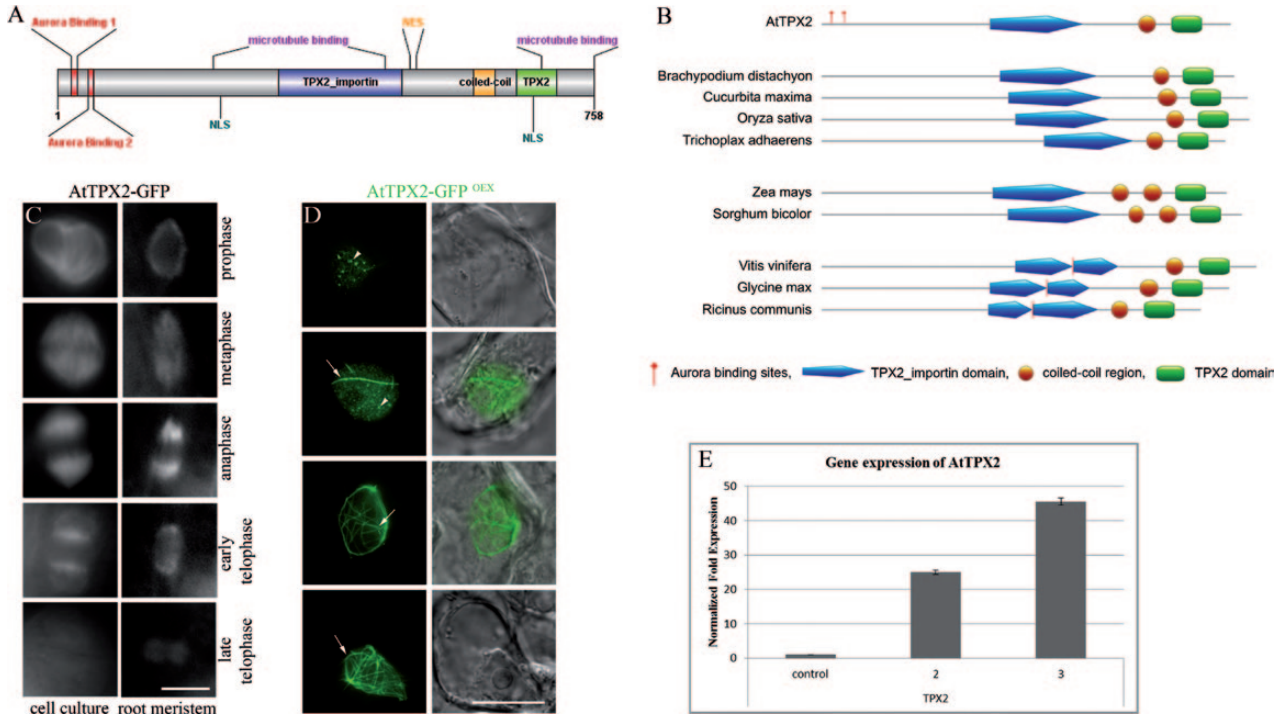

Fig. 1. Localization of AtTPX2 protein in *Arabidopsis* cells overexpressing AtTPX2. (A) Domains, putative interaction sites, and binding motifs in the amino acid sequence of *Arabidopsis* TPX2 protein. For putative interaction sites, binding motifs as well as domain graph, ELM (Dinkel *et al.*, 2012), and SUMOsp 2.0 (Ren *et al.*, 2009) databases were used. Schematic drawing was prepared in DOG (Domain Graph; Xao and Xue, 2009). (B) Domain organization of *Arabidopsis* TPX2 protein and its plant homologues: AtTPX2–targeting protein for Xklp2–TPX2 (*Arabidopsis thaliana*, AGI no. At1g03780), *Brachypodium distachyon* uncharacterized protein LOC100842911 (acc. no. XP_003560168), *Cucurbita maxima* hypothetical protein (AEK84224), *Oryza sativa* hypothetical protein OsJ_24381 *Oryza sativa Japonica Group* (EEE67237), *Trichoplax adhaerens* hypothetical protein TRIADDRAFT_55817 (XP_002112111), *Zea mays* uncharacterized protein LOC100383389 (NP_001169515), *Sorghum bicolour* hypothetical protein SORBIDRAFT_02g034250 (XP_002462908), *Vitis vinifera* uncharacterized protein LOC100262517 (XP_002274918), *Glycine max* uncharacterized protein LOC100801192 (XP_003526269), *Ricinus communis* protein with unknown function (XP_002517880). Schematic drawing of proteins was prepared in MyDomains (Prosite, http://prosite.expasy.org/cgi-bin/prosite/mydomains/). (C) Localization of AtTPX2-GFP in dividing cell in cell culture with stable expression and in root meristematic zone of *A. thaliana*. AtTPX2-GFP was localized with mitotic microtubular arrays from prophase until early telophase. Bar, 10 μm. (D) Localization of AtTPX2-GFP in *Arabidopsis* cell suspension culture overexpressing AtTPX2-GFP. AtTPX2-GFP was predominantly localized with 'dots' or 'seeds' (arrowheads), later elongated into bundled fibres around (cage-like structures) and inside the nuclei (arrows). Frequency of overexpressing cells ranged between 10–40% depending on the transformation event, bar, 10 μm. (E) Relative expression of AtTPX2 in two representative samples of dividing *Arabidopsis* suspension cultures overexpressing AtTPX2 (samples 2, 3) showed a significant increase (25-fold, 45-fold, respectively) in transcript level compared to the control cells.

Subnuclear Compartments Prediction System 2.0 (Lei and Dai, 2005, 2006), the subnuclear localization of *Arabidopsis* TPX2 protein was predicted to be nuclear speckle and nuclear lamina, respectively.

Fibres heavily decorated with TPX2 are formed in the vicinity of the nuclear envelope and in the nuclei of cells overexpressing AtTPX2

Cultured cells and seedlings of *A. thaliana* were transformed with plasmids containing full-length C- and N- terminal AtTPX2 protein fusions with GFP, under the control of the cauliflower mosaic virus (CaMV) 35S constitutive promoter. After selection, stable cell lines were derived (Petrovská *et al.*, 2012). Both C- and N- terminal AtTPX2-GFP fusion proteins showed similar localizations. The AtTPX2-GFP was observed in nuclei, the perinuclear region and, in a cell cycle-specific manner, with mitotic microtubular arrays. The localization of AtTPX2-GFP fusion protein was similar for dividing cultured cells and cells of *Arabidopsis* seedlings (Fig. 1C). The AtTPX2-GFP signal was present with perinuclear microtubules in preprophase, with kinetochore microtubular fibres in metaphase and anaphase, and with early phragmoplast in cytokinesis. It was previously found that the cell cycle-specific microtubular localization of AtTPX2-GFP requires Aurora binding (Petrovská *et al.*, 2012). TPX2 knockout in *Arabidopsis* is lethal and only heterozygous plants could be obtained for the T-DNA inserts (Vos *et al.*, 2008; data not shown). Therefore, the current study was not able to test functionality of the AtTPX2-GFP protein under

the most stringent conditions by complementation of null mutants. However, the localization data and interaction of AtTPX2-GFP with Aurora kinase (Petrovská *et al.*, 2012) suggest that the AtTPX2-GFP fusion protein is functional.

As early as 48 h after transformation, expressed fusion protein showed diffused distribution in cytoplasm with accumulation in nuclei; a later AtTPX2 signal was predominantly associated with dots and patches in nuclei and as fibrillar structures located inside the nuclei and in the perinuclear area (Fig. 1D). The effects of overproduction of AtTPX2 N- and C-terminal GFP fusion proteins were similar, suggesting that the GFP moiety did not interfere with the function of AtTPX2 in fibre formation. Overexpression of AtTPX2-GFP was confirmed by real-time qPCR (Fig. 1E). Microscopic analysis of AtTPX2-overexpressing cells showed that the dots of AtTPX2-GFP in interphase nuclei (Fig. 1D, arrowheads) were formed within a period of 3 d from transformation. AtTPX2-GFP dots and patches were gradually built into thick fibrillar structures (Fig. 1D, arrows). The AtTPX2-GFP signal was attached to filamentous structures reminiscent of microtubules that were arranged into cage-like structures surrounding the nuclei (Fig. 1D, arrows).

To prove whether AtTPX2-decorated fibres in AtTPX2-overexpressing cells represent cytoskeletal polymers, this study performed a series of double immunofluorescence experiments. Fibres were positive for α-tubulin (Fig. 2A, B), but they were not recognized by anti-actin antibody (Supplementary Fig. S1). The signal for α-tubulin localized with the AtTPX2 foci in nuclei (Fig. 2A, arrow), and with forming fibres (Fig. 2A, arrowheads). In AtTPX2-overexpressing cells containing more prominent fibrillar arrays, α-tubulin was associated with thinner AtTPX2-positive fibres along their entire length (Fig. 2B, arrow). Thicker bundles, heavily labelled with AtTPX2-GFP, showed a weaker signal for α-tubulin (Fig. 2A, arrowheads), probably due to the lower accessibility of the epitope to the anti-α-tubulin antibody.

Treatment of cells overexpressing AtTPX2 with the microtubule-depolymerizing drug APM showed that the nuclear and perinuclear microtubular bundles were resistant to drug-induced depolymerization. As shown in Fig. 2C, the microtubular arrays persisted in 98% of 5 µM APM- treated cells (n = 112), showing overexpression of AtTPX2-GFP. Microtubules were largely depolymerized by the same dose of APM in cells with stable expression of AtTPX2-GFP (Fig. 2C, inset I) and only remnants of kinetochore microtubules decorated with AtTPX2-GFP were observed (Fig. 2C, inset II). Taxol treatment did not result in further bundling or stabilization of ectopic microtubules in AtTPX2-GFP-overexpressing cells (Supplementary Fig. S2).

Serial sections of nuclei and 3-D reconstructions showed a network of microtubules decorated with AtTPX2 inside the nuclei and in the area adjacent to the nuclei (Fig. 3A). Thick intertwined bundles of microtubules were present around the nuclei (Fig. 3A, arrow) and anchored to the nucleus at the cell periphery (Fig. 3A, arrowheads). More detailed analyses using Imaris sectioning of nuclei of AtTPX2-GFP-overexpressing cells confirmed the formation of fibrillar structures inside the nuclei, in the vicinity of chromatin (Fig. 3B, cross I) and also inside the nucleoli (Fig. 3B, cross II). To prove nuclear location of ectopic arrays, cells overexpressing AtTPX2-GFP *in vivo* were analysed. As shown in Fig. 3C, fibres decorated with AtTPX2-GFP were observed, together with intranuclear foci of overexpressed TPX2.

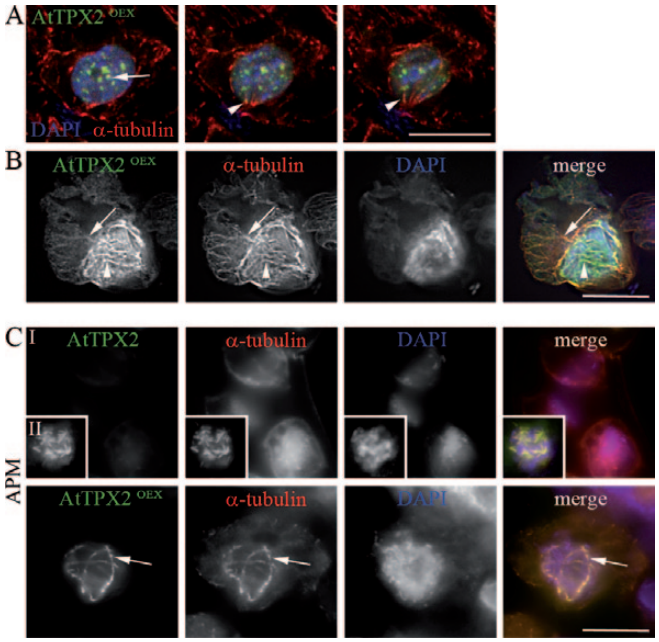

Fig. 2. AtTPX2 localization with ectopic perinuclear and nuclear microtubules in *Arabidopsis* cells overexpressing AtTPX2-GFP. (A) AtTPX2-GFP foci localized with α-tubulin in the nucleus (arrow) and AtTPX2-GFP partially loaded on α-tubulin fibres (arrowheads) in cells with foci of overexpressed AtTPX2-GFP. Serial sections (0.4 µm z-steps) of the cell immunostained by anti-GFP antibody (green) and anti-α-tubulin antibody (red) with chromatin stained by DAPI (blue) are shown. (B) α-Tubulin signal with thinner AtTPX2-GFP-decorated fibres (arrow) was stronger compared to the signal with thick bundles (arrowhead); 99% of analysed cells showed the corresponding pattern (194 analysed cells). (C) While microtubules were depolymerized, with exception of kinetochore stubs, in cells expressing AtTPX2-GPF after the treatment with 5 µM APM for 2.5 h, microtubular arrays in cell overexpressing AtTPX2-GFP ($AtTPX2^{OEX}$) were stable after the same treatment. Bars, 10 µm.

These data showed that overexpressed AtTPX2-GFP protein was initially present in nuclear foci and patches, and later, fibrillar microtubular arrays were formed in nuclei and the perinuclear area. The ectopic microtubular fibres, heavily decorated with AtTPX2-GFP, were resistant to microtubular drugs.

Interaction of importin with AtTPX2-GFP suggests involvement of the Ran cycle in TPX2-mediated formation of microtubular arrays.

Importin binds TPX2 protein and imports it into the nucleus, and RanGTPase sequesters the TPX2 nuclear pool before breakdown of the nuclear envelope (Gruss *et al.*, 2001). Binding of animal importin to recombinantly expressed plant TPX2 protein in a RanGTPase-dependent manner was

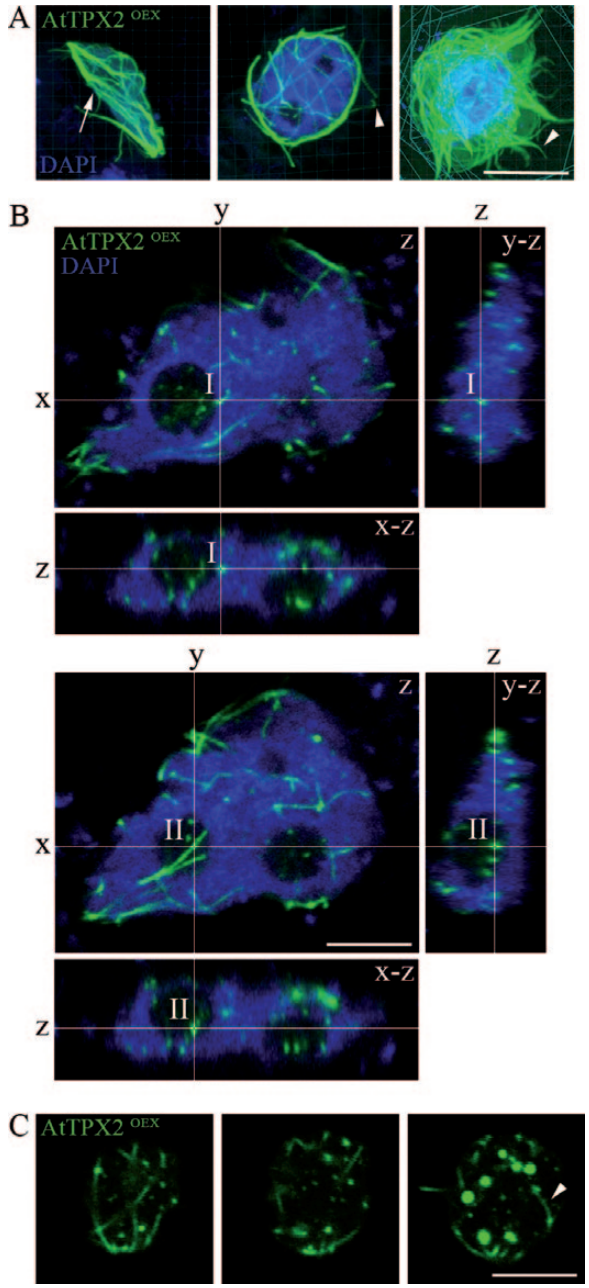

Fig. 3. Three-dimensional reconstruction and analysis of microtubular arrays in *Arabidopsis* cells overexpressing AtTPX2-GFP. (A) Representative images from 3D reconstruction of cells with AtTPX2-decorated fibres around and inside nuclei; Microtubular fibres in the perinuclear area are twisted (arrow), the microtubular fibres anchored nuclei to cell periphery (arrowheads); bar, 10 μm. (B) Two Imaris sections of the nucleus with AtTPX2-decorated fibres; AtTPX2-decorated fibres were present inside the nucleus (cross I) and the nucleolus (cross II) overexpressing AtTPX2; main panel *z* shows a single z-stack of the nucleus; right panel *y-z* shows cross-section by *y* plane perpendicular to *z* plane in the main panel; lower panel *x-z* shows cross-section by *x* plane perpendicular to *z* plane in the main panel; bar, 5 μm. (C) Serial z-stacks of nucleus of cell overexpressing AtTPX2-GFP analysed *in vivo*. Sections from nuclear surface to the centre (left to right) showed perinuclear fibres and intranuclear foci and fibres decorated by AtTPX2 (arrowhead); bar, 5 μm.

shown *in vitro* by Vos *et al.* (2008). To determine whether the RanGTPase pathway is involved in the process of AtTPX2-mediated formation of microtubular arrays, the current work analysed the immunolocalization of importin and Ran in AtTPX2-GFP-overproducing cells (Fig. 4 and Supplementary Fig. S3). The signal for importin was observed in nuclei, associated with the nuclear envelope, and with intranuclear and perinuclear microtubular fibres (Fig. 3). Quantitative co-localization analyses of AtTPX2 and importin were performed using the ImageJ plugin, JACoP (Bolte and Cordeliéres, 2006). The analyses showed high degrees of co-localization of AtTPX2-GFP, with patchy patterns of importin on perinuclear fibres (Fig. 4A, arrowhead), and with microtubular fibres extending from the perinuclear area to the periphery (Fig. 4B, arrow). All coefficients correctly reported a strong overlap between the two channels (Supplementary Fig. S5).

Previous experiments used GFP co-immunoprecipitation to show an interaction of AtAurora1-RFP with AtTPX2-GFP (Petrovská *et al.*, 2012). The current work performed GFP co-immunoprecipitation to provide evidence for an interaction between AtTPX2-GFP and importin. As shown in Fig. 4C, importin was co-purified with AtTPX2-GFP from low-speed supernatants. Negative controls as well as probing of purified TPX2 complexes with the relevant anti-actin antibody confirmed that interaction of importin with AtTPX2 was specific (Fig. 4C, Supplementary Fig. S4). It is known that AtTPX2 protein is highly unstable in plant cell extracts or under conditions of electrophoretic protein separation, and this makes detection by Western blotting difficult (Vos *et al.*, 2008; Petrovská *et al.*, 2012). In agreement with data shown by Vos *et al.* (2008) and Petrovská *et al.* (2012), several bands for the AtTPX2-GFP protein and its degradation products were detected with anti-GFP antibody in a sample of immunopurified AtTPX2-GFP (Fig. 4C).

Next, Ran protein was immunolocalized in AtTPX2-overexpressing cells. The signal for Ran was stronger in nuclei with AtTPX2-GFP nuclear dots and patches, where 83% of nuclei (n = 132) showed higher levels of the Ran signal compared to the untransformed controls (Supplementary Fig. S3). On the other hand, only 23% of nuclei (n = 94) showed any signal above that of control untransformed nuclei in cells with nuclear/perinuclear microtubular arrays (Fig. 4E). This finding suggests that the Ran nuclear signal was weakening during microtubular array assembly. Multiple labelling showed that, similarly to Ran, the importin signal was enriched in nuclei of overexpressing cells where it co-localized with some of the AtTPX2-GFP foci and accumulated around NE (Fig. 4D, arrow and arrowhead). In cells with assembled fibres, diminution of the nuclear signal for importin was even more pronounced compared to that observed for Ran (Fig. 4D, E). A smaller proportion of the importin signal was observed associated with the AtTPX2-GFP-decorated microtubular arrays.

Plant γ-tubulin is associated with microtubular arrays, around the nuclear envelope, and in nuclei (Dryková *et al.*, 2003). Petrovská *et al.* (2012) showed that γ-tubulin is localized with AtTPX2 and AtAurora1 on mitotic microtubular arrays. Therefore, this work analysed the localization of γ-tubulin in cells overexpressing AtTPX2-GFP. Signals for γ-tubulin were

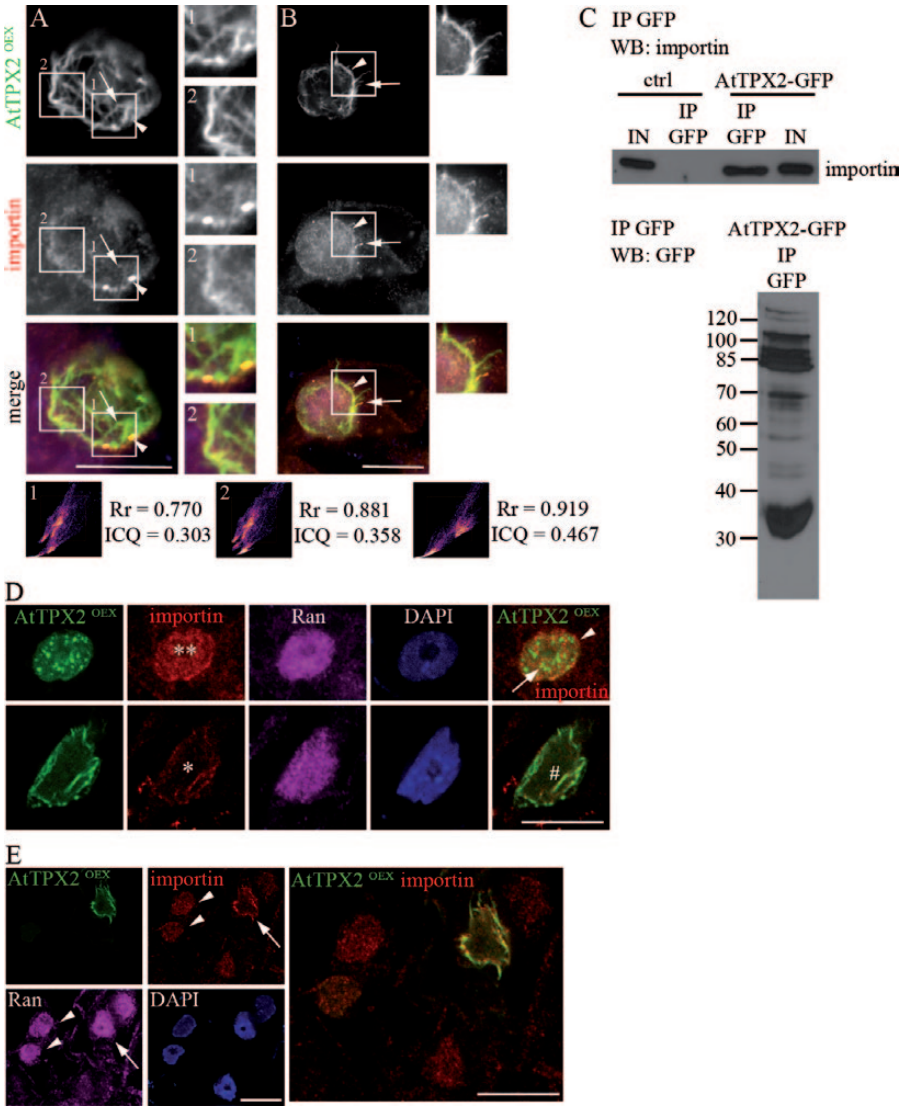

Fig. 4. Immunolocalization of importin and Ran in AtTPX2-GFP-overproducing cells. (A, B) AtTPX2 and importin colocalized in the vicinity of the nuclei (B, arrowhead), with the AtTPX2-decorated microtubular fibres (A, B, arrows), and in intranuclear foci (A, arrowhead). Colocalization was analysed by ImageJ software with JACoP plugin (Bolte and Cordeliéres, 2006). The similar labelling pattern was observed in 97% of analysed overexpressing cells (n = 112). (C) Importin was copurified with AtTPX2-GFP by GFP trap (AtTPX2-GFP IP GFP) from extracts of *Arabidopsis* cells. GFP immunoprecipitate from wild-type Ler *Arabidopsis* culture was used as a negative control (ctrl IP GFP). Immunoblotting of GFP immunoprecipitate from AtTPX2-GFP expressing cell culture with anti-GFP antibody showed several bands corresponding to the full-length molecule of AtTPX2-GFP above 100 kDa and several degradation products. (D, E) Representative images of immunofluorescence labelling of the cells overexpressing AtTPX2-GFP (green), immunostained with anti-importin (red), with anti-Ran (magenta); chromatin stained by DAPI (blue). (D) Importin signal was nuclear (two asterisks), localized around nuclei (arrowhead) and colocalized with some of the AtTPX2 foci (arrow) in overexpressing cells with AtTPX2-GFP foci and patches. Nuclear signal for importin was reduced (asterisk) in cells where AtTPX2-GFP fibres were formed and it localized with the fibres around nuclei and in the nuclei (hash mark). (E) Signal for Ran in the nuclei of cells with AtTPX2 perinuclear fibres was not above the level of signal found in untransformed cells (arrowheads). Importin nuclear signal declined in the cells with AtTPX2-GFP fibres (arrow) while a portion of signal for importin localized with AtTPX2 perinuclear fibres. Bars, 10 μm.

observed in a patchy pattern with microtubular bundles formed in the vicinity of the nuclei and extending to the cell periphery (Fig. 5A). Quantitative co-localization analyses of γ-tubulin and AtTPX2, using the ImageJ plugin JACoP, showed co-localization of γ-tubulin with AtTPX2-GFP-decorated microtubular arrays (Fig. 5B1, 2). However, the association of γ-tubulin with immunopurified AtTPX2-GFP could not be demonstrated.

Together, the immunolocalization and immunopurification data suggest a function for importin with plant AtTPX2. The accumulation of importin and Ran in the nuclei of overexpressing cells indicates a nuclear import of overexpressed AtTPX2-GFP. Diminution of the nuclear signal for importin, as observed in cells with microtubular arrays, indicates that microtubule formation was triggered by sequestered AtTPX2, possibly

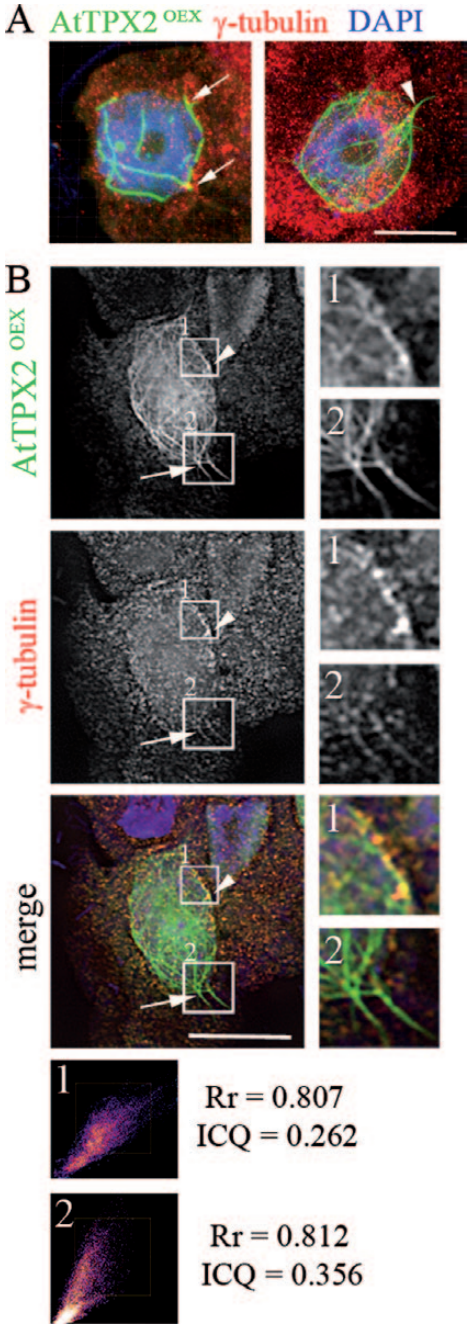

Fig. 5. Colocalization of AtTPX2 and γ-tubulin in *Arabidopsis* AtTPX2-GFP-overproducing cells. (A, B) γ-Tubulin localized together with AtTPX-GFP. (A) 3D analysis of cells overexpressing AtTPX2-GFP stained with anti-γ-tubulin antibody (red), DAPI (blue) obtained by laser scanning microscopy and 3D-reconstructed (Imaris, Bitplane). Still images from 3D reconstruction animation show γ-tubulin localized with AtTPX2-GFP-decorated fibres in vicinity of nuclear envelope (A, arrows), with intranuclear microtubules, and in patchy pattern on microtubular bundles extending from perinuclear area (arrowhead). (B) γ-Tubulin colocalized with AtTPX2 on microtubular fibres extending from perinuclear area to the cytoplasm and membranes (arrow) and on the nuclear envelope (arrowhead). Colocalization was analysed by ImageJ software with JACoP plugin (Bolte and Cordeliéres, 2006). The similar labelling pattern was observed in 93% of analysed overexpressing cells (n = 128). Bars, 10 μm.

Ran cycle-dependent. The presence of an importin signal with AtTPX2 on ectopic microtubular fibres suggests that an excess of the overexpressed AtTPX2 may still be bound to importin.

Formation of AtTPX2-decorated microtubular fibres was neither dependent on association of Aurora kinase with TPX2 nor on mitotic status of the chromatin.

Previous data showed that AtAurora1 binds to AtTPX2 and that binding is required for cell cycle-specific localization of TPX2 on mitotic microtubular arrays (Petrovská *et al.*, 2012). In cells with stable expression of AtTPX2-GFP and AtAurora1-RFP, both proteins colocalized with microtubular arrays from preprophase to early telophase (Fig. 6A) and were co-purified (Supplementary Fig. S6). However, only a weak AtAurora1 signal was found on bundled AtTPX2-decorated microtubules in overexpressing cells (Fig. 6B). To determine whether binding of Aurora kinase was required for TPX2-mediated formation of ectopic microtubular arrays, this work overexpressed a truncated version of AtTPX2 that lacked two conserved Aurora kinase binding sites (ΔN-AtTPX2; Petrovská *et al.*, 2012). Microtubular arrays were formed in the vicinity of and in the nuclei, and were associated with ΔN-AtTPX2 (Supplementary Fig. S7). This work analysed the effect of the Aurora kinase inhibitor, ZM447439 (Ditchfield *et al.*, 2003) that affected microtubular mitotic arrays in wild-type *Arabidopsis* cells (Supplementary Fig. S8). Contrary to the wild-type cells, ZM447439 treatment had no visible effect on the formation, stability, and arrangement of nuclear and perinuclear microtubular bundles in cells overexpressing AtTPX2 (Fig. 6C). These data suggest that binding of Aurora kinase with AtTPX2, which is required for localization of TPX2 on mitotic microtubules, is dispensable for the formation and organization of the ectopic peri/intranuclear microtubules in AtTPX2-overexpressing cells.

To understand whether the formation of ectopic microtubules was cell cycle specific, anti-phosphohistone H3 (Ser10) antibody was used to monitor the mitotic status of the chromatin, from pre-prophase to metaphase (Fig. 7A). Cells (n > 100) with AtTPX2-decorated fibres did not show phosphohistone staining (Fig. 7B). Further proof that fibres were not formed in preparation for mitosis was provided by DAPI staining that showed diffuse interphase chromatin but not pre-mitotic condensed chromatin in cells with the TPX2-decorated microtubular arrays. The formation and arrangement of ectopic microtubular bundles was not affected by treatment with the mitotic kinase inhibitor roscovitine (Supplementary Fig. S9), which previously showed a severe effect on microtubular arrays (Binarová *et al.*, 1998). These findings suggest that ectopic nuclear and perinuclear microtubules were not formed specifically during the transition from interphase to mitosis.

AtTPX2-stabilized microtubules are not found in cells with programmed cell death.

A type of AtTPX2-stabilized microtubular array similar to that observed in these experiments is formed in mammalian

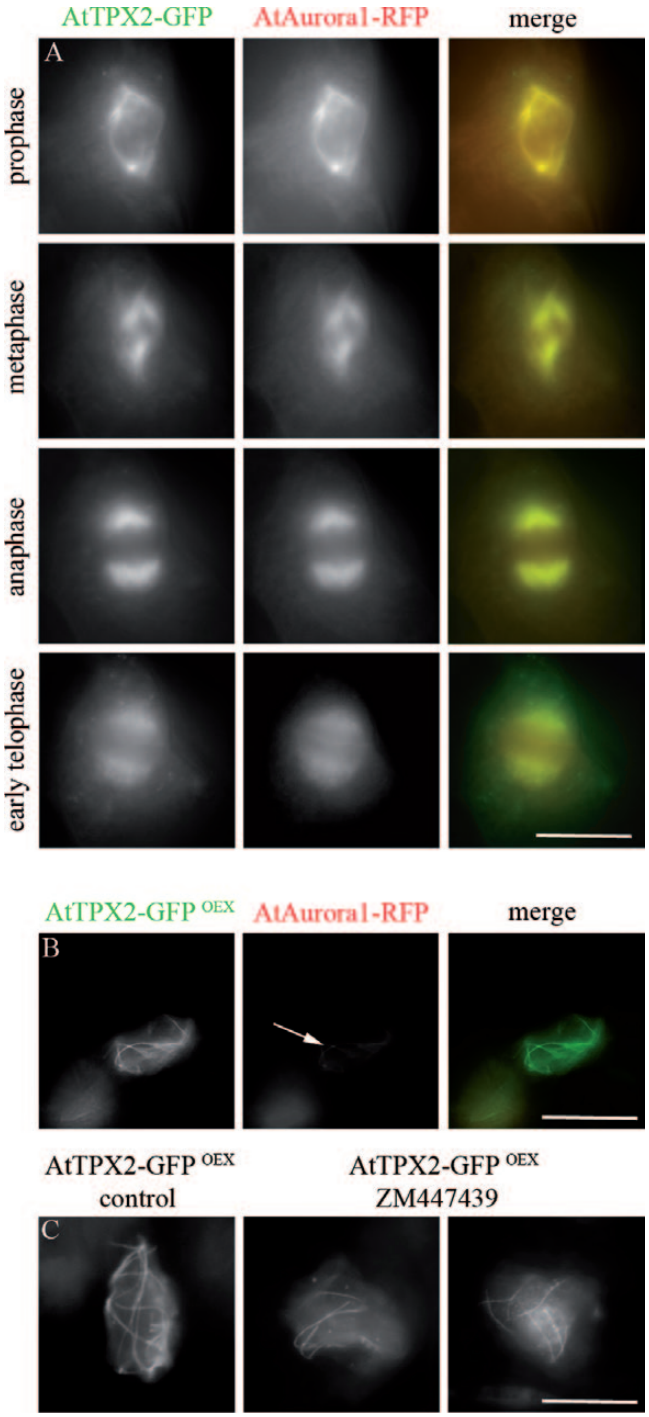

Fig. 6. Localization of AtTPX2 and AtAurora1 in control and ZM447439-treated cells overproducing AtTPX2. (A) Localization of AtTPX2-GFP and AtAurora1-RFP from the prophase until the early telophase in *Arabidopsis* cells. (B) Weak signal of AtAurora1 with AtTPX2 (arrow) in the nuclei of *Arabidopsis*. (C) AtTPX2 fibres in TPX2-overproducing cells were resistant to Aurora kinase inhibitor ZM447439 (100% of analysed cells showed resistance to ZM447439, $n = 96$). Bars, 10 μm.

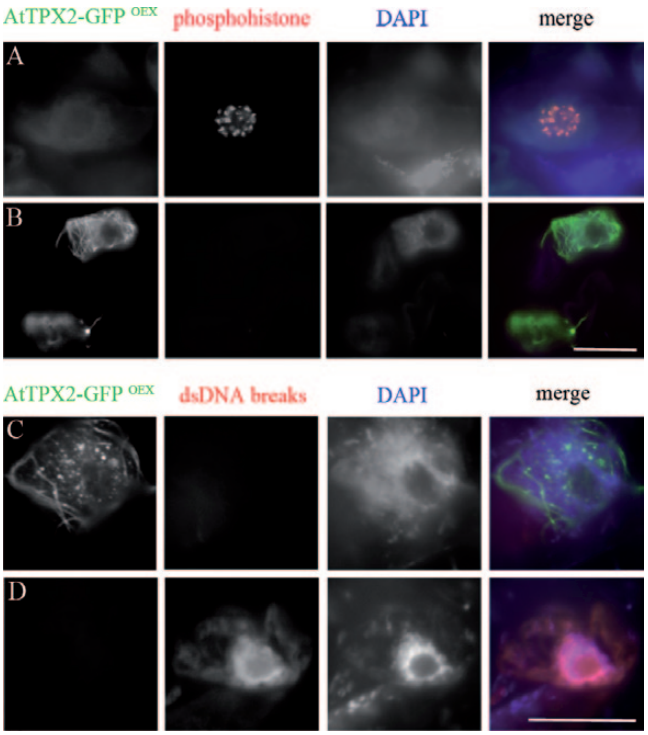

Fig. 7. Formation of AtTPX2 fibrillar structures was not dependent on mitotic status of the chromatin or connected with programmed cell death. (A, B) Immunolabelling of AtTPX2 (green) and phosphohistone (red) in prophase (A) and interphase (B) cells overexpressing AtTPX2. AtTPX2-decorated ectopic microtubules were not formed in nuclei with mitotic chromatin. (C, D) Ectopic AtTPX2-decorated microtubular arrays were not observed in cells entering programmed cell death that were positive with TUNEL labelling (red). Bars, 10 μm.

cells where it functions during apoptosis (Moss *et al.*, 2009). The current examined whether the TPX2-induced microtubules were involved in apoptosis in *Arabidopsis*. *In situ* detection of dsDNA breaks (TUNEL test) was used. The proportion of cells with TUNEL-stained nuclei was 8% in both wild-type ($n = 234$) and AtTPX2-overexpressing cells ($n = 217$). Neither AtTPX2 dots nor ectopic perinuclear or nuclear microtubules were observed in cells with TUNEL-detectable dsDNA breaks (Fig. 7C, D). To provide further evidence of programmed cell death, cell viability was analysed using Evans blue staining, which does not discriminate between apoptosis and necrosis (Danon *et al.*, 2000); however, changes in the plasma membrane recognized by Evans blue might be an early indicator of cells undergoing DNA fragmentation prior to TUNEL-detectable DNA breakage. Overexpressing cells with AtTPX2-GFP-labelled fibres were not stained with Evans blue (Supplementary Fig. S10). The formation of AtTPX2-decorated ectopic microtubules was therefore not associated with programmed cell death.

Discussion

Previous studies showed that AtTPX2 was associated with spindle microtubules and suggested a microtubular function (Vos *et al.* 2008; Petrovská *et al.*, 2012). Analysis of cells overexpressing AtTPX2 protein allowed the identification of stable microtubular arrays ectopically nucleated in the nuclei and

their periphery. Signals for overexpressed AtTPX2-GFP were initially observed in nuclear dots and patches, from which microtubules could grow and organize. Intranuclear and perinuclear AtTPX2-decorated fibrillar arrays were assembled later and formed a cage enveloping the nucleus and extending to the cell periphery.

Ran GTPase influences microtubule dynamics in mitosis by releasing spindle assembly factors from importins in the vicinity of chromatin (Joseph, 2006). There are several lines of evidence suggesting that TPX2-mediated microtubule formation observed under conditions of overproduction of AtTPX-GFP was triggered by a Ran nucleo-cytoplasmic gradient: (i) colocalization and co-immunopurification of importin with AtTPX2; (ii) accumulation of Ran and importin in the nuclei with overexpressed AtTPX2-GFP; and (iii) reduction of nuclear signals for Ran and importin that occurred simultaneously with assembly of nuclear/perinuclear microtubular arrays. Dynamic release of TPX2 from importin by Ran is active in organization of microtubules during mitosis in animals (Gruss *et al.*, 2001; Kufer *et al.*, 2002). The current data are in agreement with those on Ran GTP-dependent interaction of animal importin with recombinantly expressed plant TPX2 *in vitro* and on nucleo-cytoplasmic shuttling of the AtTPX2-GFP (Vos *et al.*, 2008). In addition to prominent nuclear and perinuclear signals for importin, the current work immunolocalized importin with TPX2-decorated ectopic microtubular arrays. While the ability of TPX2 to nucleate microtubules is abolished by binding of importin, the binding does not prevent TPX2 interaction with tubulin or with microtubules (Schatz *et al.*, 2003). The current work suggests that the importin associated with TPX2-decorated microtubules might represent the proportion of overexpressed AtTPX2 protein that was not sequestered from importin.

These data contribute to the understanding of TPX2-mediated microtubule formation in plants and suggest that the process is regulated by the Ran cycle. Most components of the Ran cycle were identified in plants and the regulatory role of Ran in cell division is, at least partially, conserved (Pay *et al.*, 2002; Jeong *et al.*, 2005). Direct visualization of the RanGTP gradient in living cells (Kalab *et al.*, 2006) has not been performed in plants. Ran FRET sensors, together with *in vivo* analysis of importin/TPX2 shuttling, are required to understand the function of the Ran cycle in microtubule formation and cell division in plants.

Microtubular nucleation in acentrosomal plants occurs from dispersed γ-tubulin-positive sites located on the nuclear envelope, in nuclei, and on pre-existing microtubules (Binarová *et al.*, 2000, 2006; Murata *et al.*, 2005; Nakamura *et al.*, 2010). The current data indicate that rearrangement from the 'seeds' through the bundled fibres might be caused by co-assembly of AtTPX2-GFP with endogenous microtubule-nucleating units, comprising γ-tubulin and TPX2 protein. A possible role of AtTPX2 with plant microtubules was indicated by previous data on colocalization of γ-tubulin with active AtAurora1/AtTPX2 on mitotic microtubular arrays (Petrovská *et al.*, 2012). Recently, it has been shown *in vitro* in *Xenopus* extracts that nucleation of branched microtubules from pre-existing microtubules requires γ-tubulin, TPX2, and augmins (Petry *et al.*, 2013). Colocalization of γ-tubulin with overexpressed AtTPX2 observed in the current work, indicating that a similar mechanism functions in TPX2-mediated formation of microtubular arrays. However, no association of γ-tubulin with immunopurified AtTPX2-GFP could be demonstrated. The low stability of plant TPX2 in extracts (Vos *et al.*, 2008; Petrovská *et al.*, 2012) might also influence the efficiency of immunopurification protocols. Furthermore, as was shown by co-immunoprecipitation by Petry *et al.* (2013), γ-tubulin is only a minor interactor with TPX2 protein. Further experiments are needed to demonstrate γ-tubulin interaction with TPX2 and to show how TPX2 cooperates with nucleation machinery in plant cells.

Similarly to animal homologues, plant TPX2 belongs to the group of cell cycle-regulated molecules that accumulate in the nuclei at the G2 phase of the cell cycle and are degraded at anaphase-telophase (Vos *et al.*, 2008). The current work found that AtTPX2-mediated microtubule formation did not require a mitotic status of chromatin, and thus is not reminiscence of Ran GTPase-dependent chromatin-mediated spindle formation in *Xenopus* extracts (Heald *et al.*, 1996). Furthermore, formation of TPX2-mediated fibres was not dependent on the binding of Aurora kinase to AtTPX2 and correspondingly was not sensitive to Aurora kinase inhibition. These data suggest that the tight tuning that depends on the cell cycle and Aurora kinase signalling to TPX2 is missing during formation of the ectopic microtubular arrays and is overdominated by AtTPX2 in microtubule nucleation and stabilization.

The TPX2-mediated microtubule nucleation pathway, regulated by Ran, is responsible for the assembly of specific acentrosomal microtubular arrays in apoptotic HeLa cells (Moss *et al.*, 2009). The TPX2-stabilized microtubular arrays functioning in apoptosis strongly resemble the stable AtTPX2-mediated microtubular arrays that was observed in plants. However, the arrays were not found in cells undergoing programmed cell death (TUNEL-positive cells) or in Evans blue-stained cells. As shown by Moss *et al.* (2009), TPX2-dependent microtubular arrays function in fragmentation of the HeLa cells nuclei during late apoptosis. While the molecular mechanisms of early apoptosis in plant and animal cells are more similar than was previously thought, the execution phase of the process differs. Electron microscopy of root initials in *Arabidopsis* showed that death of the stem cells did not show apoptotic features such as peripheral chromatin condensation and nuclear fragmentation (Fulcher and Sablowski, 2009). The TPX2-mediated formation of microtubular arrays is exploited by animal and plant cells alike, but the function of these acentrosomal microtubular arrays may reflect specific needs of the organism and cell type.

The *in silico* analysis of AtTPX2 showed the presence of a coiled-coil domain, specific motifs, interaction sites, and predicted subnuclear localization. In metazoans, coiled-coil proteins group as various cytoskeletal networks comprising intermediate filament proteins, actin-binding proteins, and microtubule-associated proteins (Burkhard *et al.*, 2001; Korolev *et al.*, 2005). Albeit several proteins closely related to the intermediate filament protein were identified *in silico* (Gardiner *et al.*, 2011), knowledge of plant coiled-coil

proteins is limited. The current work found that overproduced AtTPX2 accumulated in interphase nuclei and observed TPX2-dependent fibrillar arrays interconnecting nuclei with the nuclear periphery. A role for RanGTP in forming a lamin B spindle matrix has been reported (Tsai *et al.*, 2006) and TPX2 was shown to be required for post-mitotic nuclear envelope assembly (O'Brien and Wiese, 2006). The current study can only speculate as to whether AtTPX2 connects as-yet undefined plant cell lamina with the cytoskeleton and plays a role during interphase as a component of a plant alternative to the LINC (linker of nucleoskeleton and cytoskeleton) complex (Crisp *et al.*, 2006; Tzur *et al.*, 2006).

In summary, the overexpression of AtTPX2-GFP resulted in the formation of chromatin- and nuclei-associated microtubular arrays. The assembly of TPX2-decorated fibres was dependent on neither the mitotic status of chromatin nor the binding of Aurora kinase. The arrays were not specific to apoptotic cells. This study suggests that AtTPX2 overexpression amplified an ability of the nuclear envelope and chromatin to promote microtubule nucleation that is typical for acentrosomal plant cells. Furthermore, these findings indicate an involvement of the Ran pathway in modulation of the process.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Table S1. *In silico* analyses of AtTPX2 protein.

Supplementary Fig. S1. Immunolocalization of AtTPX2 and actin in cell cultures of *Arabidopsis thaliana.*

Supplementary Fig. S2. AtTPX2-decorated fibres are resistant to taxol in cell cultures of *Arabidopsis thaliana.*

Supplementary Fig. S3. Immunofluorescence localization of Ran in AtTPX2-GFP-overproducing cells.

Supplementary Fig. S4. Importin copurified with AtTPX2-GFP from *Arabidopsis* cultured cells.

Supplementary Fig. S5. Colocalization analyses of AtTPX2 and importin in *Arabidopsis* cultured cells.

Supplementary Fig. S6. AtAurora1-RFP copurifies with AtTPX2-GFP from *Arabidopsis* cultured cells.

Supplementary Fig. S7. Overexpression of AtTPX2 and ΔN-AtTPX2 in *Arabidopsis* nuclei.

Supplementary Fig. S8. Treatment of mitotic microtubules with Aurora kinase inhibitor ZM447439 in cell cultures of *Arabidopsis thaliana.*

Supplementary Fig. S9. Ectopic nuclear microtubular bundles were not affected by roscovitine treatment in cell cultures of *Arabidopsis thaliana.*

Supplementary Fig. S10. Evans blue viability test in *Arabidopsis* cell cultures with overproduced AtTPX2.

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Journal of Experimental Botany

Overexpressed TPX2 causes ectopic formation of microtubular arrays in the nuclei of acentrosomal plant cells.

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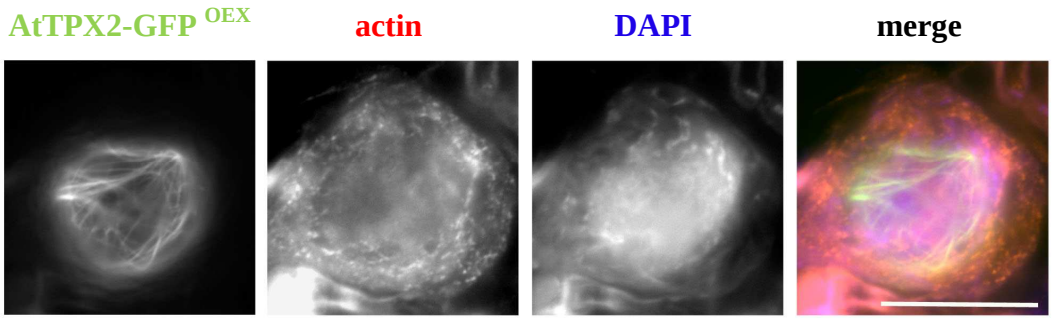
Supplementary Table S1. *In silico* analyses of AtTPX2 protein.

Name	Positions (aa)	Description
Domains:		
TPX2_importin	313-487	domain pfam: PF12214
TPX2	649-705	domain pfam: PF06886
Coiled-coil	588-619	domain
Motifs:		
Aurora	20-29, 43-51	Aurora binding sites
MTs	220-463, 684-758	Microtubule binding sites
NES	499-507	nuclear export signal
NLS	228-236, 610-758	nuclear localization signals
KEN box	148-152, 275-279, 667-671	APCC binding destruction motifs
D box	327-645	APCC binding destruction motif
Cyclin	305-309, 332-336, 516-519	cyclin recognition sites
FHA	12-18,141-147, 218-224, 291-297, 373-379, 396-402, 437-443, 581-587	FHA phosphopeptide ligands
MAD2	361-369	binding motif
MAPK	41-47, 492-502, 538-546, 723-731	MAPK (Mitogen-activated protein kinase) docking motifs
ProDKin	53-59, 154-160, 271-277, 372-378, 419-425, 723-729	MAPK phosphorylation sites
TRFH	264-268	TRFH docking motif
USP7	5-9, 55-59, 95-99, 200-204, 289-293, 371-375, 396-400, 714-718, 748-752	USP7 binding motifs (deubiquitinating enzyme)
HP1	95-99	HP1 ligand (interact with the chromoshadow domain of Heterochromatin-binding protein 1)
WW_Pin1	53-58, 154-259, 271-276, 372-377, 723-728	WW domain ligands
14-3-3	180-186, 312-318, 395-400	14-3-3 ligands (interacts with specific phosphoserine and phosphothreonine containing motifs)
COP1	628-635	COP1 binding motif (Constitutive photomorphogenesis protein)
EH1	524-528	EH ligand (endocytotic processes)
IQ	294-312	Calmodulin binding IQ motif

Name	Positions (aa)	Description
Motifs:		
SUMO	68-71, 94-98, 208-211, 497-502, 529-532, 575-578	SUMO binding sites
TRAF2	4-7, 79-82	TRAF2 binding sites
CK1	8-14, 49-55, 98-107, 139-145, 400-406, 507-513, 747-753	CK1 phosphorylation sites (for Ser/Thr phosphorylation)
CK2	1-7, 33-39, 271-277, 289-295, 335-341, 435-441, 462-468, 491-497, 509-515, 580-586	CK2 phosphorylation sites (for Ser/Thr phosphorylation)
GSK3	1-8, 7-14, 8-15, 33-40, 49-56, 111-118, 115-122, 119-126, 132-139, 136-143, 200-207, 396-403, 395-404, 415-422, 431-438, 491-498, 714-721, 719-726, 732-739, 744-751	GSK3 phosphorylation recognition sites (for Ser/Thr phosphorylation)
N-GLC	132-137, 195-200	N-glycosylation sites
PIKK	98-104, 101-107, 195-201, 325-331, 580-586	PIKK phosphorylation sites
PKA	64-70, 122-128, 431-437, 491-497, 519-525, 694-700, 736-742, 742-748	PKA phosphorylation sites
PKB	674-682	PKB phosphorylation site
PLK	1-7, 5-11, 80-86	Site phosphorylated by the Polo-like-kinase
ENDOCYTIC	21-24, 543-546, 548-551, 570-573	Y-based sorting signals
ER	330-333, 429-432, 430-433	For ER localization

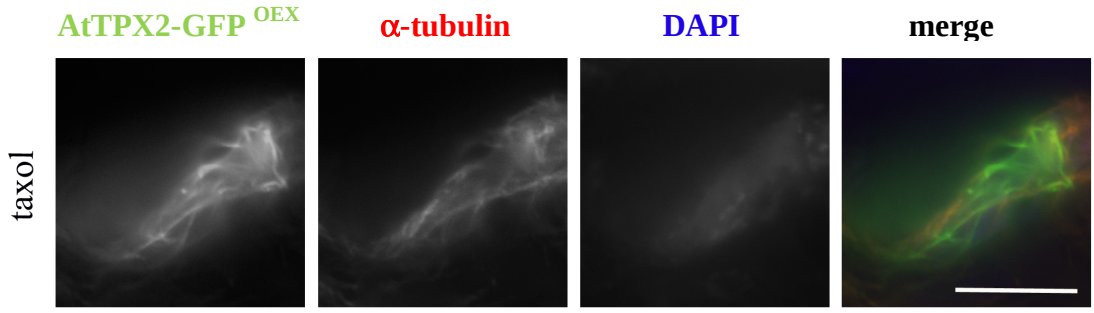
To predict putative interaction sites and binding motifs Pfam (Punta *et al*., 2012), ELM (Dinkel *et al*., 2012) and SUMOsp 2.0 (Ren et al., 2009) databases were used.

Supplementary Figure S1. Immunolocalization of AtTPX2 and actin in cell cultures of *Arabidopsis thaliana*



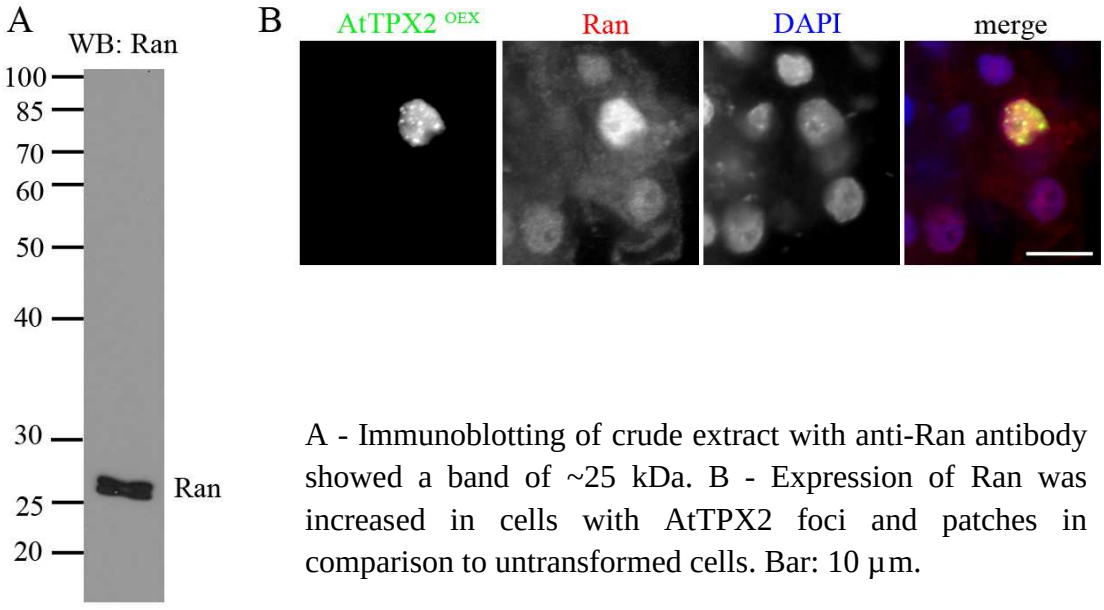
AtTPX2 fibres were not positive for actin immunolabelling. Bar: 10 µm.

Supplementary Figure S2. AtTPX2 decorated fibres were resistant to taxol in cell cultures of *Arabidopsis thaliana*



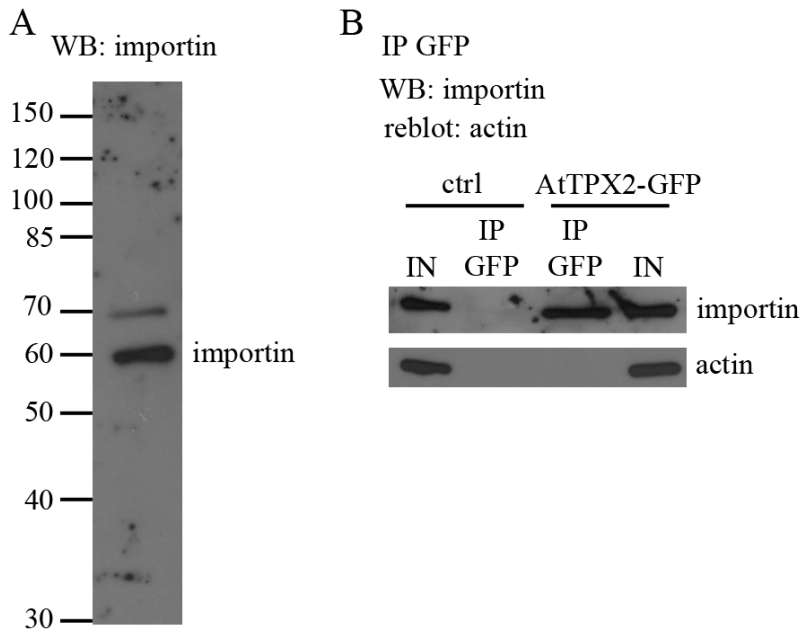
Further bundling of AtTPX2 decorated microtubules was not observed after treatment with 5 µM taxol for 3 hours. Bar: 10 µm.

Supplementary Figure S3. Immunofluorescence localization of Ran in AtTPX2-GFP overproducing cells.



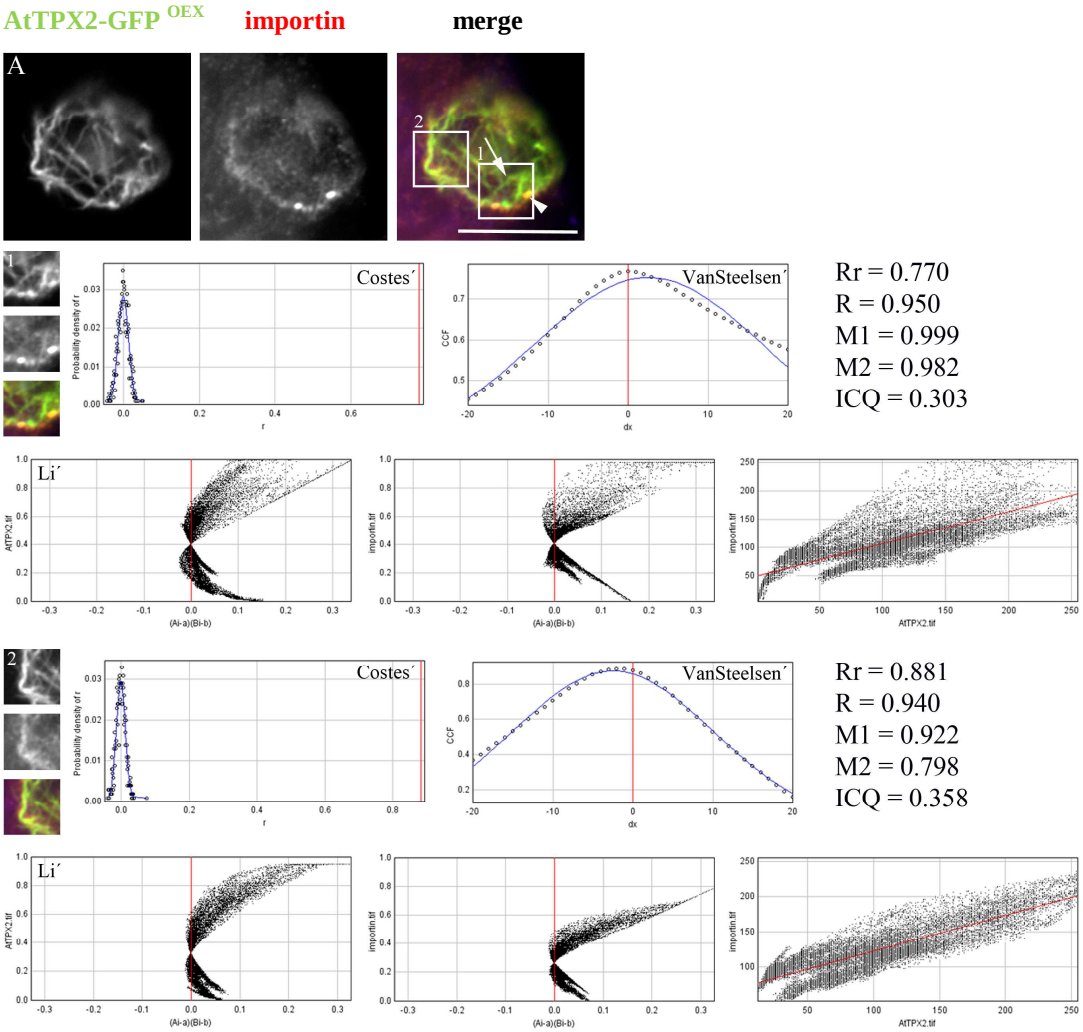
A - Immunoblotting of crude extract with anti-Ran antibody showed a band of ~25 kDa. B - Expression of Ran was increased in cells with AtTPX2 foci and patches in comparison to untransformed cells. Bar: 10 µm.

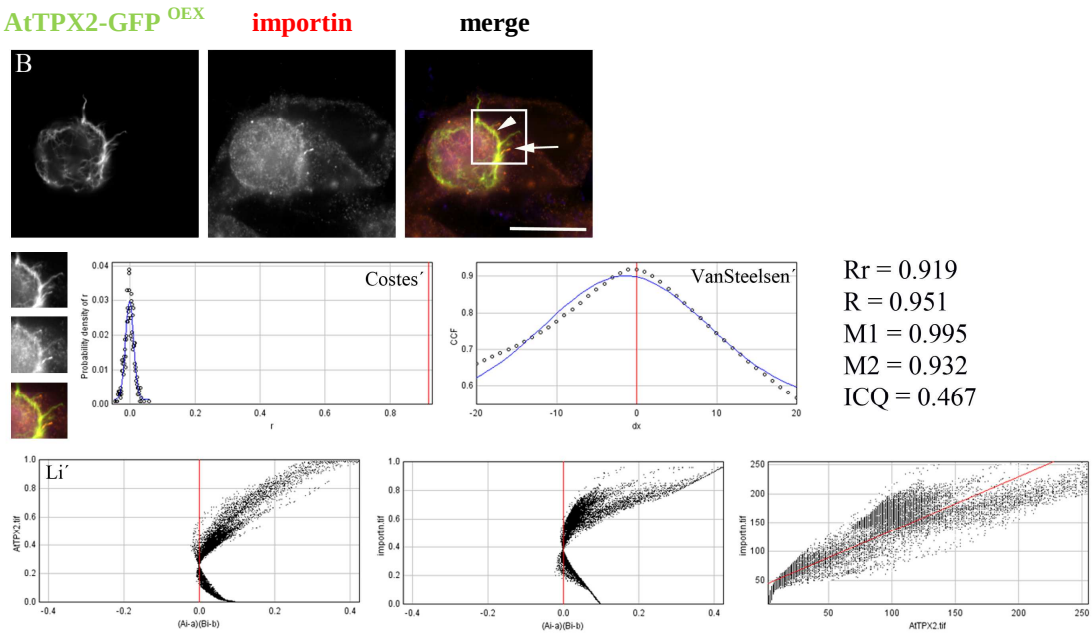
Supplementary Figure S4. Importin copurified with AtTPX2-GFP from *Arabidopsis* cultured cells.



A - Immunoblotting of crude extract with anti-importin antibody showed a band of ~60 kDa. B - Immunoblotting with anti-importin antibody showed that importin was copurified with AtTPX2-GFP using GFP trap (IP GFP) from extract prepared from cell culture expressing AtTPX2-GFP. Untransformed wild type cell culture Ler was used as a negative control for GFP immunopurification. Immunoblotting with irrelevant antibody anti-actin was used as a second negative control.

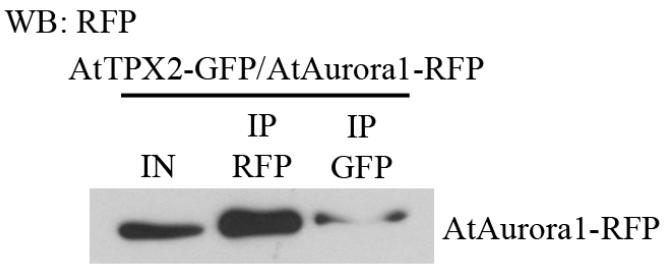
Supplementary Figure S5. Colocalization analyses of AtTPX2 and importin in *Arabidopsis* cultured cells.





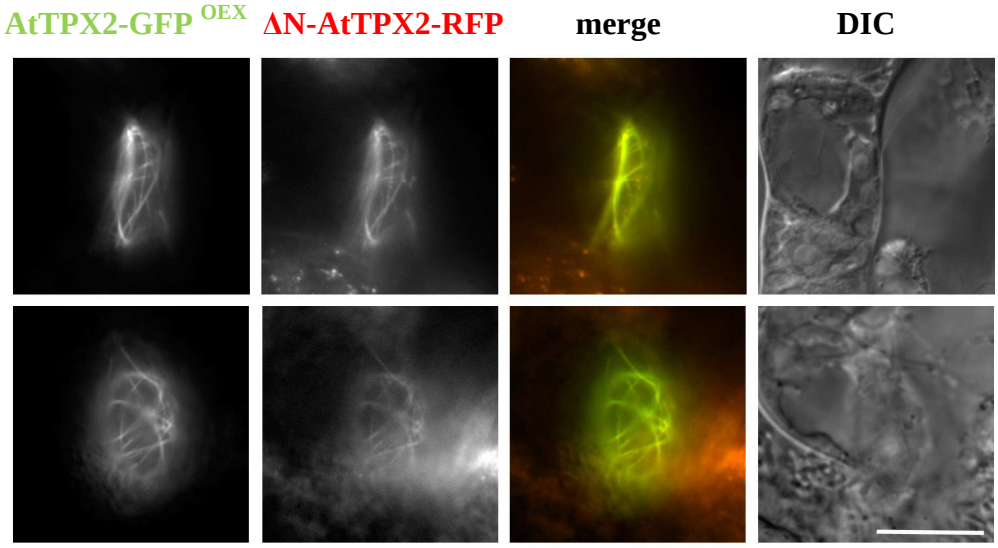
AtTPX2-GFP decorated fibres were present in close vicinity to the importin decorated nuclear envelope (A, B, arrowhead). Importin was present with AtTPX2 in dots on the nuclear envelope (A, B, arrowhead) and in the fibres (A, B, arrows). Pearson´s correlation coefficients (Rr) in Figure S8A (insets 1, 2) and S8B (0.770, 0.881, and 0.919, respectively) demonstrated colocalization of AtTPX2 and importin on the nuclear envelope and in the fibres in plant nuclei. However, Pearson´s coefficient in Figure S8A, inset 1 seemed to be complete colocalization with different intensities. The results of the Pearson´s correlation coefficient were confirmed with Manders´ coefficient (M1, M2). Figure S8 showed the completely colocalizing structure peak at the dx = 0 and bell-shaped curve. However, a difference in fluorescence intensity led to the reduction of the height of the bell-shaped curve, whereas the peak was still at dx = 0 (B). Non-colocalizing pixels in the Li´s approach are shown on the left side of the plots. The quantitative colocalization analyses of AtTPX2 and importin showed high degree of colocalization especially on the nuclear envelope. Colocalization analyses of AtTPX2 and importin was performed with ImageJ plugin JACoP (Bolte and Cordeliéres, 2006). Bars: 10 µm.

Supplementary Figure S6. AtAurora1-RFP copurified with AtTPX2-GFP from *Arabidopsis* cultured cells.



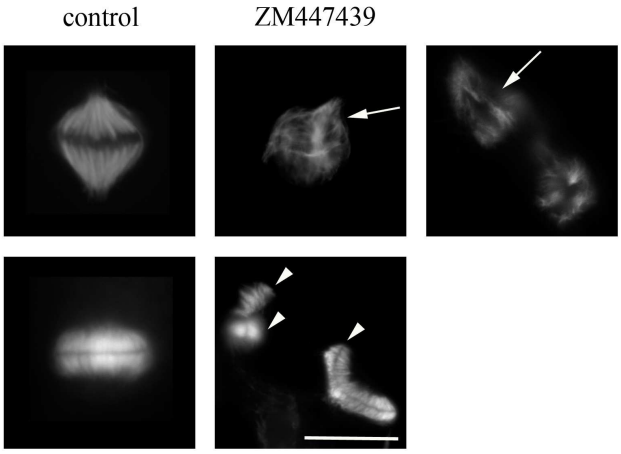
Immunoblotting with anti-RFP antibody showed that AtAurora1-RFP was copurified with AtTPX2-GFP using GFP trap (IP GFP) from extract prepared from cell culture co-expressing AtAurora1-RFP and AtTPX2-GFP. AtAurora1-RFP was purified by RFP trap (IP RFP).

Supplementary Figure S7. Overexpression of AtTPX2 and ΔN-AtTPX2 in *Arabidopsis* nuclei



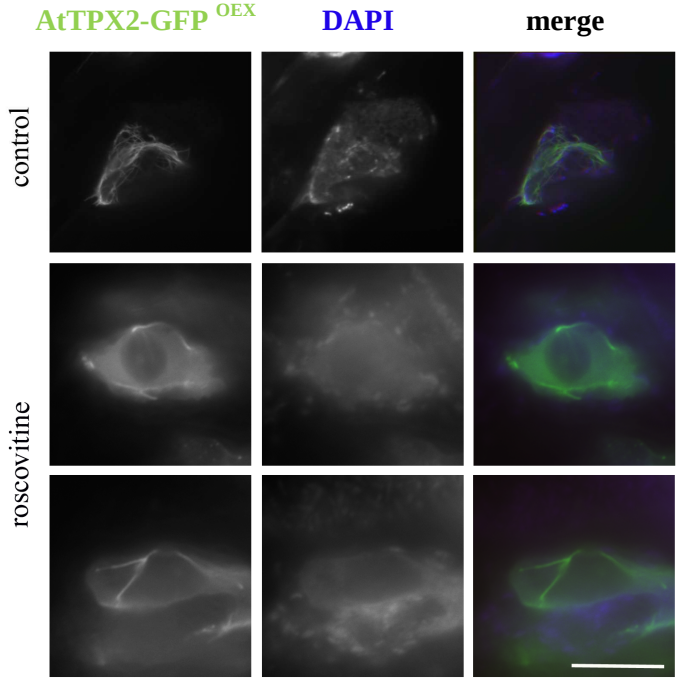
AtTPX2-GFP and its truncated version ΔN-AtTPX2-RFP localized together on microtubular array in the *Arabidopsis* nuclei. Bar: 10 µm.

Supplementary Figure S8. Treatment of mitotic microtubules with Aurora kinase inhibitor ZM447439 in cell cultures of *Arabidopsis thaliana*



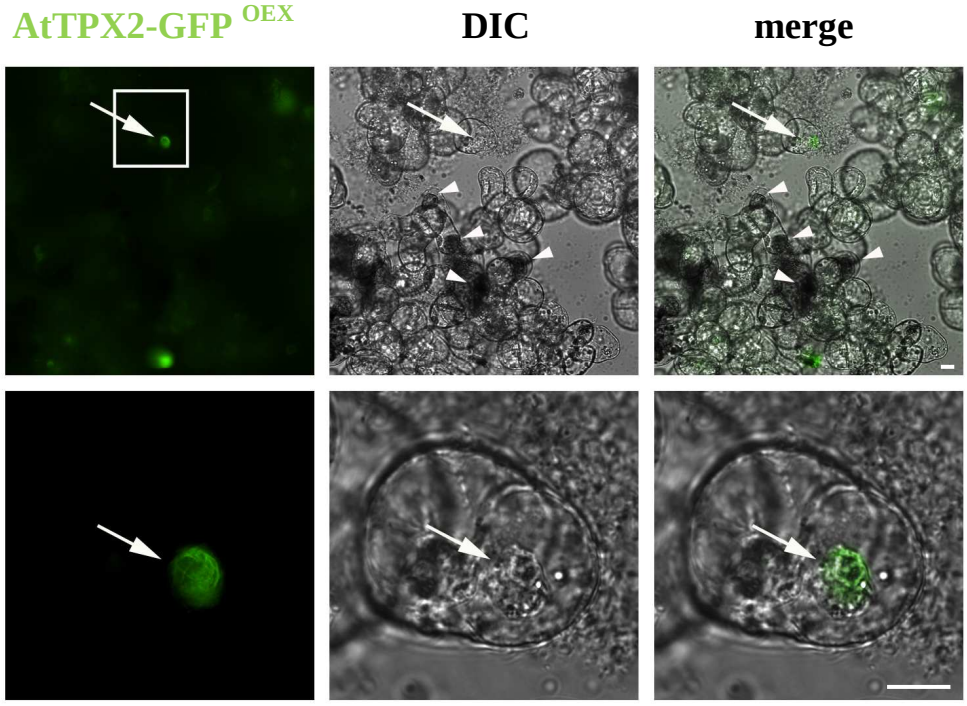
Microtubular arrays in the control (wild type) cells were affected after to the Aurora kinase inhibitor ZM447439 treatment. Multipolar mitotic spindle (arrows), fragmented phragmoplast (arrowheads) were often observed. Bar: 10 µm.

Supplementary Figure S9. Ectopic nuclear microtubular bundles were not affected by Roscovitine treatment in cell cultures of *Arabidopsis thaliana*



Formation of AtTPX2 decorated microtubular fibres was not affected with 100 µM Roscovitine, a cyclin dependent kinases inhibitor for 2.5 hours. Bar = 10 µm.

Supplementary Figure S10. Evans Blue viability test in *Arabidopsis* cell cultures with overproduced AtTPX2.



Evans Blue did not stain viable *Arabidopsis* cells with AtTPX2-GFP decorated fibres (arrows; n=68). Arrowheads showed death cells (dark grey colour). n = total number of AtTPX2-GFP analysed cells. Bars = 10 µm.

APPENDIX III

TPX2 protein of *Arabidopsis* activates Aurora kinase 1 but not Aurora kinase 3 *in vitro*

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In: Ready for submission

TPX2 protein of *Arabidopsis* activates Aurora kinase 1, but not Aurora kinase 3 *in vitro*

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Abstract

Background

Aurora kinases are involved in various mitotic events, including chromosome segregation and bipolar mitotic spindle assembly. In animals, Aurora A is activated and protected by microtubule-associated protein TPX2. It role in plants is not known. Here we have assessed the ability of TPX2 of *Arabidopsis* to regulate Aurora family members *in vitro*.

Results

*At*TPX2 acts as substrate as well as activator of *At*Aurora1, but not *At*Aurora3. Truncated version of *At*TPX2 lacking the Aurora binding domain is unable to activate the kinases; however, it is still phosphorylated. *At*TPX2-induced activation of *At*Aurora1 results in a dramatically increased phosphorylation level of downstream targets, particularly histone H3.

Conclusions

The differences in the activation mechanism of *At*Aurora1 and 3 point to a specific regulation of both kinases, which may play an important role in cell cycle regulation and signaling cascade transduction in plants.

Background

Proper chromosome segregation is one of the most critical steps in cell division. This process is mediated by mitotic spindle in all eukaryotes (Karsenti and Vernos, 2001). The spindle is assembled at the beginning of mitotic prophase and in metazoans depends on the microtubule nucleation from centrosomes, the main microtubule organizing centers. As microtubules emanating from centrosomes are captured by kinetochores, the bipolar mitotic spindle is formed. Higher plant cells are characterized by acentrosomal spindle. Nucleation of microtubules occurs at dispersed

sites localized within nuclei, on membranes and on existing microtubules via activity of gamma tubulin (Murata *et al.*, 2005; Binarová *et al.*, 2006).

TPX2 (targeting protein for *Xenopus* plus-end directed kinesin-like protein 2), is one of the downstream effectors of RanGTP (Gruss *et al.*, 2004). TPX2 is localized to cell nucleus during interphase and it is released from importin by RanGTP after nuclear envelope breaks down. Free TPX2 then stimulates microtubule nucleation at kinetochores and around chromosomes (Gruss and Vernos, 2004). TPX2 localization within mitotic spindle especially at the plus end microtubules in proximity to chromatin was observed in *Xenopus* (Witmann *et al.*, 2000). In human cells, TPX2 is phosphorylated by kinase Aurora A, a member of the serine/threonine family of kinases (Kufer *et al.*, 2002). TPX2 binding to Aurora A increases its *in vitro* autophosphorylation activity and prevents Aurora A from dephosphorylation by protein phosphatase 1 (PP1) (Bayliss *et al.*, 2003). Due to amino acid sequence similarities with other members of Aurora kinase family, similar mechanism might be employed also for Aurora B. TPX2-activated Aurora A kinase is responsible for spindle assembly stimulated by Ran GTPase in either presence or absence of centrosomes (Tsai and Zheng, 2005).

Similarly to its animal counterparts, *Arabidopsis thaliana* TPX2 (*At*TPX2) is localized in interphase nuclei and with kinetochore microtubules during mitosis (Vos *et al.*, 2008). *At*TPX2 is actively transported to cell nucleus together with importin β. After dissociation from the complex by RanGTP, *At*TPX2 is activated and promotes bipolar mitotic spindle assembly (Vos *et al.*, 2008). Overexpression of *At*TPX2 in *Arabidopsis* cell cultures results in formation of ectopic microtubules in nucleus and around nuclear envelope (Petrovská *et al.*, 2013). A TPX2 homologue is also present in G1-phase nuclei of barley (Petrovská *et al.*, 2014). Therefore it is likely, that in

addition to the role in mitotic microtubule organization, *At*TPX2 might have other nuclear functions.

Two types of Aurora kinase family are present in *Arabidopsis*: alpha (Aurora 1 and 2) and beta (Aurora3) (Demidov *et al.*, 2005; Kawabe *et al.*, 2005). All of them are capable to *in vitro* phosphorylate histone H3 at serine10 and serine 28, which is a modification associated with chromosome segregation. During interphase, *At*Aurora1 and 2 are localized in nucleus. Upon the entry into mitosis they localize at perinuclear region and associate with spindle microtubules, similarly to mammalian Aurora A. The analog of Aurora B, *At*Aurora3, is located at centromere (Demidov *et al.*, 2005, 2009; Kurihara *et al.*, 2006, Petrovská *et al.*, 2011). Nevertheless, not only AtAurora3 shows centromere localization (Kawabe *et al.*, 2005; Demidov *et al.*, 2009). During mitosis also *At*Aurora1, mostly connected with microtubules, overlap with *At*Aurora3 at the centromere, similar to the chromosomal passenger kinase Aurora B of mammals. One of the crucial substrates for Aurora kinase at centromere is histone H3, which undergoes cell cycle dependent phosphorylation with Aurora kinases (Kurihara *et al.*, 2006).

Here, we evaluate TPX2 of *Arabidopsis* as *in vitro* regulator of members of the Aurora family. Based on sequence similarities between plant alpha and beta Aurora kinases and TPX2 with its human homologues, we determined putative interaction sites in *Arabidopsis* proteins. A set of kinase assays with *At*Aurora1 and 3 revealed that only *At*Aurora1 phosphorylates *At*TPX2. Furthermore, we found that autophosphorylation activity of *At*Aurora1 is dramatically increased after addition of *At*TPX2. Increased autophosphorylation results in overall increase in the kinase activity towards histone H3, one of its most important mitotic targets. Our data

suggest that a similar mechanism of Aurora kinase activation occurs in plants and metazoans.

Results

Putative TPX2 interaction sites are present in both *At*Aurora1 and 3, but at some positions show isoform-specific amino acid composition

To analyze putative TPX2 interaction sites in *At*Aurora1 and 3, the amino acid sequences of homologous proteins from other plant species were aligned and conserved protein regions were identified (Additional file 1, 2). Then the conserved regions were compared with known TPX2 interaction sites in human Aurora A and B kinases (Bibby *et al.*, 2009). Indeed, plant *At*Aurora1 homologues contain conserved amino acids, which are likely involved in interaction with TPX2 (Additional file 1). Some potential TPX2 interaction sites were observed in plant *At*Aurora3 homologues as well (Additional file 2). At the same time, some of the amino acids in such interaction sites differ from those of *At*Aurora1 and 2 (Additional file 1, 2). For both groups of plant Aurora kinases, putative TPX2 interacting amino acids are distributed over the N-terminal region. Sequence alignment of *Arabidopsis* and human Aurora kinases revealed high homology especially in the deduced TPX2 interaction region (Additional file 3), suggesting that TPX2 acts as activator and/or regulation of plant Aurora kinases too.

*At*TPX2 family contains putative Aurora-binding sites well conserved between plants and animals

Next, we wanted to determine whether the Aurora binding site is conserved in various members of the *Arabidopsis* TPX2 family. Homologues of *At*TPX2 were identified, aligned and conserved Aurora A binding sites were deduced based on similarities with Aurora binding site in human TPX2 (Bayliss *et al.*, 2003). Amino acids involved in potential binding of *At*TPX2 to Aurora A kinase were predicted (Additional file 4).

Aurora A binding sites in *At*TPX2 have already been described by by Evrard *et al.*(2009) and additionally, 5 of 9 family members of TPX2-like proteins exhibit potential binding sites (Figure 1). Different splicing variants were found and not all splicing products of the *At*TPX2 family contain such binding site. Importantly, the TPX2-like protein At4g22860 produces a full size protein with Aurora kinase binding domain and TPX2 domain. At the same time, this gene can be represented in four different splicing variants. One of them is lacking a TPX2/Xklp2 motif; on the other hand, the second variant is without the Aurora kinase binding domain and contains only a TPX2 domain (Figure 1, Additional file 5). Since there is a similar domain composition among TPX2 and above mentioned proteins we suggest to rename them as TPX2-like protein 1 (TPXL1, At3g01015), TPX2-like protein 2 (TPXL2, 4g11990), TPX2-like protein 3 (TPXL3, At4g22860), TPX2-like protein 4 (TPXL4, At5g07170), TPX2-like protein 5(TPXL5, At5g15510), TPX2-like protein 6 (TPXL6, At5g37478), TPX2-like protein 7 (TPXL7, At5g44270) and TPX2-like protein 8 (TPXL8, At5g62240) (Figure 1). Based on *in silico* analysis of predicted Aurora binding domain of *At*TPX2 we assume, that similarly to human TPX2 and Aurora A, activation of *At*Aurora1 and 3 by *At*TPX2 might occur also in *Arabidopsis thaliana*.

***At*TPX2 is *in vitro* phosphorylated by *At*Aurora1, but not by *At*Aurora3**

Human TPX2 is phosphorylated by Aurora A both *in vitro* and *in vivo* (Kufer *et al.*, 2002). Based on the high similarity between the predicted TPX2-interacting amino acid of *At*Aurora1 and 3, we decided to test whether *Arabidopsis* TPX2 is phosphorylated by *At*Aurora1 and 3. Recombinant proteins were expressed in *E. coli* and purified by affinity chromatography. In addition to full-length *At*TPX2-His of about 110 kDa (758 aa, Additional file 6B), we prepared a truncated version of the protein, lacking the Aurora binding sites and one of the microtubule binding domains

(Petrovská *et al.*, 2011). The truncated version of the protein, *At*TPX2ΔN covers amino acids 442 – 758 (around 45 kDa, Additional file 6B) of the full length protein. Although we tested different expression vectors and cultivation conditions, due to the high degradation rate of TPX2, it was complicated to obtain full length *At*TPX2. TPX2 protein is highly unstable with a half time of degradation of around 10 min (Stewart *et al.*, 2005). Nevertheless, we were able to purify enough *At*TPX2 for our kinase assays (Additional file 6B).

The kinase assay showed that *At*Aurora1 phosphorylates *At*TPX2 *in vitro*. The phosphorylation was also observed for *At*TPX2ΔN, indicating that the phosphorylation sites of *At*TPX2 are located not only at N-terminal part of the protein. In addition to full length proteins, *At*Aurora1 phosphorylated also all degradation products of *At*TPX2 and *At*TPX2ΔN (Figure 2A). Therefore the *At*TPX2 possesses Aurora binding and phosphorylation sites. In comparison with *At*Aurora1, *At*Aurora3 phosphorylates TPX2 only weakly. For example a full-size variant of TPX2 shows no phosphorylation in kinase assay (Figure 2B).

*At*TPX2 increases autophosphorylation activity of *At*Aurora1

One of the modes of Aurora kinase activation is through autophosphorylation of a conserved threonine residue, which results in a conformation change critical for Aurora A activity (Marumoto *et al.*, 2005). TPX2 is a well-known coactivator of Aurora A in human (Bayliss *et al.*, 2003). To test whether plant TPX2 also acts as coactivator, a kinase assay was performed with *At*Aurora1 and three different substrates – *At*TPX2, *At*TPX2ΔN and *Arabidopsis* histone H3. *At*Aurora1 autophosphorylation activity is increased after addition of full-length *At*TPX2 but neither by *At*TPX2ΔN nor *Arabidopsis* histone H3 (Figure 2C).

Increased *At*Aurora1 activity towards histone H3 after coactivation of kinase by *At*TPX2

Next we tested whether the *At*TPX2-induced autophosphorylation of *Arabidopsis* Aurora might increase the phosphorylation level of downstream targets. Histone H3 in core histone mix from calf thymus was selected as downstream target for testing *At*Aurora1 activity (Demidov *et al.*, 2005). Kinase assay was performed with or without addition of recombinant *At*TPX2. Consistently with our previous findings, the autophosphorylation of *At*Aurora1 increased strongly after addition of full-length *At*TPX2 and hyperphosphorylation of histone H3 was observed in the presence of *At*TPX2 (Figure 3A). In contrast, elevated levels of H3 phosphorylation were not found for *At*Aurora3. Hence, *At*TPX2 is a potent activator of *At*Aurora1 and upstream regulator of histone H3 phosphorylation *in vitro*.

Discussion

Aurora kinases are regulated by phosphorylation and dephosphorylation as well as by association with activators and negative regulators (Walter *et al.*, 2000; Eyers *et al.*, 2003). In the present study we identified the microtubule-associated protein *At*TPX2 (At1g03780) as a new substrate and activator of *Arabidopsis* Aurora1.

Protein sequence alignment of *At*TPX2 to other plant TPX2 homologues and human TPX2 revealed the presence of a conserved Aurora A-binding domain and TPX2 motif. This allowed us to predict Aurora kinase binding domains and to compare our results with previously published data (Bayliss *et al.*, 2003). Additionally, we revealed a high sequence conservation of plant TPX2 protein within Aurora kinase binding domains and TPX2 motif- regions as well as relatively high variability outside of these domains.

TPX2 family members in *Arabidopsis* have different domain structures depending on alternative splicing (Figure 1). Some splicing variants are characterized by the

absence of Aurora binding domain (Figure 1, At4g22860 of 306 amino acids and At5g62240 of 327 amino acids) and some by the absence of a TPX2 motif (Figure 1, At4g22860 of 501 amino acids or At5g15510, At3g01015, At5g37478, At5g44270). These structural differences may determine the functionality of splicing variants. TPX2 protein variants without Aurora binding domain could be involved in formation of TPX2-tubulin complex that does not depend on phosphorylation by Aurora kinase. On the other hand, a protein with Aurora binding domain, but lacking TPX2 motif, could be involved in the activation of Aurora only, and is not needed for localization of Aurora to microtubules. These data allow us to assume that TPX2 variants have a physiological importance not only for the function of spindle microtubules during cell division (Tsai and Zheng, 2005), but also have additional function. Recent data obtained in human showed that Aurora B is not only interacting with TPX2, but TPX2 is needed for the activation of Aurora B (Iyer and Tsai, 2012). It was suggested that TPX2 activates Aurora B via stabilization of Aurora B/Survivin or Aurora B/INCENP complexes. However, a direct activation cannot be excluded. Alignment of protein sequences of alpha (1 and 2) and beta (3) Aurora kinases of plants with overlay of human TPX2 interaction sites identified by Bayliss *et al.*(2003), allowed to determine functionally conserved amino acids potentially interacting with TPX2 (Additional file1, 2). Also the alignment of beta type of plant Aurora protein sequences permitted identification of sequence differences between *Arabidopsis* Aurora1, 2 and 3 involved in interaction with TPX2 (Additional file 3). No members of plant proteins of chromosomal passenger complex (CPC) still have been identified, especially INCENP as *At*Aurora3 activator. Because AtAurora3 also contains amino acids potentially involved in interaction with *At*TPX2, it can be assumed that its activation is also implemented by *At*TPX2 protein or other family members.

We demonstrated that *At*TPX2 is a substrate for *At*Aurora1, while *At*Aurora3 phosphorylates *At*TPX2 *in vitro* with low efficiency. Truncated version of *At*TPX2 lacking the Aurora kinase binding domain was also phosphorylated by *At*Aurora1 and slightly by *At*Aurora3. In human, TPX2 is also a substrate for Aurora A and phosphorylation occurs on serine residues (Kufer *et al.*, 2002). Particular residues and exact function of TPX2 phosphorylation in animals as well as in plants is unknown, however it might be related to mitosis, as *At*Aurora1 and *At*TPX2 colocalize with spindle microtubules from preprophase up to telophase (Petrovská *et al.*, 2012). TPX2 also acts as activator of Aurora kinase. After TPX2 binding, the autophosphorylation activity of human Aurora A at threonine 288 is increased and dephosphorylation is prevented (Bayliss *et al.*, 2003). Aurora A is activated at the vicinity of chromatin where it regulates localization and activity of parts of the microtubule organizing complexes (Katayama *et al.*, 2008). Similarly to human homologues, *At*TPX2 stimulates autophosphorylation of *At*Aurora1 *in vitro*. *At*TPX2 has no effect on *At*Aurora3 activity, pointing toward divergent functions for both types of kinases that are probably determined by different regulators. Indeed diverse roles for *At*Aurora1, 2 and 3 were demonstrated previously (Van Damme *et al.*, 2011). Furthermore, the lack of activation in case of *At*TPX2ΔN suggests that it is probably Aurora binding domain in *At*TPX2 molecule that is responsible for kinase activation. As low amounts of *At*TPX2 are sufficient to activate *At*Aurora1, *At*TPX2 must be considered a potent activator of the kinase. These observations indicate conservation of the mechanism of Aurora kinase activation in plants and animals.

Increased autophosphorylation activity of *At*Aurora1 is followed by increased histone H3 phosphorylation. Phosphorylation of histone H3 at serine 10 by *At*Aurora1 depends on preexisting modifications (epigenetic crosstalk). It is tempting to

speculate that *At*TPX2 coactivation of *At*Aurora1 changes the kinase activity towards differently modified histone H3 and therefore influences only subset of substrates, e.g. in a specific phase cell cycle. *At*Aurora1 is known to regulate proper kinetochore / microtubule attachment (Kurihara *et al.*, 2008). Furthermore TPX2 has a role in cellular response to DNA damage through H2AX phosphorylation at serine 139 (γH2AX). TPX2 might regulate γH2AX through remodeling of DNA double strand break flanking chromatin by association between TPX2 and histone deacetylase SIRT1 (Neumayer *et al.*, 2012). Therefore a nuclear and chromatin-related role of *At*TPX2 is plausible.

Conclusions

*At*TPX2 is regulates *At*Aurora1 activity not only by targeting the kinase to mitotic spindle (Petrovská et al., 2012), but also by increasing its autophosphorylation activity towards histone H3. Activation of *At*Aurora1 by *At*TPX2 could be a mechanism for translation of RanGTP signaling to phosphorylation cascade performed by Aurora kinases at kinetochores. The increased *At*Aurora1 activity might also activate various downstream targets such as histones and spindle assembly factors. Such hyperactivation cascade could stimulate fast and precise reconstruction of mitotic chromatin and/or mitotic spindle formation. The fact that particular splicing variants of TPX2-related proteins do not contain TPX2/Xklp2 motif, points to other than microtubule-related role of these proteins. Such proteins might act only as activators of the kinase, e.g., when the kinase is not localized at microtubules, such as during phosphorylation of histone H3. Interestingly, Neumayer *et al.*(2012) showed that TPX2 is also involved in phosphorylation of H2AX. This is a new function of TPX2, since previously it was assumed that TPX2 colocalizes only with microtubules and is not involved in regulation of chromatin function.

Methods

In silico analysis of putative interaction sites between TPX2 and *plant* Aurora kinases

The Basic Local Alignment Search Tool (BLAST, Altschul *et al.*, 1990) was used to determine potential TPX2, Aurora1 and Aurora3 homologues in the other plant genomes. The T-Coffee tool (EMBL-EBI, Notredame *et al.*, 2000) was used for multiple sequence alignment.

Plasmid construction for expression of recombinant proteins in *E. coli*

*At*TPX2 and *At*TPX2ΔN (truncated version of *At*TPX2 without the N-terminal part of the protein with Aurora-binding site) coding sequences were obtained as described previously (Petrovská *et al.*, 2011). For expression of recombinant His-tagged proteins, *At*TPX2- and *At*TPX2ΔN-specific PCR products were cloned into the Gateway expression vectors pET55DEST (Novagen, Madison, WI, USA) and pETG10A (EMBL, Heidelberg, Germany), respectively.

*At*Aurora1 and *At*histone H3 were cloned as described previously (Demidov *et al.*, 2009). *At*Aurora3 was cloned into pDEST566 for recombinant expression of MBP-tagged protein (New England Biolabs, Ipswich, MA, USA).

Production of recombinant proteins

Recombinant *At*TPX2, *At*TPXΔN and *At*histone H3 were produced in *E. coli* BL-21 and purified under denaturating conditions. *E. coli* BL-21 with appropriate vectors were inoculated into 50 ml LB medium at 37°C, 180 rpm and grown until OD_{600} = 0.6 when protein expression was induced with 1 mM IPTG. Cells were incubated for additional 3 hours, 37°C, 180 rpm. Recombinant proteins were purified using Protino NiNTA Agarose (Macherey-Nagel, Düren, Germany) with pH step elution to pH 2.5. GST-*At*Aurora1 and MBP-*At*Aurora3 were produced in *E. coli* C43 and purified under native conditions. *E. coli* C43 (DE3) cells containing pDEST15-*At*Aurora1 and

pDEST566-*At*Aurora3 constructs were inoculated into 50 ml LB medium at 37°C. When cultures reached a density OD_{600}=0.4 – 0.6, protein expression was induced by adding 1 mM IPTG. After further incubation at 18°C, 180 rpm for 12 – 16 hours, cells were harvested. Cell lysis was processed as described in Demidov *et al.*(2009). Soluble GST-*At*Aurora1 was affinity purified using glutathione agarose 4B resin (Macherey-Nagel, Düren, Germany), eluted by 50 mM Tris-HCl, 30 mM reduced glutathione, pH 8.0. Soluble MBP-*At*Aurora3 was affinity purified with amylose resin (New England Biolabs, Ipswich, MA, USA), eluted by 200 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, 1 mM β-mercaptoethanol and 20 mM maltose.

In vitro **kinase assay**

Purified recombinant proteins were desalted in kinase buffer (10 mM Tri HCl, 50 mM KCl, 2 mM MgCl x 6 H_2O, 0.2 mM EGTA, pH 7.5) using 7 K MWCO Zeba Spin Columns (Thermo Scientific, Waltham, MA, USA). *At*Aurora1 or 3 were preincubated at 30°C, 30 min with 0.5 x kinase buffer and 0.1 mM ATP for activation of the kinases. Subsequently, [^{32}P]ATP and substrates (*At*TPX2 ~ 0.5-1 µg, *At*TPXΔN ~ 1-2 µg, *At* histone H3 ~10 µg, core histone mix from calf thymus = 4 µg, Roche Applied Science, Mannheim, Germany) were added and incubated for additional 60 min at 30°C. Kinase reactions were terminated and processed for imaging as described previously (Demidov *et al.*, 2009).

Accession numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers BAE00019 (AGI locus identifier At4g32830), BAE00020 (At2g25880), NP_182073 (At2g45490) for *At*Aurora1, 2 and 3 (respectively), NP_973754 (At1g03780) for *Arabidopsis At*TPX2, and NC_003070.9 (At1g09200) for *Arabidopsis* histone H3.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ET and DD prepared the recombinant proteins, performed the kinase assays and wrote the manuscript. DD interpreted experimental results. HJ cloned the *At*TPX2 expression vectors. JD and AH conceived and obtained funding for the research. PB interpreted experimental results. BP designed and interpreted experimental results and wrote the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1 - Different variants of *Arabidopsis* TPX2-related proteins and their domains

*At*TPX2 and TPX2 related proteins and their splicing variants were analyzed for their domain composition. At4g22860 (306 amino acids) and At5g62240 (327 amino acids) do not possess TPX2/Xklp2 motif. These proteins probably acts only as regulators of Aurora kinase activity and are not involved in *At*Aurora1 localization at microtubules.

Figure 2 - Phosphorylation of *At*TPX2 and *At*TPX2ΔN by *At*Aurora 1 and *At*Aurora3 *in vitro*

Radioactive kinase assays were performed with *At*Aurora1 and *At*Aurora3 kinase using full-length and truncated *At*TPX2 proteins. A, B – CBB staining (1 – 4) and autoradiographs (5 – 8) of kinase assays. Phosphorylated *At*TPX2 and *At*TPX2ΔN are marked with red arrows.

A – *At*Aurora1 is able to phosphorylate both *At*TPX2 (lane 7) and *At*TPX2ΔN (lane 8). Lane 5, 6 – negative control without kinase. Phosphorylated degradation products of *At*TPX2 are marked with white asterisks.

B – The kinase assay with *At*Aurora3 indicates that only truncated version of *At*TPX2 (lane 7) and not full-length protein (lane 8) is substrate for the kinase. Lane 5, 6 – negative control without kinase.

C – *At*TPX2 is coactivator of *At*Aurora1 autophosphorylation activity. Kinase assay revealed, that after addition of *At*TPX2 (lane2) but not *At*TPX2ΔN (lane 1) kinase activity of *At*Aurora1 is increased in comparison to control – histone H3, well-known substrate for Aurora kinases (lane 3).

Figure 3 - *In vitro* activation of *At*Aurora1 by *At*TPX2 causes increased phosphorylation of downstream targets

A – *In vitro* phosphorylation of histone H3 is markedly increased after activation of *At*Aurora1 by *At*TPX2. Comparison of *At*Aurora1 activity without (lane 3) and with (lane 7) addition of *At*TPX2 as kinase coactivator.

B – *In vitro* phosphorylation of histone H3 is not increased after *At*Aurora3 incubation with *At*TPX2.

Additional files

Additional file 1 – Analyses of putative TPX2-interaction motif in *At*Aurora1 plant homologues

Multiple sequence alignment revealed high level of similarity in the TPX2 interaction motif between *At*Aurora1 and its plant homologues. Black arrows indicate the conserved amino acids. Blue arrows indicate the amino acids involved in interaction with TPX2 which are not conserved.

Additional file 2 – Analyses of putative TPX2-interaction motif in *At*Aurora3 plant homologues

Multiple sequence alignment revealed high level of similarity in the TPX2 interaction motif between *At*Aurora3 and its plant homologues. Black arrows indicate the conserved amino acids. Blue arrows indicate the amino acids involved in interaction with TPX2 which are not conserved. Red frame indicated differences between *At*Aurora 1 and *At*Aurora3 homologues.

Additional file 3 – Alignment of *Arabidopsis* and human Aurora kinases

Multiple sequence alignment revealed high level of similarity in the TPX2 interaction motif between *Arabidopsis* and human Aurora kinase genes. Black arrows indicate conserved amino acids at potential TPX2-interaction region. Blue arrows indicate the amino acids which are not conserved. In the red frame are amino acids different between *At*Aurora1 and *At*Aurora3.

Additional file 4 – Analyses of putative AuroraA-binding sites in *At*TPX2 plant homologues

Multiple sequence alignment revealed high level of similarity in the AuroraA binding site of *At*TPX2 and its plant homologues. Black arrows indicate conserved amino acids for AuroraA binding region, based on analyses of non-plant TPX2 proteins. Green arrows indicate conserved amino acids exclusively in plant TPX2 Aurora1 binding region.

Additional file 5 – Analyses of putative AuroraA-binding sites in Arabidopsis TPX2-related proteins

Multiple sequence alignment revealed absence of AuroraA-interacting region in different splicing variants of TPX2-related proteins.

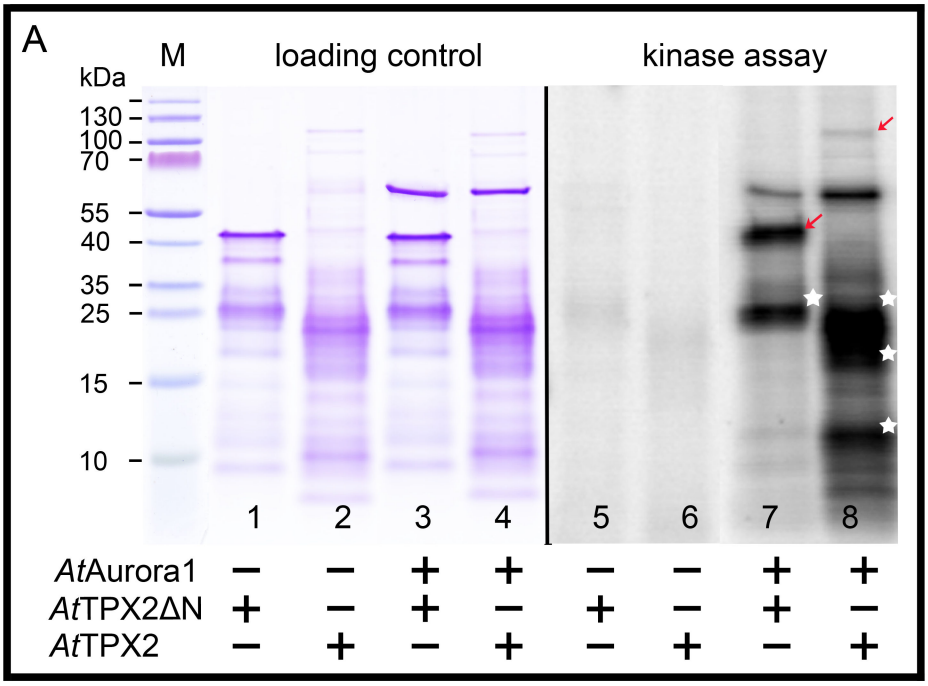
Additional file 6 – Expression and purification of recombinant proteins

A – SDS-PAGE separation of purified MBP-tagged *At*Aurora3 (lane 1) and GST-tagged *At*Aurora1 (lane 2) after Commasie Brilliant Blue (CBB) staining. The positions and respective size of recombinant proteins are indicated.

B – SDS-PAGE separation of purified His-tagged *At*TPX2ΔN (truncated version of *At*TPX2 without Aurora binding domain) and full length *At*TPX2 protein. The positions and sizes of recombinant proteins are indicated. Degradation products of *At*TPX2 are marked with asterisks.

C – SDS-PAGE separation of purified His-tagged *At* histone H3 after CBB staining.

Gene	Proposed name	Protein length	Aurora BD	TPX2/importin motif	TPX2/Xklp2 motif
At4g22860	TPX2-like protein 3	509	+	+	-
	TPXL3	501	-	+	-
		487	+	+	-
		306	+	-	-
At4g11990	TPX2-like protein 2	521	+	+	-
	TPXL2	501	+	+	-
At5g07170	TPX2-like protein 4	542	+	+	-
	TPXL4	394	+	+	-
At5g62240	TPX2-like protein 8	377	+	+	-
	TPXL8	327	+	+/-	-
At1g03780		790	+	+	+
		758	+	+	+
		743	+	+	+
		687	+	+	-
At5g15510	TPX2-like protein 5	519	-	-	+
	TPXL5	497	-	-	+
At3g01015	TPX2-like protein 1 TPXL1	488	-	-	+
At5g37478	TPX2-like protein 6	96	-	-	+
	TPXL6	178	-	-	+
At5g44270	TPX2-like protein 7 TPXL7	309	-	-	+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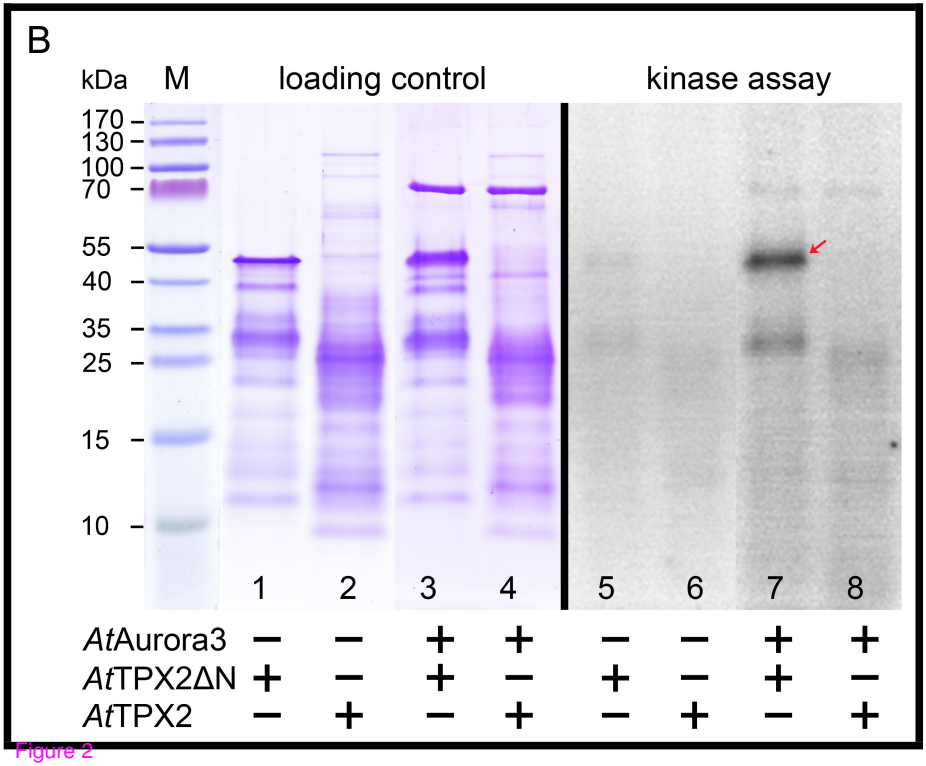
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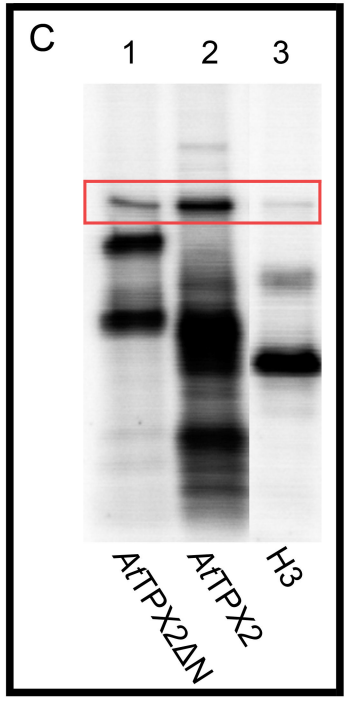
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Figure 2

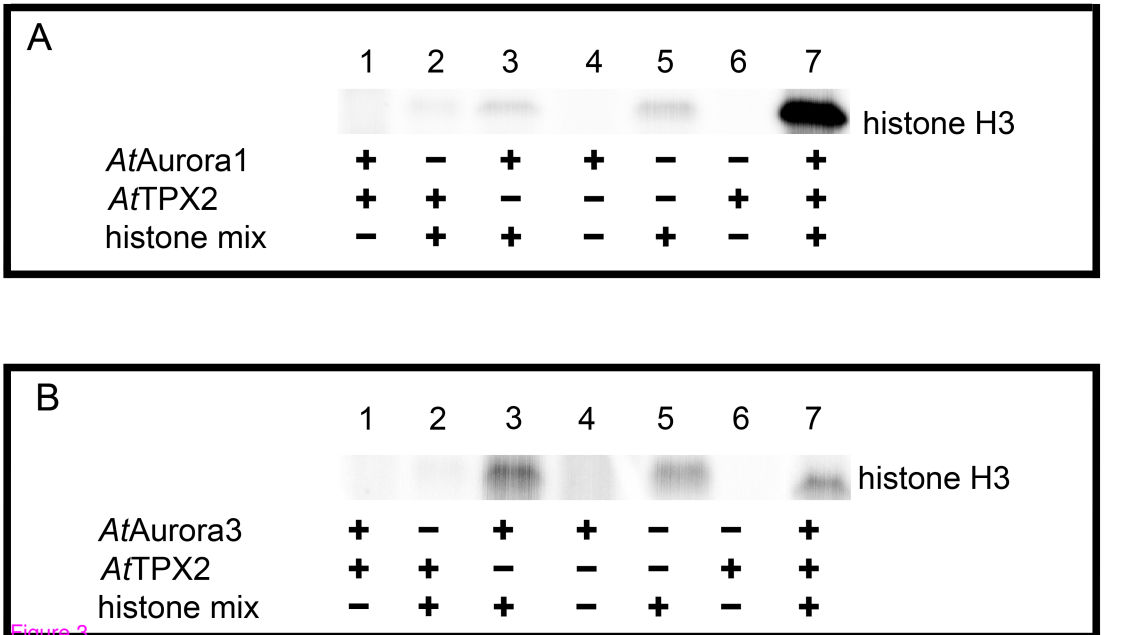
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Figure 3

Additional files provided with this submission:

Additional file 1: Additional file 1.pdf, 15183K
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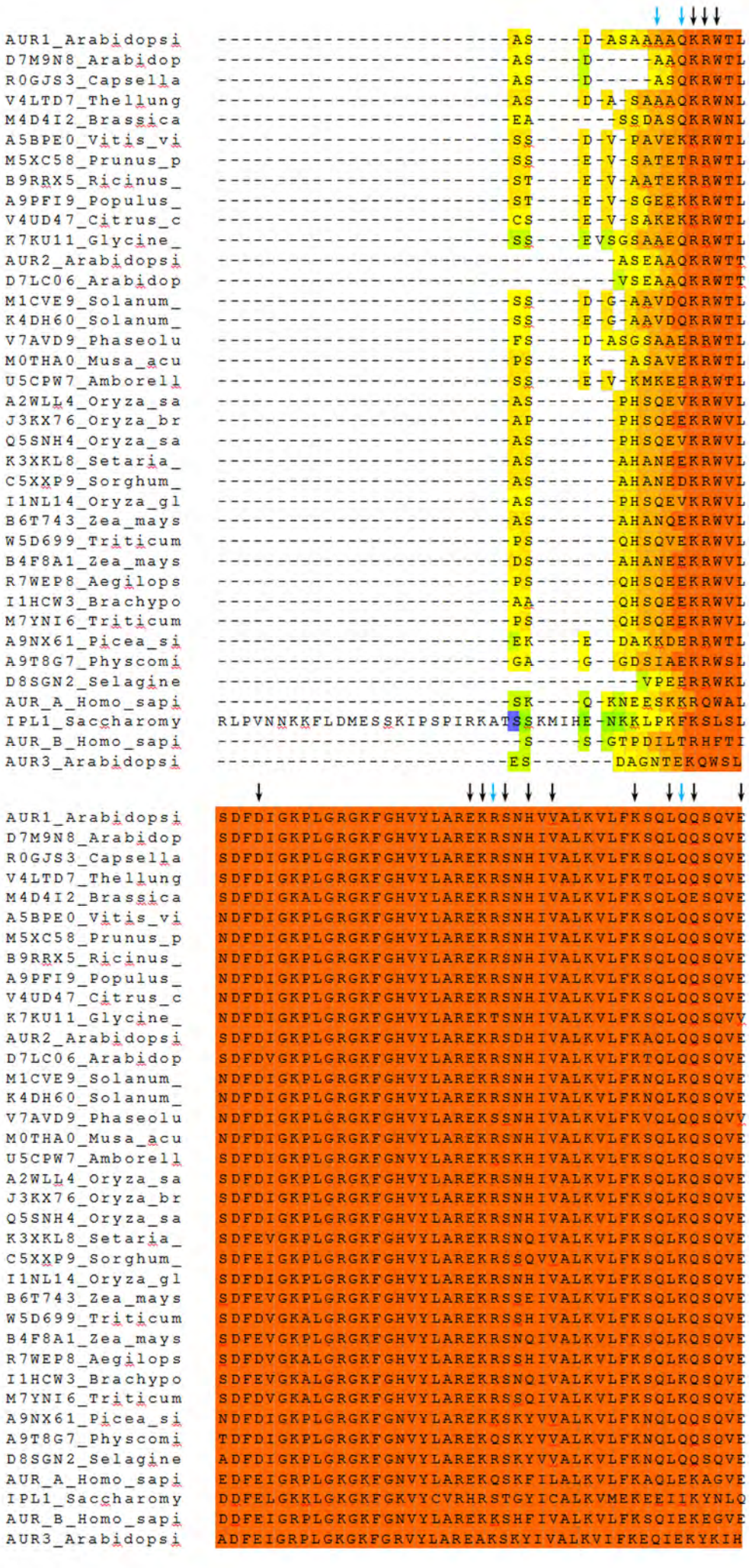
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AUR_A_Homo_sapi ELQKLSKFDEQRTATYITELANALSYCHSKRVIHRDIKPENLLLG
IPL1_Saccharomy LLRLHGPFNDILASDYIYQIANALDYMHKKNIIHRDIKPENILIG
AUR_B_Homo_sapi ELQKSCTFDEQRTATIMEELADALMYCHGKKVIHRDIKPENLLLG
AUR3_Arabidopsi VLKQNGHLTEQQAATYIASLSQALAYCHGKCVIHRDIKPENLLLD


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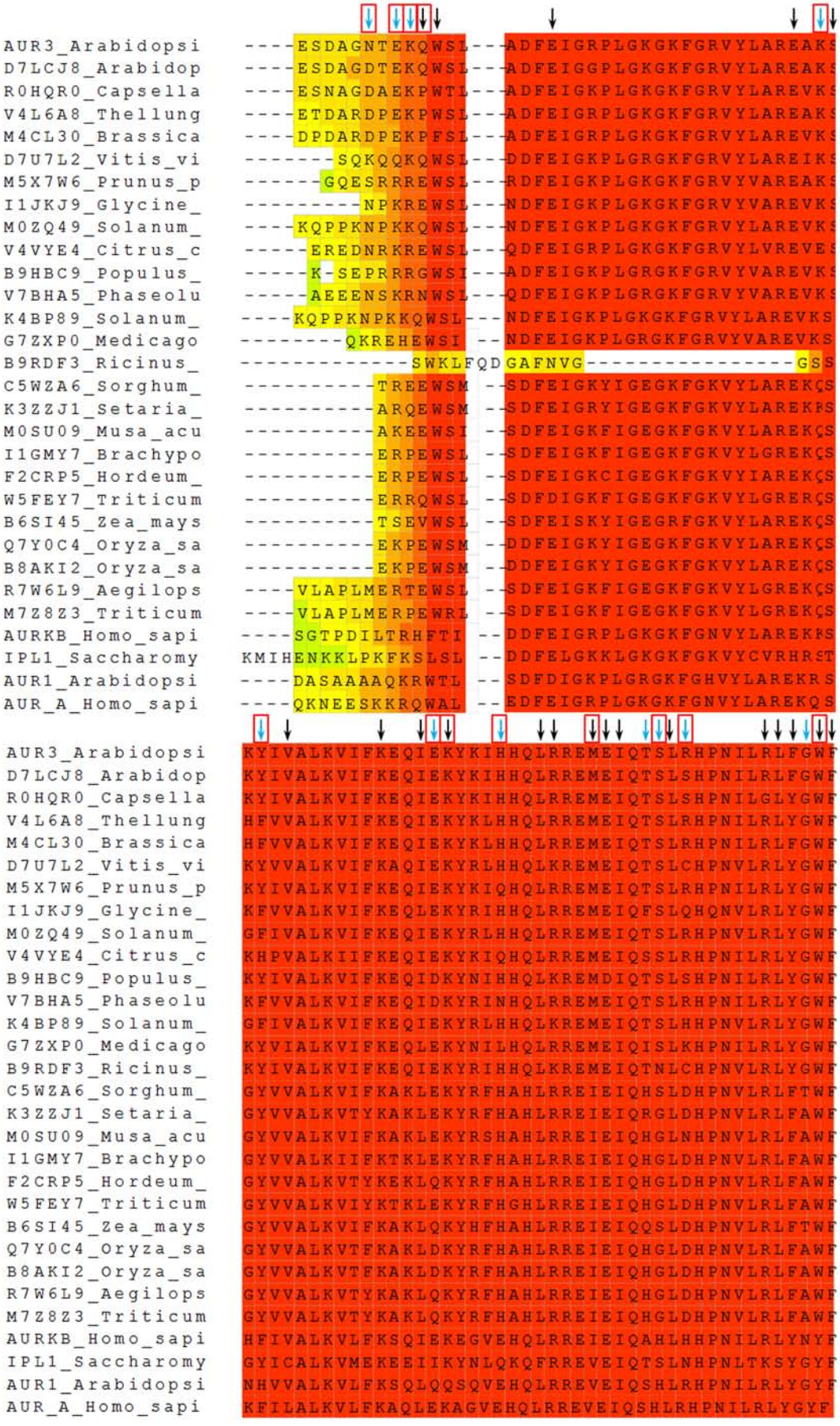
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AURKB_Homo_sapi
IPL1_Saccharomy
AUR1_Arabidopsi
AUR_A_Homo_sapi


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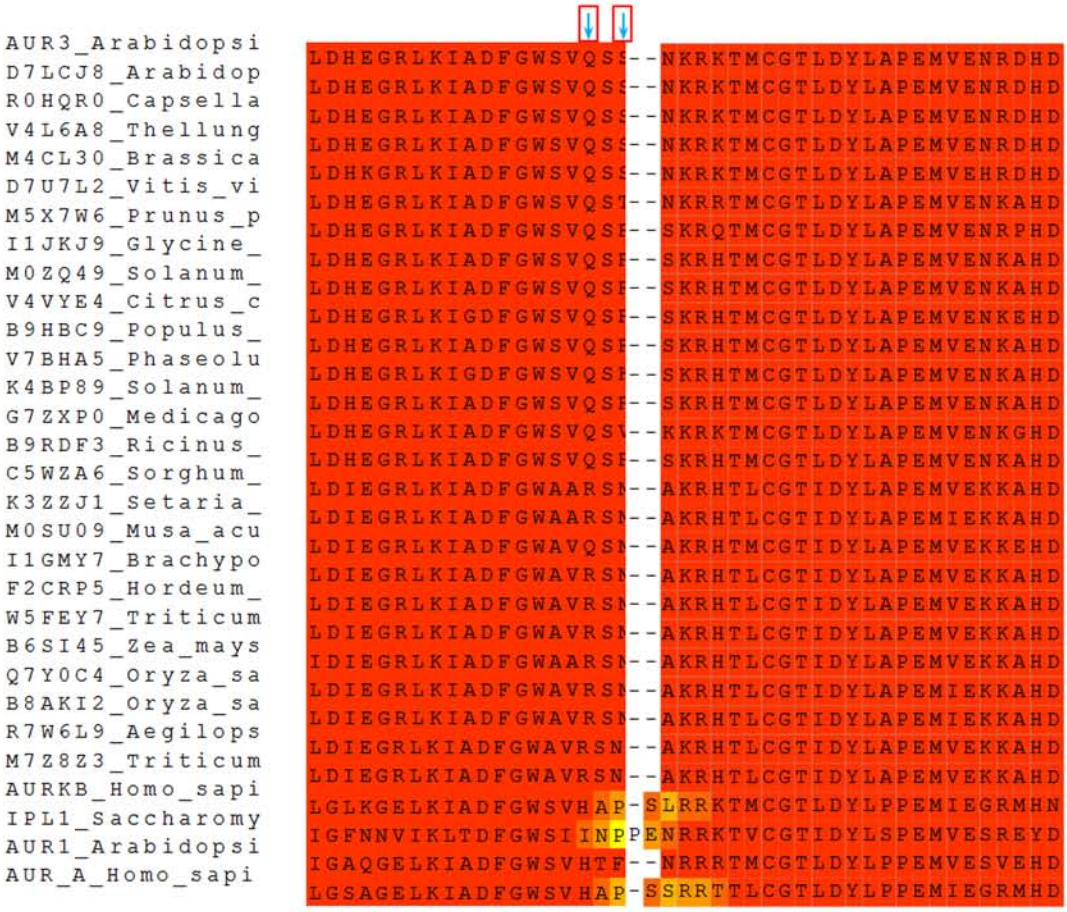
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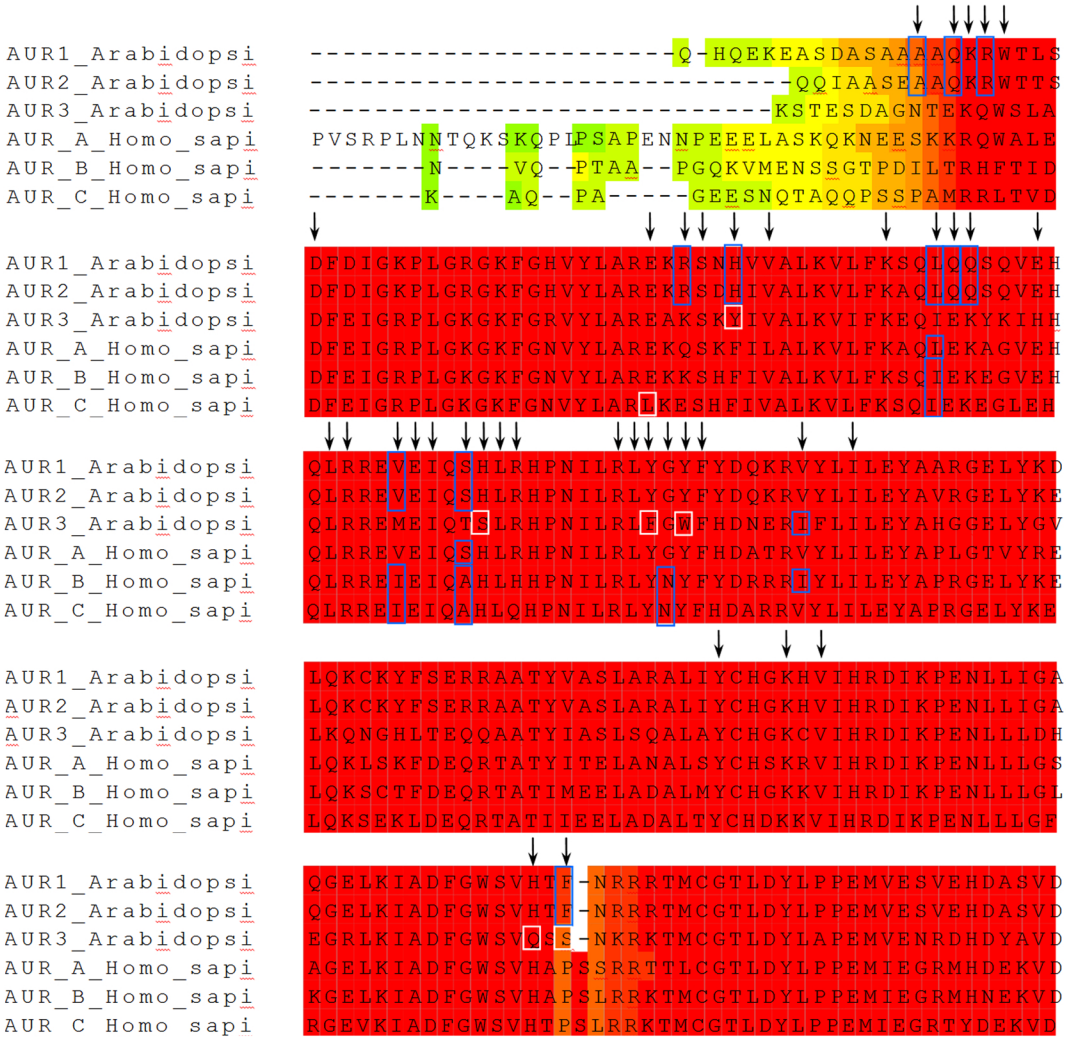
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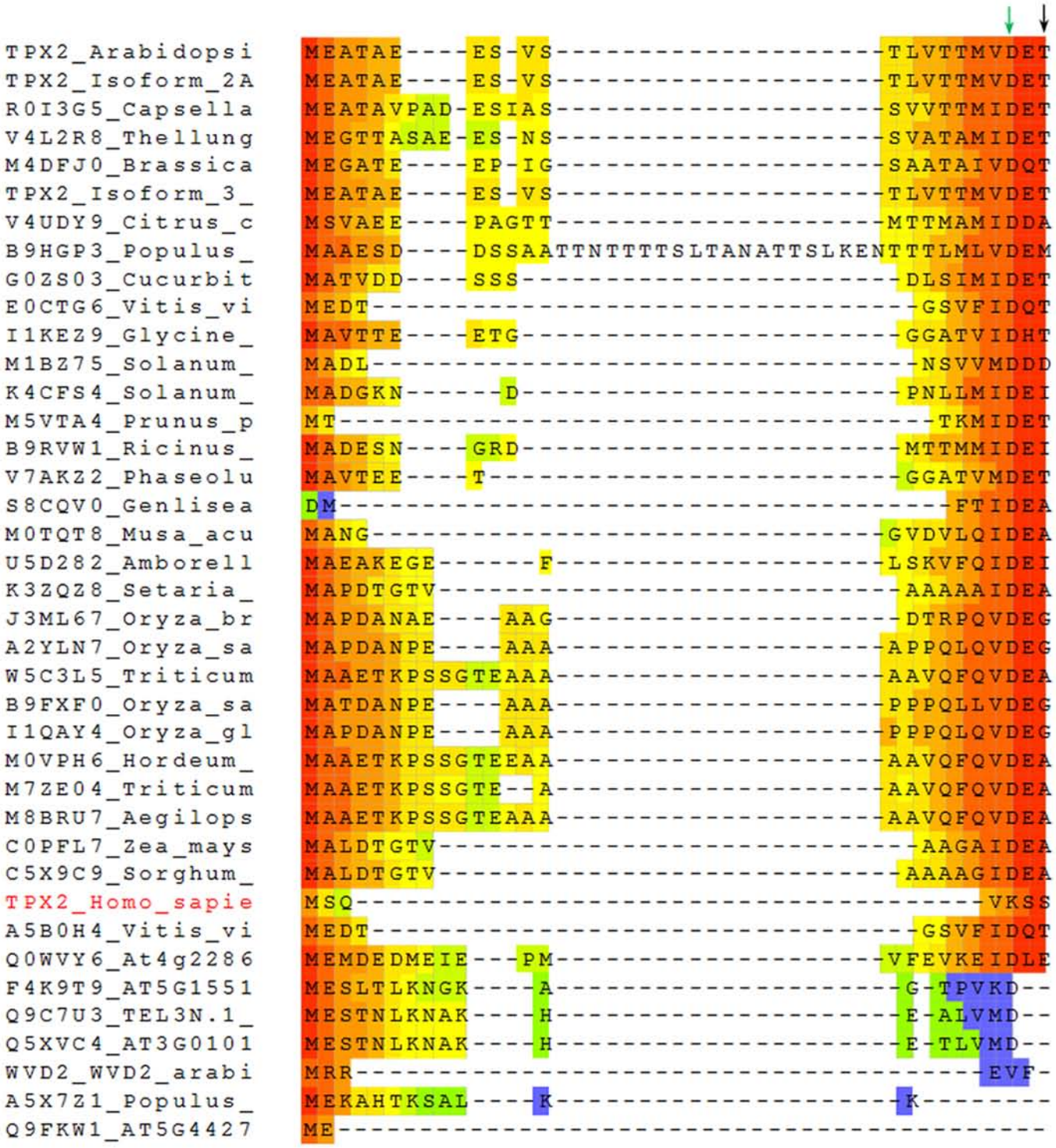
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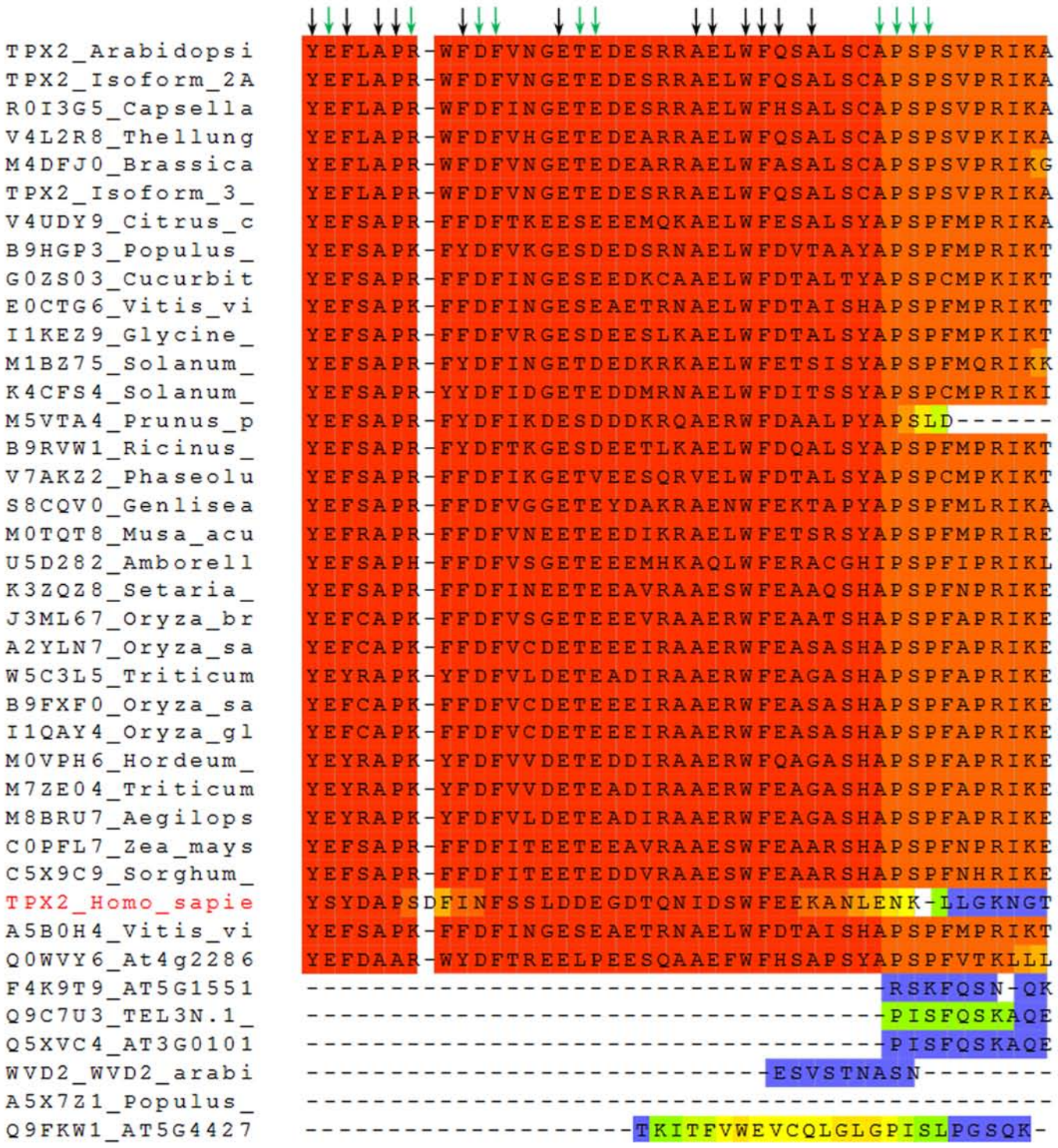
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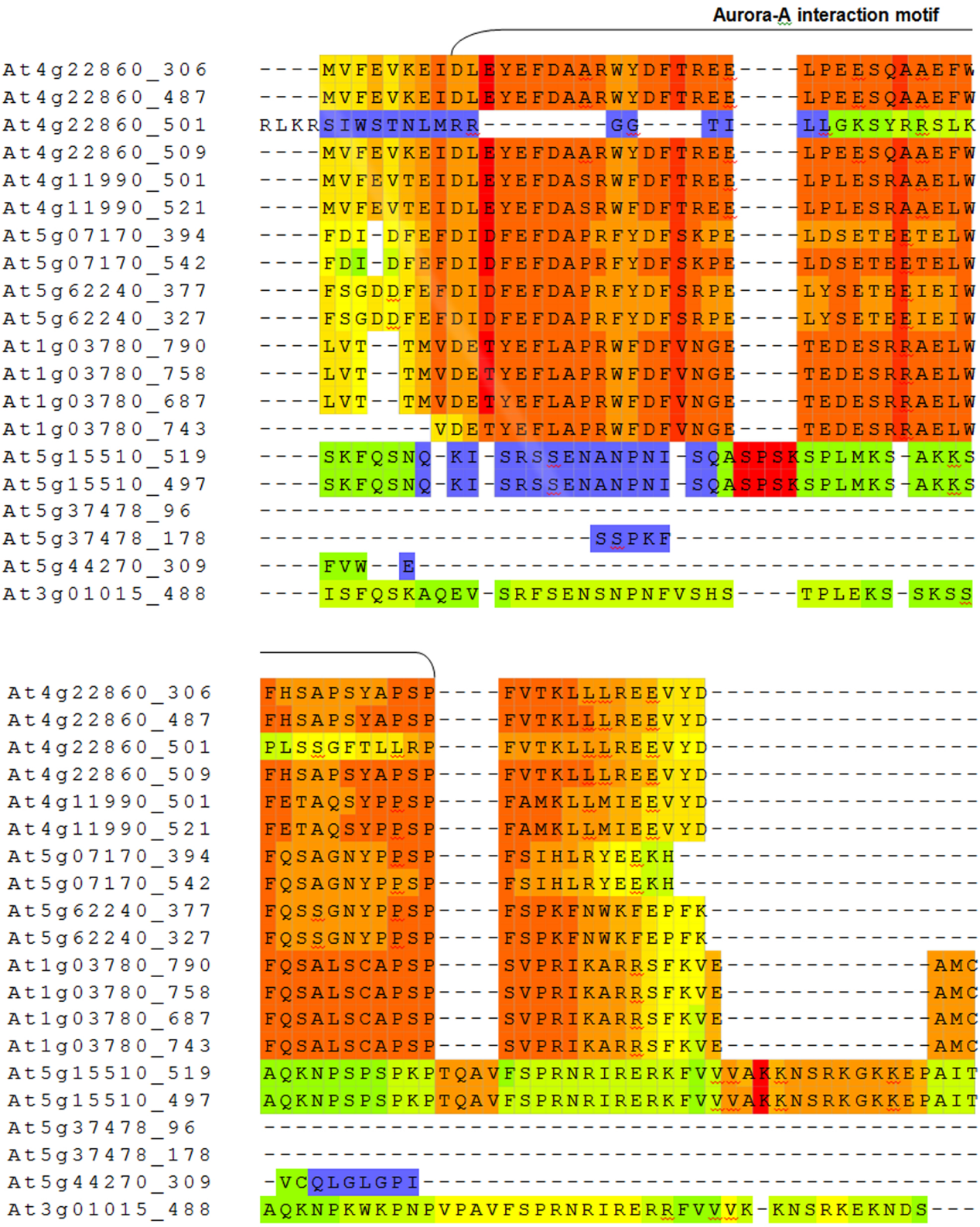
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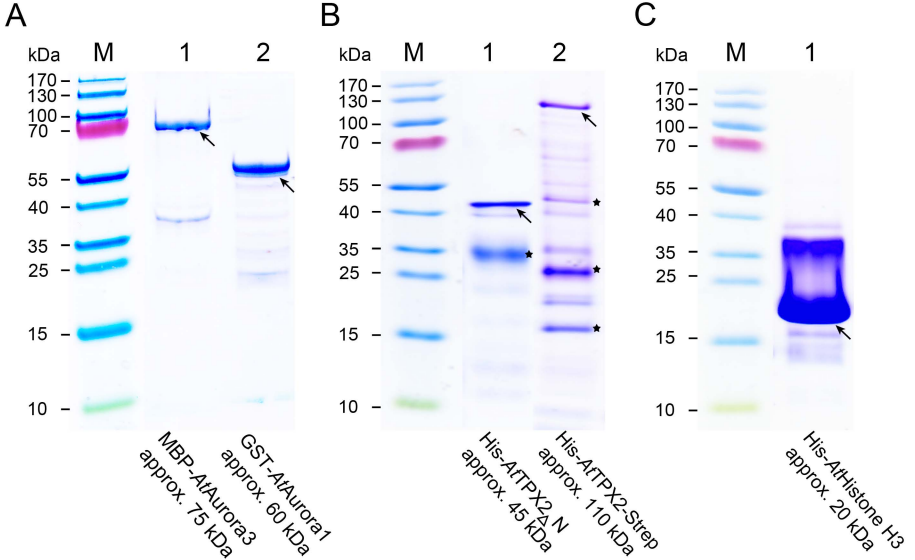
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kDa
M
1
2
170
130
100
70
55
40
35
25
15
10
MBP-AtAurora3
approx. 75 kDa
GST-AtAurora1
approx. 60 kDa
B
kDa
M
1
2
170
130
100
70
55
40
35
25
15
10
His-AtTPX2_N
approx. 45 kDa
His-AtTPX2-Strep
approx. 110 kDa
C
kDa
M
1
170
130
100
70
55
40
35
25
15
10
His-AtHistone H3
approx. 20 kDa

APPENDIX IV

Význam Aurora kináz u rostlin

Tomaštíková E

In: Vesmír 2012, 91 (142):327 – 328.

pohled se může zdát, že vazby mezi takovým množstvím druhů jsou čistě náhodné a výskyt i četnost jednotlivých „etnik" nemá hlubší význam. Ovšem stejně jako v případě lidského osídlení Země se při podrobnějším zkoumání ukáže, že právě rovnováha sil na mnoha frontách, jejich počet, potenciální možnosti i strategické umístění tvoří křehký a komplikovaný ekosystém vazeb, jenž – nemá-li dojít ke krizi – se musí neustále udržovat. Taková společenství nazýváme mikrobiomy[2] a jejich komplexní struktura dává tušit, že navzdory obecné typologii jednotlivých „pracovních skupin" se v detailech jedinec od jedince liší. A právě mikrobiomy jsou klíčem k pochopení toho, jak nejspíš tradiční medicína funguje.[3] Principiálně existují dva způsoby. Společenstvo mikrobiomu může buď přímo modifikovat účinné chemické látky obsažené v bylinách a následně je potom „přepouštět" v podobě, s níž si již naše tělo umí poradit, anebo naopak některé látky obsažené v bylinných medikamentech ovlivňují rovnováhu v zastoupení jednotlivých bakteriálních populací, a tím mění „pravidla hry".

Lano by tu tedy bylo – a světová vědecká obec už po něm i začíná šplhat vzhůru, k čemuž jí dopomáhají především nové možnosti efektivnější sekvenace DNA. Americký Národní institut zdraví v minulém roce spustil projekt lidského mikrobiomu,[4] který je koordinován s evropským projektem MetaHIT.[5] Výstup na Olymp úplného poznání lidského zdraví je asymptotou vzdálené budoucnosti, ale každá velká cesta začíná jedním krokem. Stejně jako v případě sekvenování lidského genomu, jež bylo přímou příčinou zrychleného vývoje mnoha inovativních technologií a bez nadsázky odstartovalo novou éru biologického výzkumu, i tady se biologie a spolu s ní i medicína dostávají na práh nových možností poznání. Práh, za nímž leží možná poslední velká *Terra incognita*. Kdoví, jak budou za deset let vypadat léčebné praktiky u mnoha dosud těžko uchopitelných civilizačních chorob a s čím vším si lékaři budou umět poradit lépe než dnes. Do té doby možná přibude na příbalových letáčcích mnoha syntetických, ale i přírodních léčiv nenápadné oznámení: „Bakterie nejsou součástí balení." Zatím.

1) Jaroslav Flegr: Úvod do evoluční biologie, Academia, Praha 2007, s. 33–34.

2) Pozor, nejde o ekologický termín *biom* s předponou mikro (i když významově by dával smysl), nýbrž o pojem pro souhrn veškerých mikrobiálních populací žijících v prostředí hostitelského makroorganismu – podobně jako termín genom označuje souhrn veškeré genetické informace v buňce.

3) J. M. Crow: That healthy gut feeling. Nature 480, 888–889, 2011/7378.

4) Human Microbiome Project; viz http://commonfund.nih.gov/hmp/.

5) Metagenomics of the Human Intestinal Tract; viz http://www.metahit.eu/.

Význam **aurorakináz** u rostlin

EVA TOMAŠTÍKOVÁ

Aurorakinázy jsou proteiny významné pro regulaci buněčného dělení, jejichž funkce byla popsána zejména u živočichů. Svůj název získaly podle lokalizace v průběhu dělení buňky – vyskytují se na pólech mitotického vřeténka – podobně jako polární záře – *aurora borealis* – na severním pólu Země. Je známo, že fosforylací nejrůznějších proteinů řídí segregaci sesterských chromatid, a tím přesné rozdělení genetického materiálu do dceřiných buněk. Aurorakinázy hrají také významnou roli ve správném načasování cytokineze. U živočichů může vést nadměrné množství proteinu či jeho zvýšená aktivita ke vzniku nádorů, proto jsou aurorakinázy častými cíli protinádorové terapie.

Rostlinné aurorakinázy nejsou tak podrobně prozkoumány jako ty živočišné, ale v posledním roce byly publikovány dvě významné práce zabývající se úlohou aurorakináz v regulaci buněčného dělení u modelové rostliny *Arabidopsis thaliana*; jejich autoři dospěli různými metodami k podobným závěrům. Skupina vědců z olomouckého pracoviště Ústavu experimentální botaniky AV ČR zkoumala funkci aurorakináz u rostlin s využitím techniky posttranskripčního umlčování genové exprese metodou RNA interference (RNAi), tedy pomocí exprese krátkých molekul RNA, které jsou komplementární s částí sekvence genu, jejž chceme umlčet. Vzniklé dvouřetězcové uspořádání je pak enzymaticky rozštěpeno. U RNAi rostlin, které měly sníženou expresi všech tří aurorakináz přítomných u *A. thaliana*, byla pozorována řada poruch buněčného dělení. Rostliny měly zakrslé hlavní kořeny; pokud se hlavní kořen vyvinul, buněčné přepážky v něm byly nepravidelně uspořádány. Růst primárních meristémů byl zastaven, kořenové vlásky měly poškozeny polární růst, bylo pozorováno seskupování průduchů jako důsledek narušeného dělení buněk. Analýza RNAi rostlin průtokovou cytometrií ukázala i změny míry endoreduplikace (zdvojení chromozomů bez jejich rozdělení do dvou jader, zvyšující množství genetické informace v buňce). Narušenou kontrolou procesu endoreduplikace autoři vysvětlují i poruchy větvení trichomů či různé velikosti jader buněk.

Druhá skupina vědců z Gentu využila ke studiu role aurorakináz T-DNA inzerční mutanty *A. thaliana*. Tyto rostliny mají v genu pro příslušnou aurorakinázu vložený krátký úsek cizorodé DNA (tzv. T-DNA), který pak znemožňuje její přepis a následnou syntézu proteinu. Rostliny, které měly zmutovanou

K DALŠÍMU ČTENÍ

Petrovská B. et al.: Plant Aurora kinases play a role in maintenance of primary meristems and control of endoreduplication. New Phytologist 193, 590–604, 2011.

Van Damme D. et al.: Arabidopsis α Aurora kinases function in formative cell division plane orientation. The Plant Cell 23, 4013–4024, 2011.

pouze jednu kinázu, neprojevovaly žádné změny fenotypu. Pokud však byly tyto rostliny zkříženy, výslední dvojití mutanti, kteří měli nefunkční aurorakinázy 1 i 2, dosahovali menšího vzrůstu nadzemní části rostlin, starší rostliny měly kratší kořeny a méně bočních kořenů. Po podrobné analýze uspořádání buněk a buněčných dělení v místech, kde jsou zakládány boční kořeny, autoři prokázali, že i tyto mutantní rostliny, podobně jako výše zmíněné RNAi rostliny, mají nesprávně orientované buněčné dělení a následně i přepážku, která se vytváří mezi dceřinými buňkami.

Jak je zřejmé, živočišné a rostlinné aurorakinázy mají mnoho společného, zejména pokud jde o regulaci buněčného dělení. Bude tedy zajímavé sledovat, jaké další úlohy budou pro rostlinné proteiny objeveny. Vysoký potenciál by mohlo mít studium signálních drah zapojených do regulace aurorakinázami, které může pomoci odhalit u eukaryot obecně se uplatňující mechanismy vedoucí k potlačení tumorogeneze.

Preteky
o rýchle sekvenovanie DNA naberajú obrátky

ŠTEFAN VILČEK

Rýchle a lacné sekvenovanie molekúl DNA, t. j. čítanie poradia báz (A, T, G, C) v DNA, je aktuálnou výzvou na rozvoj genomiky, biomedicínskych vedných oblastí a na využitie v medicínskej praxi. Vyvíjajú sa nové metódy sekvenovania, ktoré majú nahradiť staršie s cieľom efektívne získať čo najviac sekvenčných údajov. To je veľmi dôležité napr. pre získanie relevantných údajov v najmodernejšej diagnostickej praxi a efektívnej liečbe. Jedna z novších metód sekvenovania DNA je založená na princípe pretláčania reťazca molekuly DNA cez nanopórový otvor z bielkovinových molekúl. Na čítanie poradia báz v DNA využíva zmenu elektrického impulzu vyvolanú prechodom báz DNA cez nanopórový otvor (Vesmír 91, 163, 2012/2), čím sa umožňuje sekvenovanie neobyčajne dlhých fragmentov DNA.

Hoci sekvenovanie na princípe využitia nanopórov bolo publikované v odbornej literatúre už v polovici 90. rokov, skutočné využitie tejto technológie v praxi sa črtá až v súčasnosti. Firma Oxford Nanopore Technologies začiatkom tohto roka oznámila, že vyvinula dve zariadenia, jedno ako príručný minisekvenátor, druhé ako väčší laboratórny sekvenačný prístroj, ktoré sú schopné rýchle sekvenovať dlhé fragmenty DNA. Minisekvenátor vo forme modifikovaného USB kľúča, ktorý môže byť pripojený k notebooku alebo klasickému PC, má 500 nanopórových čipov na sekvenovanie DNA. Nanopórový čip číta poradie báz v molekule DNA rýchlosťou 20–400 báz za sekundu, pričom prečíta fragmenty DNA dlhé až desiatky tisíc nukleotidov. Napríklad genóm bakteriofága lambda dlhý 48 000 nukleotidov spracovalo toto zariadenie ako jeden kompletný fragment DNA. Pripomeňme, že terajšie sekvenátory dokážu prečítať fragmenty DNA dlhé iba niekoľko desiatok až tisíc nukleotidov. Príručný nanopórový minisekvenátor je síce len na jedno použitie, ale jeho cena sa pohybuje iba okolo 900 dolárov, čo je v porovnaní s terajšími megacenami sekvenátorov nízka položka. Väčší laboratórny sekvenátor má 2000 nanopórových čipov a za 24 hodín spracuje desiatky GB sekvenčných údajov. Už na budúci rok sa plánuje dokončenie vývoja sekvenátora s 8000 nanopórmi. Odborníci predpokladajú, že s využitím nanopórových sekvenátorov bude celý ľudský genóm prečítaný za 15 minút za 1500 dolárov a už na budúci rok môže cena tejto operácie klesnúť pod 1000 dolárov (Biotechniques, 23 February 2012). A to je už prijateľná suma aj pre praktické využitie nových sekvenátorov, napr. v medicíne.

Prvé sekvenovanie celého ľudského genómu, ktoré bolo v projekte HUGO dokončené v r. 2000, trvalo desať rokov a pracovalo na ňom množstvo špecializovaných vedeckých tímov z celého sveta. Ambiciózny projekt zhltol viac ako 3 miliardy dolárov. Odvtedy ale vývoj sekvenovania DNA pokračuje míľovými krokmi. Už v roku 2008 bolo možné uskutočniť sekvenciu celého ľudského genómu za niekoľko týždňov, pričom jej cena klesla na milión dolárov. Onedlho budeme nanopórovými sekvenátormi celý genóm poznať za desiatky minút za cenu asi 1000 dolárov. Problémom pri nanosekvenovaní zostáva ešte pomerne veľká chyba pri čítaní poradia báz v DNA (okolo 4 %), ale pracuje sa na jej znížení pod 1 %.

Ukazuje sa, že v priebehu niekoľkých rokov budú vytvorené podmienky na to, aby sa pacientovi pri návšteve lekára po odobratí krvi rýchle izolovala DNA z buniek, prečítal celý genóm a vyhodnotil jeho genetický profil. Táto genetická informácia poslúži na podstatne cielenejšiu a presne definovanú liečbu. Personalizovaná medicína je na prahu nemocníc a zaiste čoskoro vstúpi aj do lekárskych ordinácií.

Prof. Ing. Štefan Vilček, DrSc., (*1950) vyštudoval chémiu na ČVUT v Prahe. Zaoberá sa detekciou a charakterizáciou vírusov na genetickej úrovni. Vyvinul viaceré PCR testy na diagnostiku infekčných chorôb hospodárskych zvierat a venuje sa aj molekulárnej epizootológii. Teraz pracuje na Univerzite veterinárskeho lekárstva a farmácie v Košiciach.

APPENDIX V

Characterization of *Arabidopsis At*RanSPRY protein, a new member of RanGTPase pathway

Tomaštíková E, Binarová L, Cenklová V, Petrovská B, Gelová, Z, Pochylová Ž, Tyčová M, Binarová P

In: 19. Cytoskeletal Club 2011, Vranovská Ves, Czech Republic.
(oral presentation abstract)

Characterization of *Arabidopsis At*RanSPRY protein, a new member of RanGTPase pathway

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The Ran GTPase proteins (Ras related nuclear proteins) are important regulators of nucleocytoplasmic transport that function as a molecular switch, in which the binding of GDP or GTP regulates its interaction with effectors. In addition to their role in nucleocytoplasmic transport, Ran GTPases are involved in regulation of cell division and nucleation of microtubules. Compare to the well understood function of Ran GTPases in chromatin driven microtubule nucleation in metazoan cells not much is known about their possible role in plants. Here we provide data on a yet uncharacterized protein At RanSPRY that shows homology with RanBPM protein involved in microtubule nucleation in animal cells. We prepared GFP fusion and analysed At RanSPRY-GFP *in vivo* in *Arabidopsis* cultured cell and in plants. Our data revealed that At RanSPRY-GFP is localized in cytoplasm and accumulated around interphase nuclei and dispersed around mitotic spindle during mitosis. Our *in vivo* analysis of *Arabidopsis* plants showed expression of At RanSPRY-GFP in perinuclear area of interphase root meristematic cells as well as in differentiated root and hypocotyl cells. Only weak GFP signal was observed in dividing cells. Next we raised antibody and analyzed endogenous At RanSPRY. Immunolocalization studies confirmed cellular localization and its dynamics during cell cycle progression as it was observed for GFP-fused protein. Futhermore, we found that At RanSPRY colocalized around mitotic spindle together with one of the Ran-binding protein, RanBP1 in mitosis, while another proteins of RanGTPase pathway RCC1 and RanGTP were compare to At RanSPRY localized closer to chromatin. Our data pointed to an implication of yet uncharacterized At RanSPRY in RanGTPase signalling pathway. Its possible role with microtubules as indicated in our experiments will be studied further.

This work was supported by by Grants 204/07/1169, 204/09/P155 from GAČR, grant LC06034 and LC545 from MŠMT, grant IAA500200719 from the Grant Agency of the Czech Republic, the MŠMT and the European Regional Development Fund (Operational Programme Research and Development for Innovations No. CZ.1.05/2.1.00/01.0007) for ET, BP, MT and IGA UP Agency No. PřF_2011_003 for ET, MT.

APPENDIX VI

*At*RanSPRY is a new member of proteins of RanGTPase regulatory pathway

Cenklová V, Petrovská B, Binarová L, Gelová Z, Tomaštíková E, Tyčová M, Volc J, Binarová P

In: Plant Biotechnology: Green for Good 2011, Olomouc, Czech Republic
(poster abstract)

_At_RanSPRY is a new member of proteins of RanGTPase regulatory pathway

Cenklová V[2], Petrovská B[1], Binarová L[2], Gelová Z[2], Tomaštíková E[1], Tyčová M[1], Volc J[3], Binarová P[3]

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Nucleo-cytoplasmic transport of proteins and RNAs and mitotic spindle assembly are ubiquitously regulated by GTP/GDP cycles of highly conserved Ran GTPase proteins (Ras-related nuclear proteins) from Ras superfamily of proteins. We studied yet uncharacterized member of Ran GTPases a SPRY domain containing protein At RanSPRY that is homologous to RanBPM protein in animals. At RanSPRY-GFP showed higher expression in dividing cells of *Arabidopsis* plants and in dividing cultured cells. At RanSPRY protein was localized in cell cycle specific manner in cytoplasm and accumulated in perinuclear region. Localization pattern as revealed by *in vivo* analysis of At RanSPRY-GFP was confirmed by immunolocalization studies of endogenous protein performed with anti-At RanSPRY antibody that we prepared. At RanSPRY localized together with RanGTP, RanBP1, RCC1 in the cytoplasm and in the perinuclear area. However, neither we observed colocalization of At RanSPRY with Ran GTP and RCC1 in close vicinity of chromatin nor it colocalized with RanBP1 on microtubules. Our data pointed out to the role of At RanSPRY in Ran GTPases driven nucleo-cytoplasmic transport.

This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic and the European Regional Development Fund (Operational Programme Research and Development for Innovations No. CZ.1.05/2.1.00/01.0007) for BP, ET, and MT, by Grants 204/07/1169, 204/09/P155 from the Grant Agency of the Czech Republic, grants LC06034 and LC545 from Ministry of Education Youth and Sports of Czech Republic, and grant IAA500200719 from the Grant Agency of the Czech Republic.

At RanSPRY is a new member of proteins of plant Ran GTPase regulatory pathway

Eva Tomaštíková[1], Lenka Binarová[2], Věra Cenklová[2], Beáta Petrovská[1], Zuzana Gelová[2], Žaneta Pochylová[2], Michaela Tyčová[1], Jindřich Volc[3], Pavla Binarová[3]

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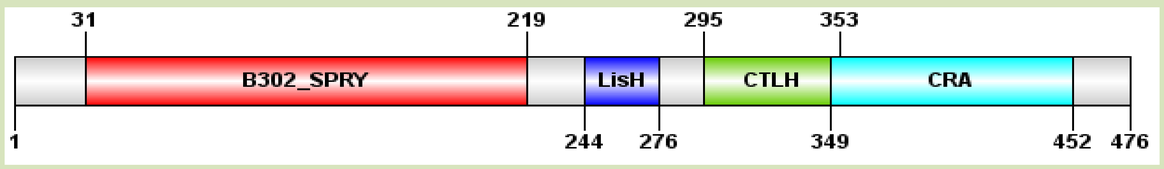
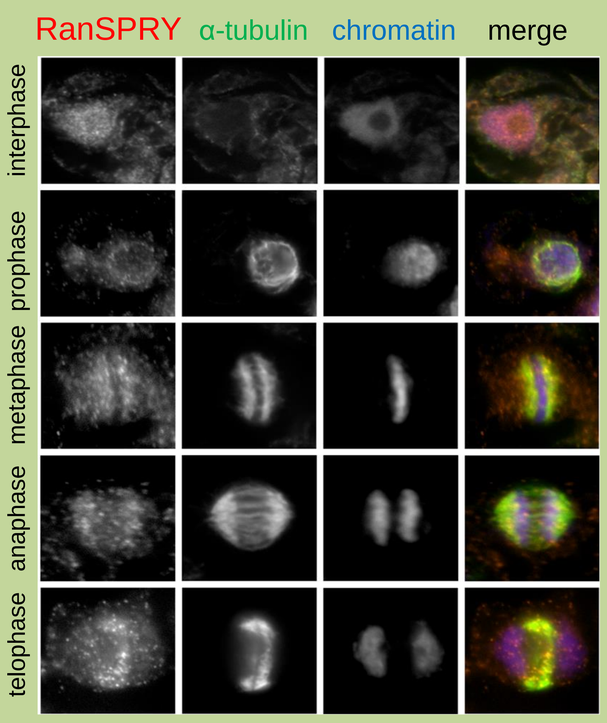
Introduction: The Ran GTPase pathway is involved in direction of nucleocytoplasmic transport that function as a molecular switch - binding of GDP or GTP to the Ran protein (Ras related nuclear protein) regulates its interaction with effectors. In addition to this role, Ran GTPase pathways is also involved in regulation of cell division and nucleation of microtubules. In metazoans cells chromatin-driven microtubule nucleation is well known. However, in plant cells this activity is poorly described. Here, we provide data about newly identified *Arabidopsis* RanSPRY protein (SPRY domain-containing protein), close homolog of human RanBPM protein (Ran-binding protein in microtubule-organizing centre). AtRanSPRY protein possess unique domain organization typical for the members of SCORPIN family of proteins (Hosono *et al*., 2010) and might act as a scaffolding protein.

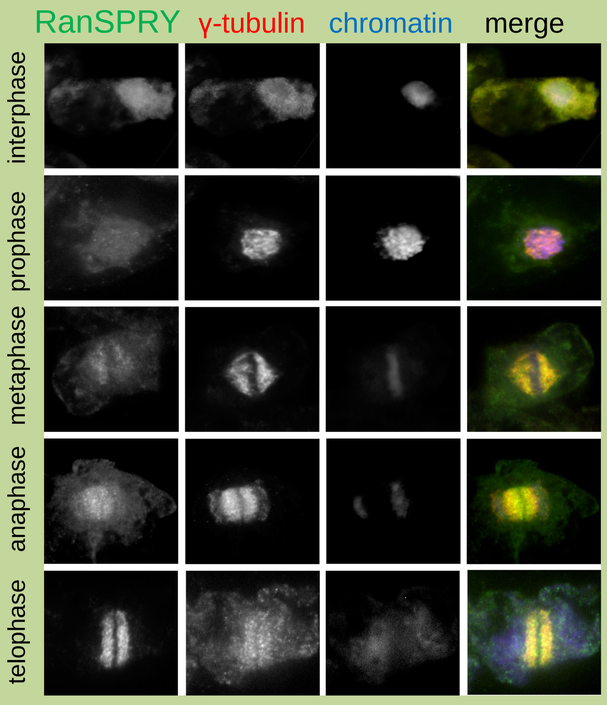


Domain organization of Arabidopsis RanSPRY protein. Domains are responsible for binding to various partners.

Double immunofluorescence labelling

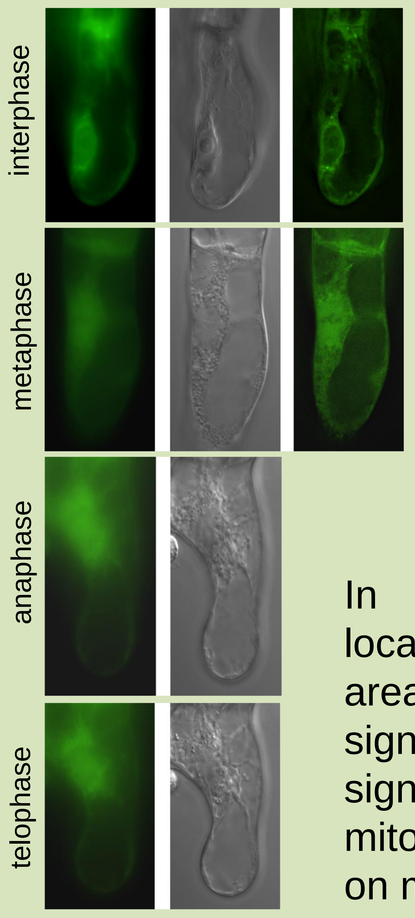


Double immunofluorescence labelling of AtRanSPRY and α-tubulin. Colocalization with microtubules was not observed. AtRanSPRY signal is dispersed around mitotic spindle and accumulate in the area of phragmoplast.

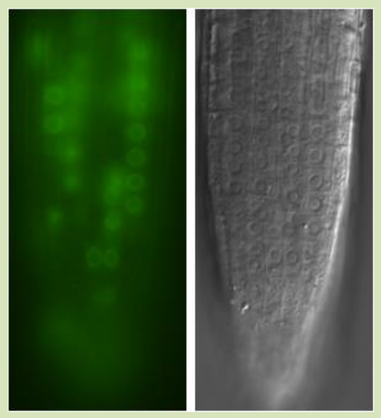


Double immunofluorescence labelling of AtRanSPRY and γ-tubulin. AtRanSPRY is not colocalized with γ-tubulin in microtubule-organizing centers of acentrosomal plant cells.

AtRanSPRY-GFP signal in *Arabidopsis* seedling and cell suspension culture



AtRanSPRY-GFP localization during the cell cycle in *Arabidopsis* cell suspension culture.

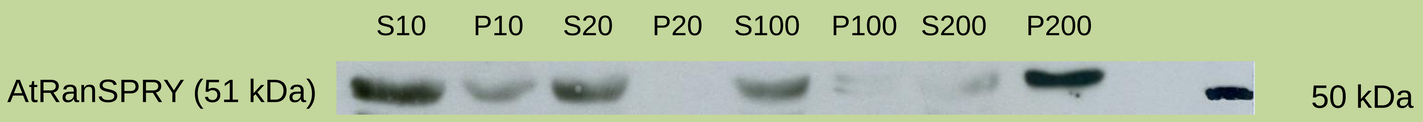


AtRanSPRY–GFP localization in *Arabidopsis* root tip.

In interphase, signal is localized to the perinuclear area and cytoplasm with weak signal in nuclei. During mitosis, signal is dispersed aroung mitotic spindle, with no signal on microtubules and chromatin.

Conclusion: During mitosis, AtRanSPRY is dispersed around mitotic spindle, however it is neither localized on microtubules, nor on chromatin. Our results suggest, that this protein does not have a role with microtubules or with γ-tubulin. Localization in cytoplasm was confirmed by distribution of AtRanSPRY after differential centrifugation. Since it is localized in perinuclear area during interphase, AtRanSPRY might have a role in nucleocytoplasmic transport or act as a scaffolding protein. These putative roles will be further tested.

Distribution of the AtRanSPRY in cellular fractions after differential centrifugation. Majority of the AtRanSPRY protein was present as a soluble pool in cytoplasm.



References:
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This work was supported by Supported by 204/07/1169, 204/09/P155, LC06034, LC545, IAA500200719, CZ.1.05/2.1.00/01.0007) for ET, BP, MT and IGA UP PřF_2011_003 for ET, MT.

APPENDIX VII

Arabidopsis Aurora1 and its activator TPX2 associate with microtubules and form a polar gradient during rearrangement of mitotic microtubules

Petrovská B, Cenklová V, Pochylová Ž, Kourová H, Tyčová M, Tomaštíková E, Binarová L, Binarová P

In: Plant Biotechnology: Green for Good 2011, Olomouc, Czech Republic
(poster abstract)

Arabidopsis Aurora1 and its activator TPX2 associate with microtubules and form a polar gradient during rearrangement of mitotic microtubules

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Aurora kinases are a family of conserved serine/threonine kinases that are major regulators of mitotic progression and cytokinesis. Human Aurora A, together with its activator TPX2, plays an important role in spindle assembly, and an interaction between both molecules is required to determine spindle length, primarily via the nucleation of microtubules. Although functions of animal and yeast Aurora kinases and TPX2 have been analyzed, interaction between plant Aurora kinases and TPX2 protein is not well understood.Our *in vivo* and *in situ* microscopical analysis revealed that plant At Aurora1 localized together with its activator At TPX2 on mitotic microtubules. Consistent with microscopical observation were data from *in vitro* taxol driven polymerization of plant microtubules that confirmed association of At Aurora1 and At TPX2 with microtubules. Furthermore we found that At Aurora1 kinase and At TPX2 protein formed gradient from spindle poles to the part of phragmoplast distal of the cell plate during anaphase/ telophase transition. Truncated version of At TPX2 that lack both Aurora binding sites showed disturbed polar localization of At TPX2 with microtubules. We found that interaction of At TPX2 with the kinase was a prerequisite for its polar localization with microtubules. Only At Aurora1 but not At TPX2 was translocated to the area of forming cell plate. Our data on the interaction of At Aurora1 with its activator At TPX2 suggested that the kinase has properties of AuroraA kinase of animal cells. .

This work was supported by Centre of the Region Haná for Biotechnological and Agricultural Research for BP, MT, and ET (Grant No. ED0007/01/01), Grant 204/09/P155, 204/07/1169, 204/09/H084 from the Grant Agency of the Czech Republic, grant LC06034 from Ministry of Education Youth and Sports of Czech Republic, and grant IAA500200719 from the Grant Agency of the Czech Republic.

AtAurora1 and its activator TPX2 associate with microtubules and form a polar gradient during rearrangement of mitotic microtubules.

Beáta Petrovská[1], Věra Cenklová[2], Žaneta Pochylová[2], Hana Kourová[3], Michaela Tyčová[1], Eva Tomaštíková[1], Lenka Binarová[2], and Pavla Binarová[3]

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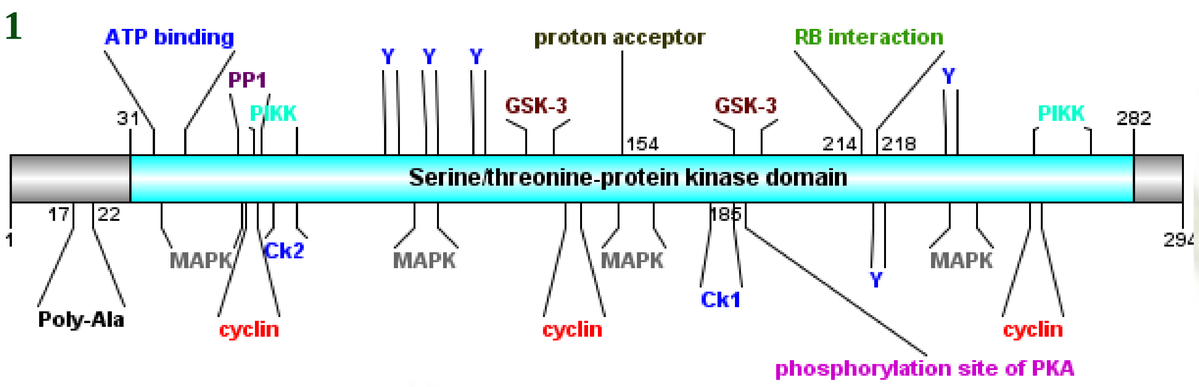
Introduction

Aurora kinases are a family of conserved serine/threonine kinases that are major regulators of mitotic progression and cytokinesis. Human Aurora A, together with its activator TPX2, plays an important role in spindle assembly, and an interaction between both molecules is required to determine spindle length, primarily via the nucleation of microtubules. Although functions of animal and yeast Aurora kinases and TPX2 have been analyzed, interaction between plant Aurora kinases and TPX2 protein is not well understood.

Results

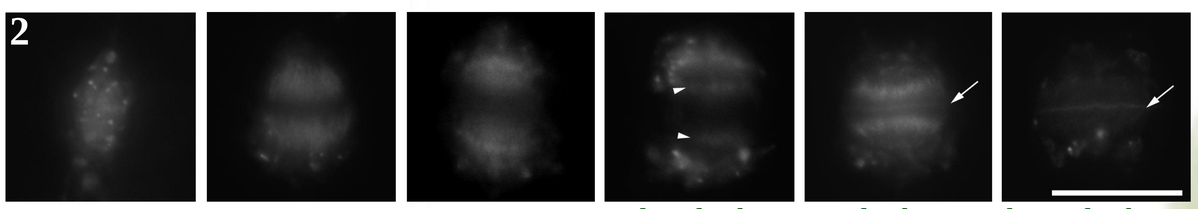
1



Detailed domain graph (Fig.1) of *Arabidopsis* Aurora1 with cyclin interaction sites (amino acids 60-63, 140-144, 256-259), Retinoblastoma (RB) interaction sites (214-218), MAPK docking motifs (39-47, 49-59, 51-59, 102-108, 153-162, 236-243), PP1 motif (58-64), Ck I and II phosphorylation sites (176-182, 67-73, respectively), GSK-3 phosphorylation sites (130-137, 182-189), PIKKs phosphorylation sites (62-68, 67-73, 257-263, 265-271), and Tyrosine (Y)-based sorting signals (95-98, 105-108, 117-120, 217-220, 235-238).

In vivo and *in situ* microscopical analysis revealed that plant AtAurora1 (Fig.2) localized together with its activator AtTPX2 (Fig.3) on mitotic microtubules.

2



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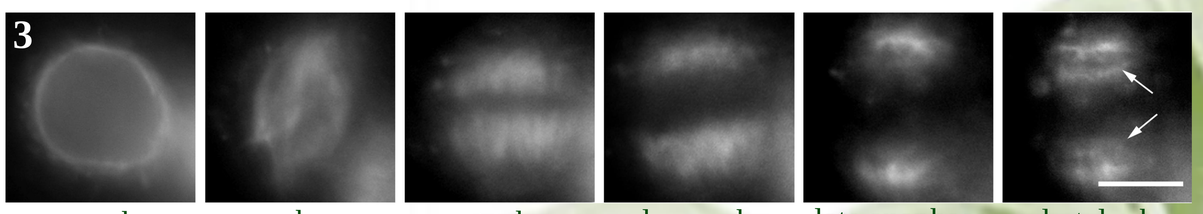

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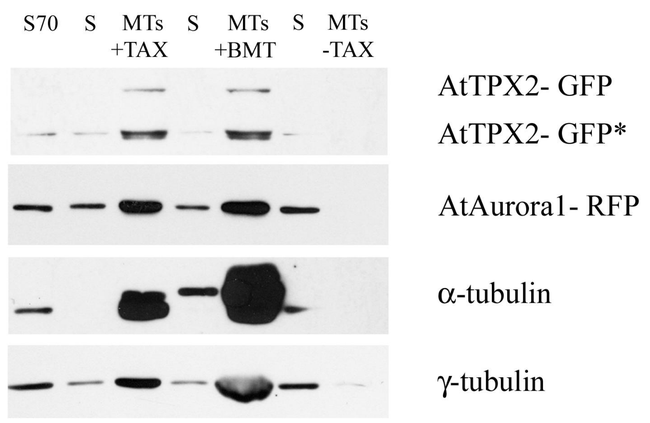

Consistent with microscopical observation were data from *in vitro* taxol driven polymerization of plant microtubules that confirmed association of AtAurora1 and AtTPX2 with microtubules (Fig.4).

AtAurora1 kinase and AtTPX2 protein formed gradient from spindle poles to the part of phragmoplast distal of the cell plate during anaphase/telophase transition (Fig.5).

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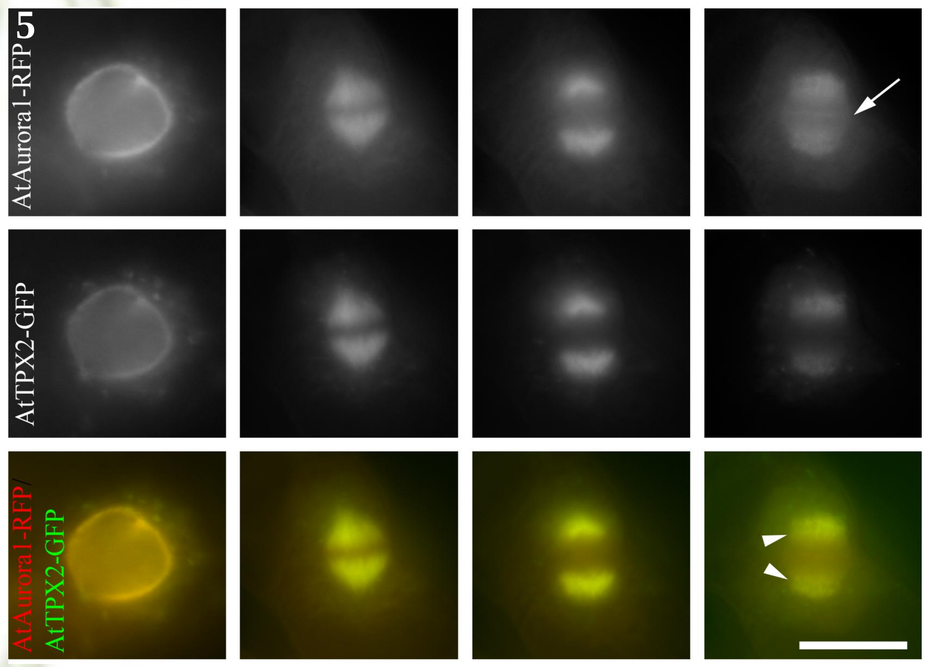

Truncated version of AtTPX2 that lack both Aurora binding sites (Fig.6) showed disturbed polar localization of AtTPX2 with microtubules (Fig.7).

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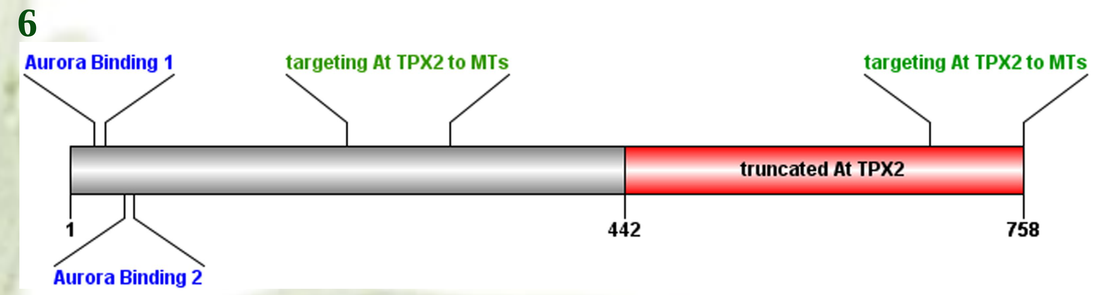

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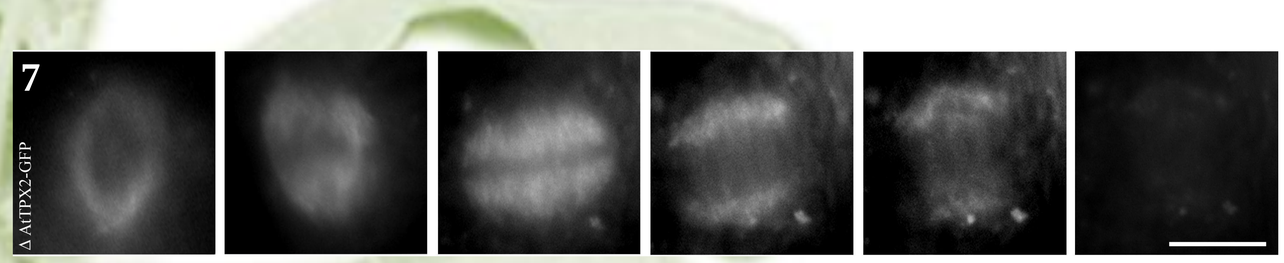

Conclusions

We found that interaction of AtAurora1 was a prerequisite for polar localization of AtTPX2 with microtubules and its stability until late anaphase/telophase transition. Only AtAurora1 but not AtTPX2 was translocated to the area of forming cell plate. Our data suggest that AtAurora1 has properties of Aurora A and B kinases of animal cells.

This work was supported by Centre of the Region Haná for Biotechnological and Agricultural Research for BP, MT, and ET (No. CZ.1.05/2.1.00/01.0007), Grant 204/09/P155, 204/07/1169, 204/09/H084 from the Grant Agency of the Czech Republic, grant LC06034 from Ministry of Education Youth and Sports of Czech Republic, and grant IAA500200719 from the Grant Agency of the Czech Republic.

APPENDIX VIII

Characterization of SPRY-domain containing protein At RanSPRY in *Arabidopsis thaliana*

Tomaštíková E, Binarová L, Cenklová V, Petrovská B, Gelová Z, Pochylová Ž, Tyčová M, Volc J, Binarová P

In: IX. International Conference of Ph.D. Students of Experimental Plant Biology „Fresh Insights in Plant Affairs“ 2011, Prague, Czech Republic.
(poster abstract)

Characterization of SPRY-domain containing protein At RanSPRY in *Arabidopsis thaliana*

Tomaštíková E[1], Binarová L[2], Cenklová V[2], Petrovská B[1], Gelová Z[2], Pochylová Ž[2], Tyčová M[1], Volc J[3], Binarová P[3]

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The RanGTPase pathway is involved in regulation of many cellular events, including microtubule nucleation, nuclear envelope and mitotic spindle assembly, and nucleocytoplasmic transport. Spatial localization of the GTP-bound form of Ran regulated by Ran-specific nuclear GEFs (guanine nucleotide exchange factors) and cytoplasmic GAPs (GTPase activating proteins) are crucial factors regulating the pathway. Although the RanGTPase pathway is well-understood in animal cells plant Ran and Ran-binding proteins are still poorly described. Here, we provide data about newly characterized *Arabidopsis* SPRY-domain containing protein (At RanSPRY, At1g35470). At RanSPRY possess same domain organization and high sequence homology with human RanBPM protein that was in a truncated version colocalized with centrosome (Nakamura *et al*., 1998). We prepared GFP fusion and analysed localization of At RanSPRY-GFP *in vivo* in *Arabidopsis* cultured cells and plants. At RanSPRY-GFP signal was localized in cytoplasm and around nuclei during interphase, in mitosis dispersed signal was found in the mitotic spindle area. In *Arabidopsis* plants At RanSPRY-GFP signal was clearly visible in perinuclear area of interphase cells of elongation root zone. We did not observe increase in intensity of GFP signal in dividing cells. Next, we raised antibody against peptide from At RanSPRY protein sequence. Immunolocalization confirmed cellular localization of GFP-fused protein observed *in vivo.* Similar localization pattern was observed for RanBP1 protein and not for RanGTP and RCC1 protein. Taken together our data suggest that compare to truncated version of animal homologue of RanBPM protein the At RanSPRY protein is not associated with microtubules or microtubule nucleation centra. The role of At RanSPRY in *Arabidopsis* will be further tested.

Supported by Grants 204/07/1169, 204/09/P155 from GAČR, grant LC06034 and LC545 from MŠMT, grant IAA500200719 from the Grant Agency of the Czech Republic, the MŠMT and the European Regional Development Fund (Operational Programme Research and Development for Innovations No. CZ.1.05/2.1.00/01.0007) for ET, BP, MT and IGA UP Agency No. PřF_2011_003 for ET, MT.

Characterization of SPRY-domain containing protein *At*RanSPRY in *Arabidopsis thaliana*

Eva Tomaštíková[1], Lenka Binarová[2], Věra Cenklová[2], Beáta Petrovská[1], Zuzana Gelová[2], Žaneta Pochylová[2], Michaela Tyčová[1], Jindřich Volc[3], Pavla Binarová[3]

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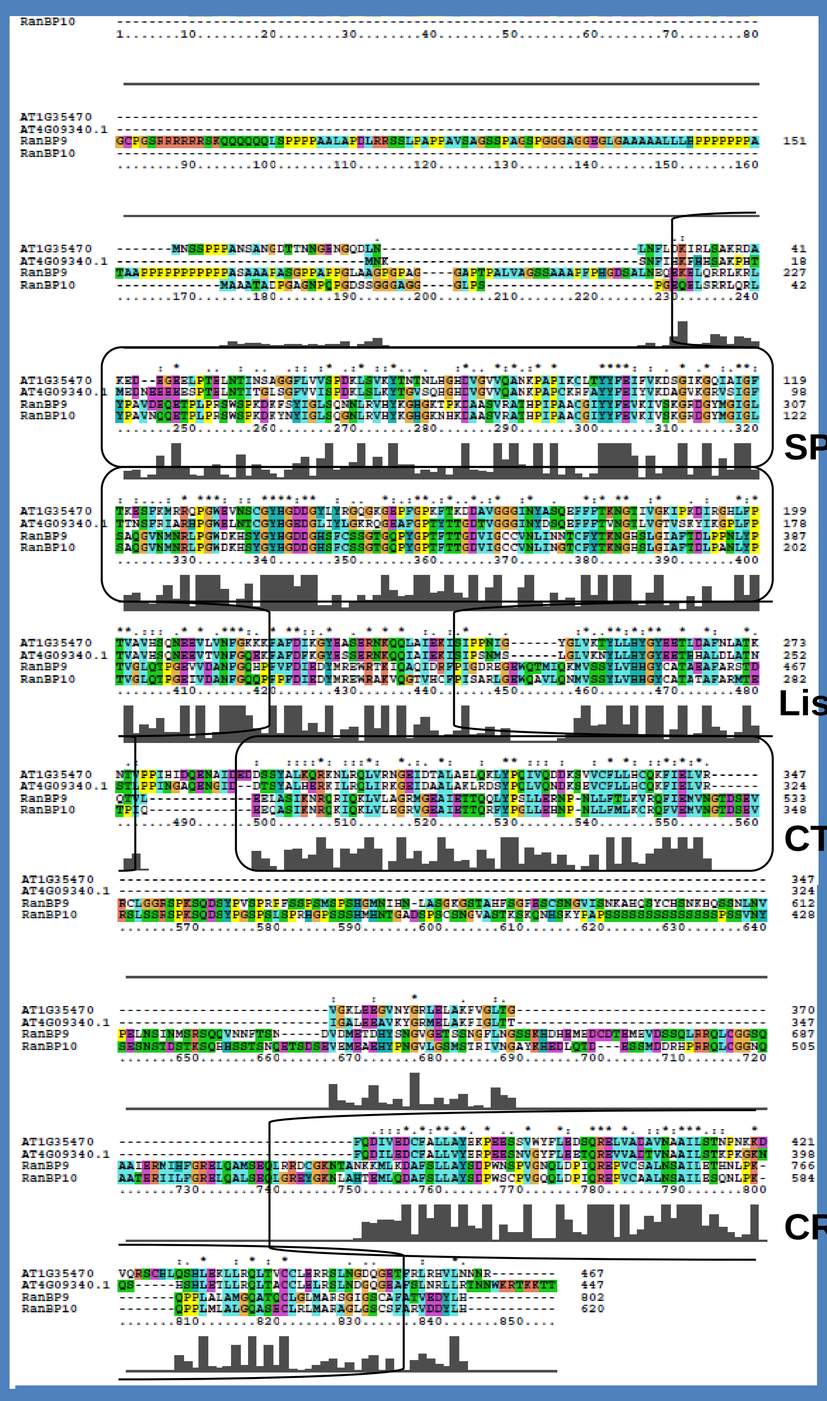
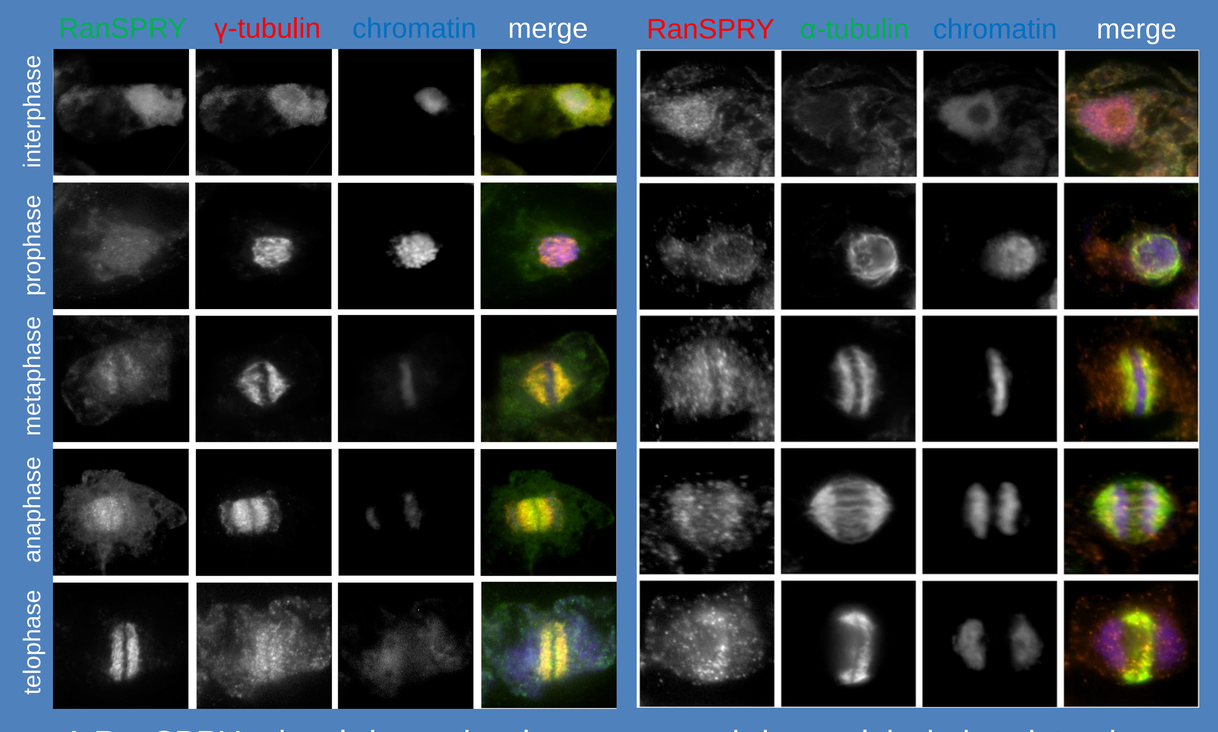
[3] Institute of Microbiology, AS CR, v.v.i., Vídeňská 1083, 142 20 Prague 4, Czech Republic.

Introduction: Ran (Ras related nuclear small GTPase) is involved in nucleocytoplasmic transport, microtubule assembly and nuclear membrane formation. RanBPM, a Ran-binding protein in microtubule organizing centre was localized in the centrosomes. Later centrosomally located protein was shown to be a truncated version of whole molecule of RanBPM that was predominantly localized extra of centrosomes in nucleus and in cytoplasm and formed protein complex of more than 670 kDa (Nishitani *et al*., 2001). We compared localization of γ-tubulin that is a marker of microtubule nucleation with cellular localization of newly identified AtRanSPRY (*Arabidopsis* RanSPRY domain-containing protein). We analysed putative role of the AtRanSPRY role in Ran GTPase pathway.

Sequence alignment of AtRanSPRY (chromosome 1), At4g09340 (segmental genome duplication on chromosome 4), human RanBP9 and RanBP10

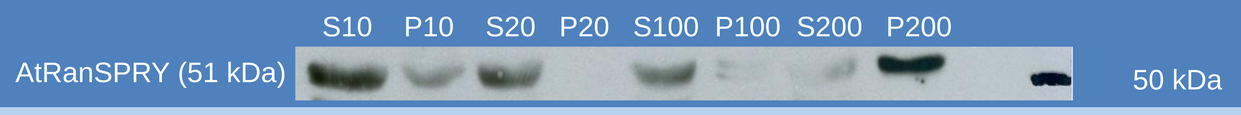


AtRanSPRY imunolocalization with γ-tubulin and α-tubulin in *Arabidopsis* cells

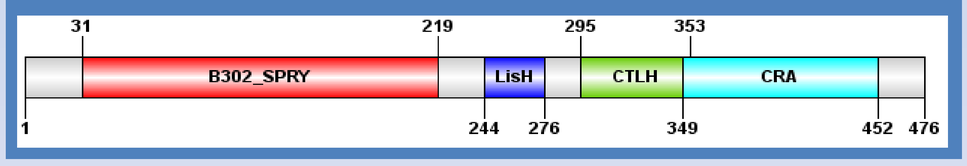


AtRanSPRY signal in perinuclear area and in nuclei during interphase, dispersed signal in area of mitotic spindle and phragmoplast during mitosis did not show colocalization with γ-tubulin

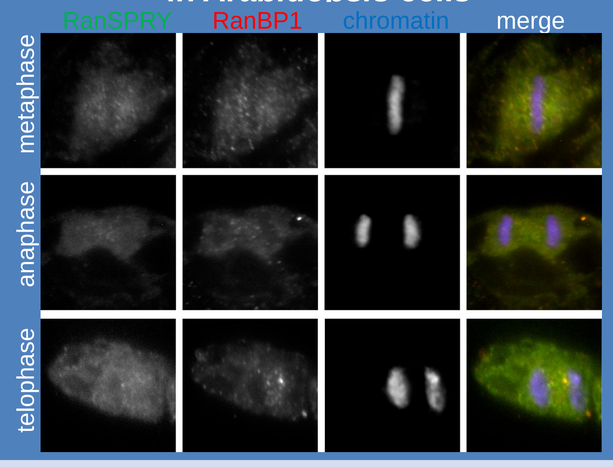
Western blot analysis of cell extracts separated by differential centrifugation showed that the majority of the AtRanSPRY protein was present in the soluble cytoplasmic S100 pool and high speed microsomal pellet (P200).



Predicted domains of AtRanSPRY molecule



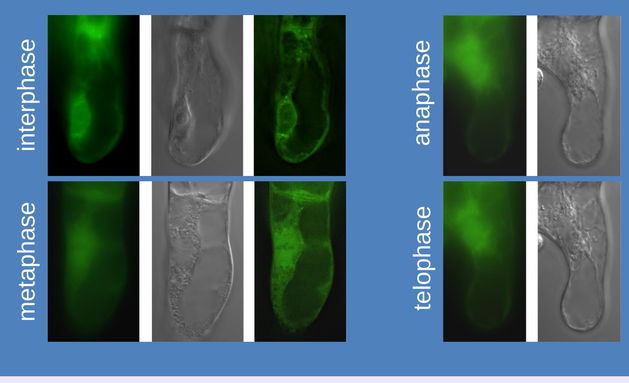
AtRanSPRY imunolocalization with RanGTPase regulator RanBP1 in *Arabidopsis* cells



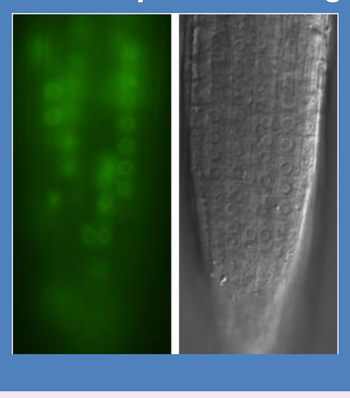
Conclusion:

- AtRanSPRY-GFP localized as dispersed signal in nuclei and perinuclear region during interphase and in the area of mitotic apparatus during cell division.
- We did not prove colocalization of AtRanSPRY with microtubules and with γ-tubulin.
- AtRanSPRY shows similar localization as RanGTPase pathway regulator RanBP1 as dispersed signal around mitotic spindle.
- Our data did not indicate function of AtRanSPRY in γ-tubulin positive nucleation sites similar to that shown for truncated version of RanBPM (Nakamura *et al*., 1998). Whether AtRanSPRY, that possess unique domain organization typical for the members of SCORPIN family of proteins with scafolding functions, (Hosono *et al*., 2010) might act similarly remains to be elucidated.

AtRanSPRY-GFP in *Arabidopsis* cultured cells



AtRanSPRY-GFP localization in *Arabidopsis* seedling



AtRanSPRY-GFP localization in perinuclear area and weak signal in nuclei during interphase, dispersed signal for AtRanSPRY-GFP in area of mitotic spindle during metaphase and anaphase and in area of phragmoplast during telophase.

References:
Hosono K *et al*., *Genomics* **96**:102-111 (2010)
Nishitani H *et al*., *Gene* **272:**25-33 (2001)
Nakamura M *et al*., *J Cell Biol* **143:**1041-1052 (1998)

This work was supported by CZ.1.05/2.1.00/01.0007 for ET, BP, MT , 204/07/1169, 204/09/P155, LC06034, LC545, IAA500200719 and IGA UP PřF_2011_003 for ET, MT.

APPENDIX IX

Protein interaction of *Arabidopsis* RanBPM homologue reveal conservation of CTLH complexes in eukaryotes

Tomaštíková E, Petrovská B, Kohoutová L, Cenklová V, Halada P, Binarová P

In: X International Conference of Ph.D. Students of Experimental Plant Biology 2012, Brno, Czech Republic

(oral presentation abstract)

Protein interaction of *Arabidopsis* RanBPM homologue reveal conservation of CTLH complexes in eukaryotes

Tomaštíková E[1], Petrovská B[1], Kohoutová L[3], Cenklová V[2], Halada P[3], Binarová P[3]

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[3]*Institute of Microbiology, AS CR, v.v.i., Vídeňská 1083, 142 20 Prague 4, Czech Republic.*

RanBPM (Ran-binding protein in the microtubule-organizing centre) was originally reported as a centrosome-associated protein in human cells. However, RanBPM protein is currently considered as a scaffolding protein with multiple cellular functions. Based on sequence similarity, we identified a homologue of the human RanBPM in *Arabidopsis thaliana* (AtRanBPM, At1g35470). AtRanBPM protein has highly conserved SPRY, LisH, CTLH and CRA domains. We prepared GFP fusion and analysed the localization and distribution of the protein in *Arabidopsis* cultures and plants. Microscopic analysis of GFP-AtRanBPM *in vivo* and immunofluorescence localization of endogenous AtRanBPM showed mainly cytoplasmic and nuclear localization. Absence of colocalization with γ-tubulin was consistent with the biochemical data and suggests another than centrosomal role of the AtRanBPM protein. Cell fractionation showed that endogenous AtRanBPM or expressed GFP-AtRanBPM are mainly cytoplasmic proteins with only a minor portion detectable in microsomal fractions. AtRanBPM was identified predominantly in the form of soluble cytoplasmic complexes. Immunopurification of AtRanBPM followed by mass spectrometric analysis identified proteins containing LisH and CRA domains. Homologues of copurified proteins are known to be components of the C-terminal to the LisH motif (CTLH) complexes in humans and budding yeast. Taken together, our data indicate that as yet uncharacterized *Arabidopsis* RanBPM protein physically interacts with LisH-CTLH domain-containing proteins and forms high molecular weight cytoplasmic protein complexes homologous to similar complexes in human and budding yeast. Although the exact functions of the CTLH complexes in scaffolding of protein degradation in protein interactions and in signalling from the periphery to the cell centre are not yet fully understood, structural conservation of the complexes across eukaryotes suggests their important biological role.

Supported by the European Regional Development Fund No. CZ.1.05/2.1.00/01.0007 for ET and BP, IGA UP PrF/2012/001 for ET, grants LC06034 and LC545 from MEYS of Czech Republic, 204/07/1169, 204/09/P155, and 204/09/H084 from the GACR, and grant IAA500200719 from GAAV.

APPENDIX X

Differential activity of *Arabidopsis* Aurora kinase members towards histone H3

Tomaštíková E, Doležel J, Houben A, Demidov D

In: 9th Plant Science Student Conference 2013, Halle, Germany

(poster abstract)

Differential activity of Arabidopsis Aurora kinase members towards histone H3

Eva Tomaštíková[1*], Jaroslav Doležel[1], Andreas Houben[2], Dmitri Demidov[2§]

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[2]*Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstr. 3, 06466 Gatersleben, Germany*

Histones are subjected to a variety of post-translational modifications (PTM). Such modifications are required for interactions with specific protein domains and regulate many aspects of chromosome activity. Phosphorylation of histone H3 is crucial for cell-cycle dependent chromosome condensation and segregation. Various H3 kinases have been identified in plant, such as Aurora and Haspin. Members of the Aurora kinase family are responsible for cell cycle-dependent phosphorylation of serine 10 and serine 28 of H3 in both plant and non-plant organisms. In non-plant species cross-talk occurs between phosphorylation of H3S10 and acetylation or methylation of H3. For plants, information on the cross-regulation between H3S10 phosphorylation and PTMs of neighboring amino acids is limited. Data for the cross-talk between phosphorylation of H3S10 by AtAurora3 and other neighboring PTMs or around H3S28 by AtAurora1 and 3 are missing.
To elucidate whether the phosphorylation activity of the Aurora kinase family members of *Arabidopsis* towards H3S10 and H3S28 is influenced by neighboring PTMs, an *in vitro* kinase assay was performed. As substrates, N-terminal peptides of H3 with various modifications close to S10 and S28, were used. Recombinant AtAurora1 and AtAurora3 were expressed in *E. coli*. Our kinase assay confirmed that both serine positions are phosphorylated by both kinases *in vitro*, although phosphorylation of H3S28 is much weaker than of H3S10. However, H3S28 phosphorylation is increased by dimethylation and acetylation of neighboring K27. H3S10 phosphorylation by AtAurora1 is increased by dimethylation of H3K4 and phosphorylation of H3T6. Decrease in kinase activity was observed for acetylated H3K4.
Taken together our data suggest that a cross-talk between different H3 modifications occurs also in plants, although in similar rather than the same manner as in non-plant species.
*Supported by ED0007/01/01 and IGA UP Prf/2013/003.
§Supported by DFG Germany (SFB 648)

Differential activity of *Arabidopsis* Aurora kinase members towards histone H3

Eva Tomaštíková[1], Jaroslav Doležel[1], Andreas Houben[2], Dmitri Demidov[2]

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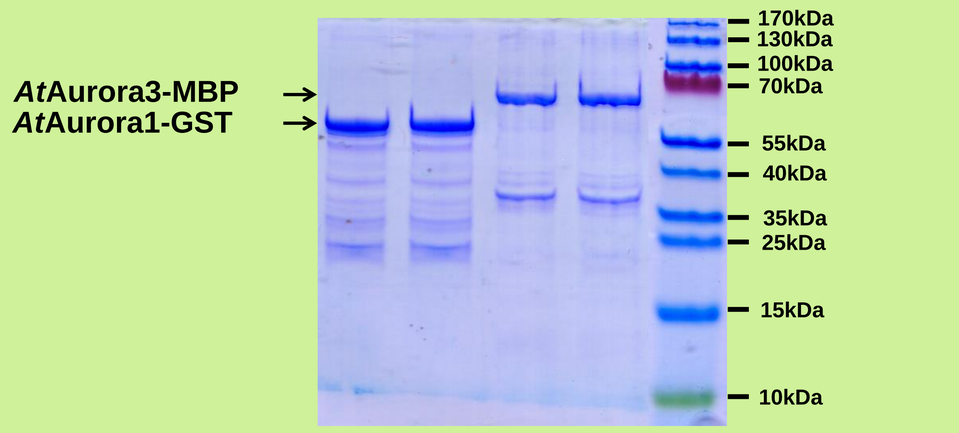
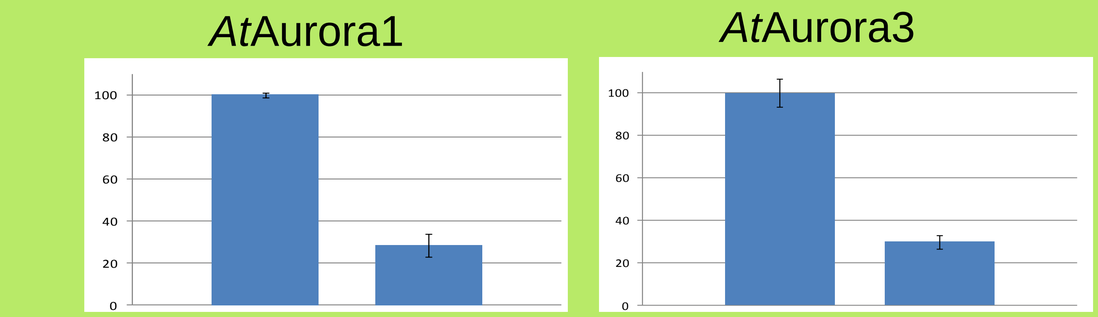
Introduction: Histones are subjected to a variety of post-translational modifications that are required for interactions with specific protein domains and regulate many aspects of chromosome activity. Phosphorylation of histone H3 is crucial for cell-cycle dependent chromosome condensation and segregation. Aurora kinases are responsible for cell cycle-dependent phosphorylation of Ser10 and 28 of H3 in both plant and non-plant organisms. To analyze whether a cross-talk between different histone modifications exists like in non-plant species, we determined whether the phosphorylation activity of the different members of the *Arabidopsis* Aurora family (*At*Aurora 1, 2 and 3) is differentially influenced by adjacent modifications..

Expression and purification of recombinant *At*Aurora kinase 1 and 3

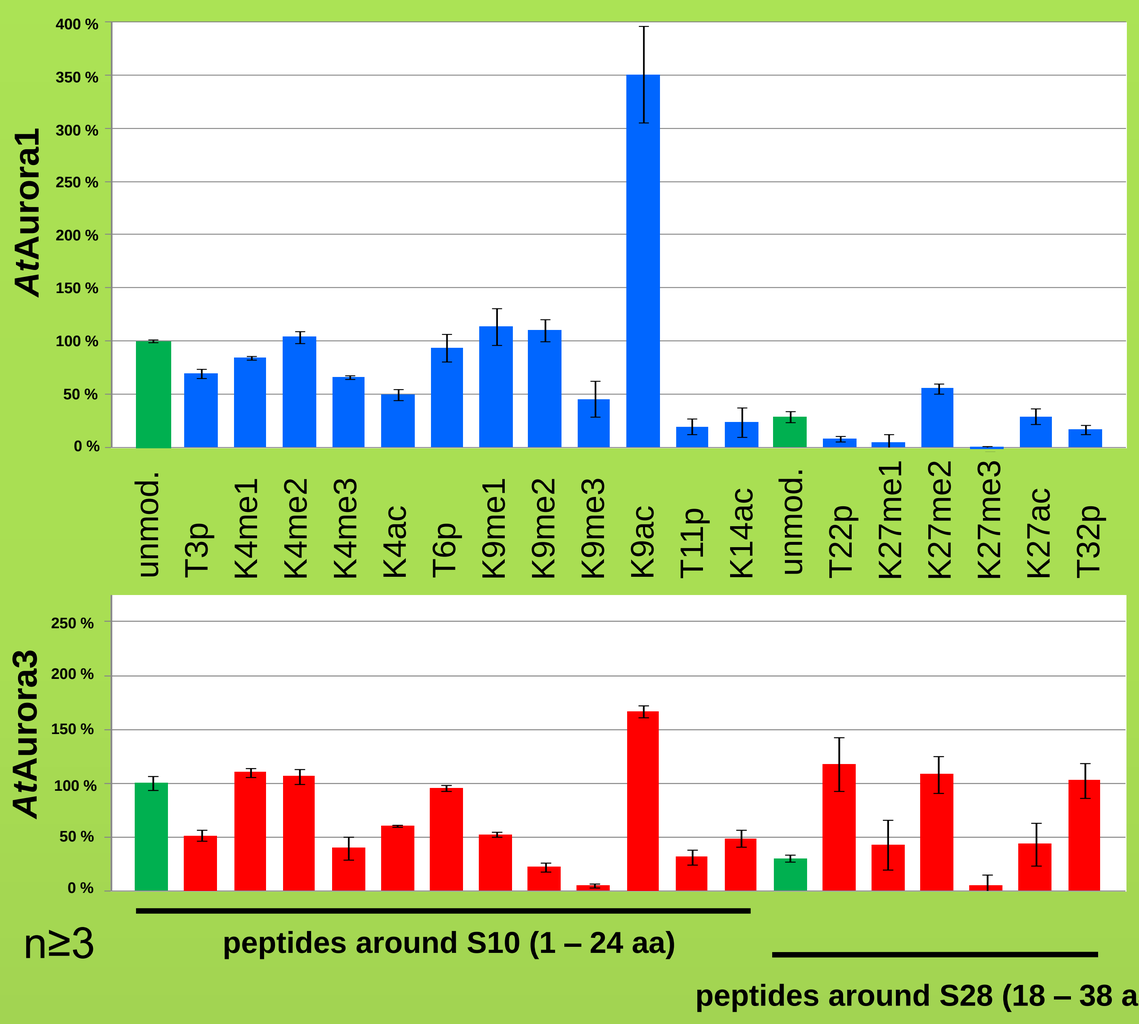


Phosphorylation of H3S28 by *At*Aurora1 and 3 is weaker than of H3S10



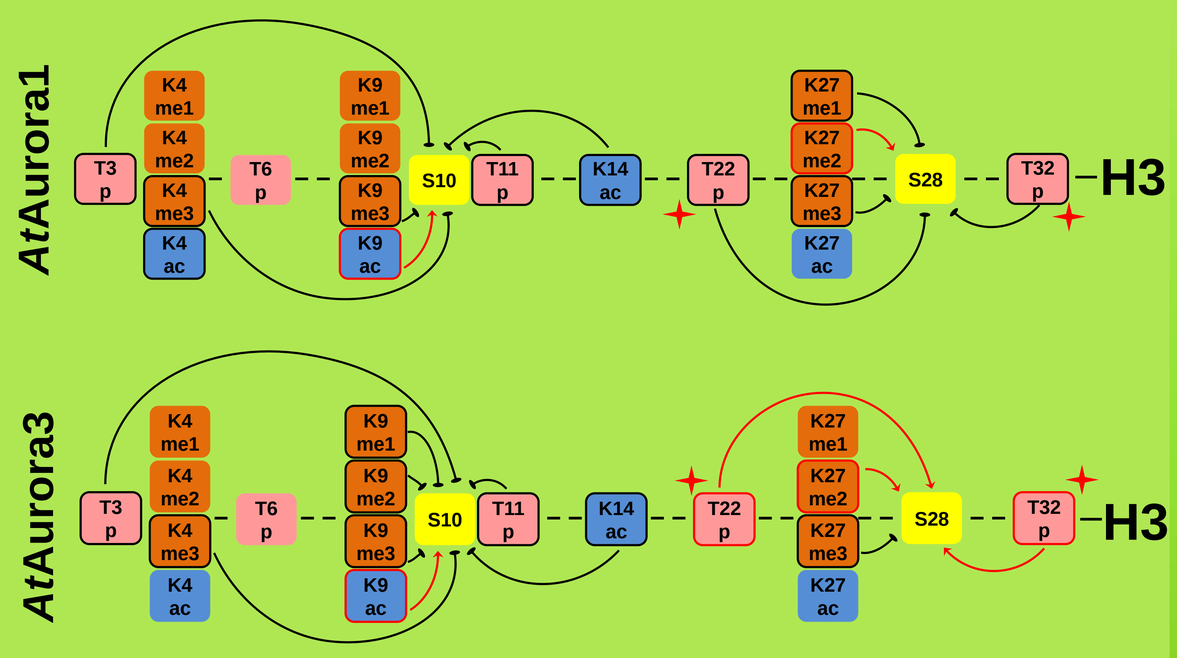
Incorporation of γATP during *in vitro* kinase assay with recombinant *At*Aurora1 and 3

Phosphorylation of H3 by *At*Aurora1/3 is influenced by neighboring post-translational modifications



In vitro kinase assays were performed with modified peptides related to H3S10 and H3S28. Activity of both Aurora kinases is influenced by various post-translational modifications on N-terminal part of histone H3 related to Ser10 and Ser28 positions *in vitro.*

Cross-talk occurs between different posttranslational modifications on histone H3



Similarly to non-plant species, cross-talk occurs between phosphorylation of Ser10 and Ser28 and acetylation or methylation of adjacent residues. *At*Aurora 1 and 3 are either negatively or positively influenced by methylation and/or acetylation of K4, K9, K14 and K27. *At*Aurora activity is also influenced by threonine phosphorylation. Differences occur (✦) between *At*Aurora1 and *At*Aurora3.

Conclusions:

- Cross-talk between different H3 modifications occurs also in plants, although in a similar rather than the same manner as in non-plant species.
- Some modifications influence activity of *At*Aurora 1 and 3 similarly, however differences between T22ph and T32ph, indicate different substrate specificity of both kinases.
- Influence of Aurora kinase phosphorylation activity by different modifications suggest that the so-called methyl/phos switch might occur also in plants.

Future perspectives: To confirm our results *in vivo*, *Arabidopsis* suspension cultures will be treated with inhibitors of various histone-modifying enzymes and the level of histone modification will be tested biochemically and microscopically.

This work was supported by ED0007/01/01 and IGA UP Prf/2013/003 for E.T. and DFG Germany (SFB 648) for D.D. and A.H.

APPENDIX XI

Differential activity of *Arabidopsis* Aurora kinase members towards histone H3

Tomaštíková E, Doležel J, Houben A, Demidov D

In: Plant Biotechnology: Green for Good 2013, Olomouc, Czech Republic
(poster abstract)

Differential activity of *Arabidopsis* Aurora kinase members towards histone H3

Tomaštíková E[1]*, Doležel J[1], Houben A[2], Demidov D[2§]

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Histones are subjected to a variety of post-translational modifications (PTM). Such modifications are required for interactions with specific protein domains and regulate many aspects of chromosome activity. Phosphorylation of histone H3 is crucial for cell-cycle dependent chromosome condensation and segregation. Various H3 kinases have been identified in plant, such as Aurora and Haspin. Members of the Aurora kinase family are responsible for cell cycle-dependent phosphorylation of serine 10 and serine 28 of H3 in both plant and non-plant organisms. In non-plant species cross-talk occurs between phosphorylation of H3S10 and acetylation or methylation of H3. For plants, information on the cross-regulation between H3S10 phosphorylation and PTMs of neighboring amino acids is limited. Data for the cross-talk between phosphorylation of H3S10 by AtAurora3 and other neighboring PTMs or around H3S28 by AtAurora1 and 3 are missing.

To elucidate whether the phosphorylation activity of the Aurora kinase family members of *Arabidopsis* towards H3S10 and H3S28 is influenced by neighboring PTMs, an *in vitro* kinase assay was performed. As substrates, N-terminal peptides of H3 with various modifications close to S10 and S28, were used. Recombinant AtAurora1 and AtAurora3 were expressed in *E. coli*. Our kinase assay confirmed that both serine positions are phosphorylated by both kinases *in vitro*, although phosphorylation of H3S28 is much weaker than of H3S10. However, H3S28 phosphorylation is increased by dimethylation and acetylation of neighboring K27. H3S10 phosphorylation by AtAurora1 is increased by dimethylation of H3K4 and phosphorylation of H3T6. Decrease in kinase activity was observed for acetylated H3K4.

Taken together our data suggest that a cross-talk between different H3 modifications occurs also in plants, although in similar rather than the same manner as in non-plant species.

*Supported by ED0007/01/01 and IGA UP Prf/2013/003.
§Supported by DFG Germany (SFB 648)

Differential activity of *Arabidopsis* Aurora kinase members towards histone H3

Eva Tomaštíková[1], Jaroslav Doležel[1], Andreas Houben[2], Dmitri Demidov[2]

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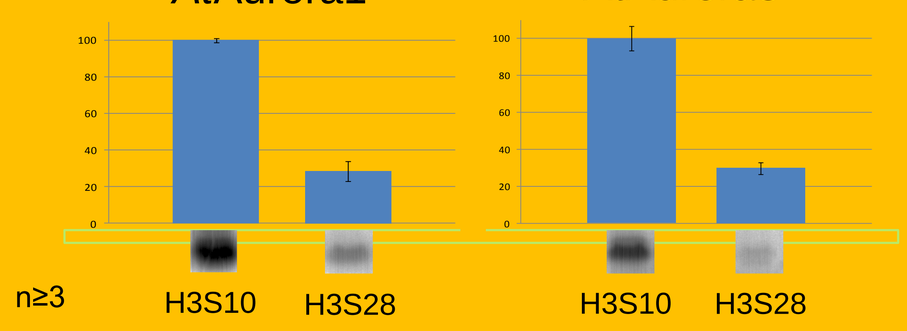
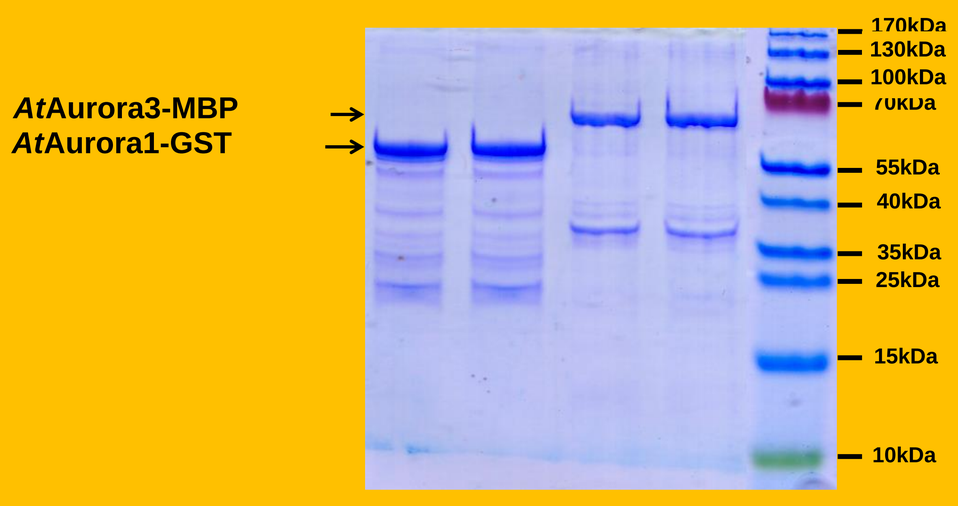
Introduction: Histones are subjected to a variety of post-translational modifications (PTM) that are required for interactions with specific protein domains and regulate many aspects of chromosome activity. Phosphorylation of histone H3 is crucial for cell-cycle dependent chromosome condensation and segregation. Members of the Aurora kinase family are responsible for cell cycle-dependent phosphorylation of serine 10 and serine 28 of H3 in both plant and non-plant organisms. In non-plant species cross-talk occurs between phosphorylation of H3S10 and acetylation or methylation of H3. For plants, information on the cross-regulation between H3S10 phosphorylation and PTMs of neighboring amino acids is limited. To elucidate whether the phosphorylation activity of the Aurora kinase family members of *Arabidopsis* towards H3S10 and H3S28 is influenced by neighboring PTMs, an *in vitro* kinase assay was performed.

Phosphorylation of H3S28 by *At*Aurora1 and 3 is weaker than of H3S10

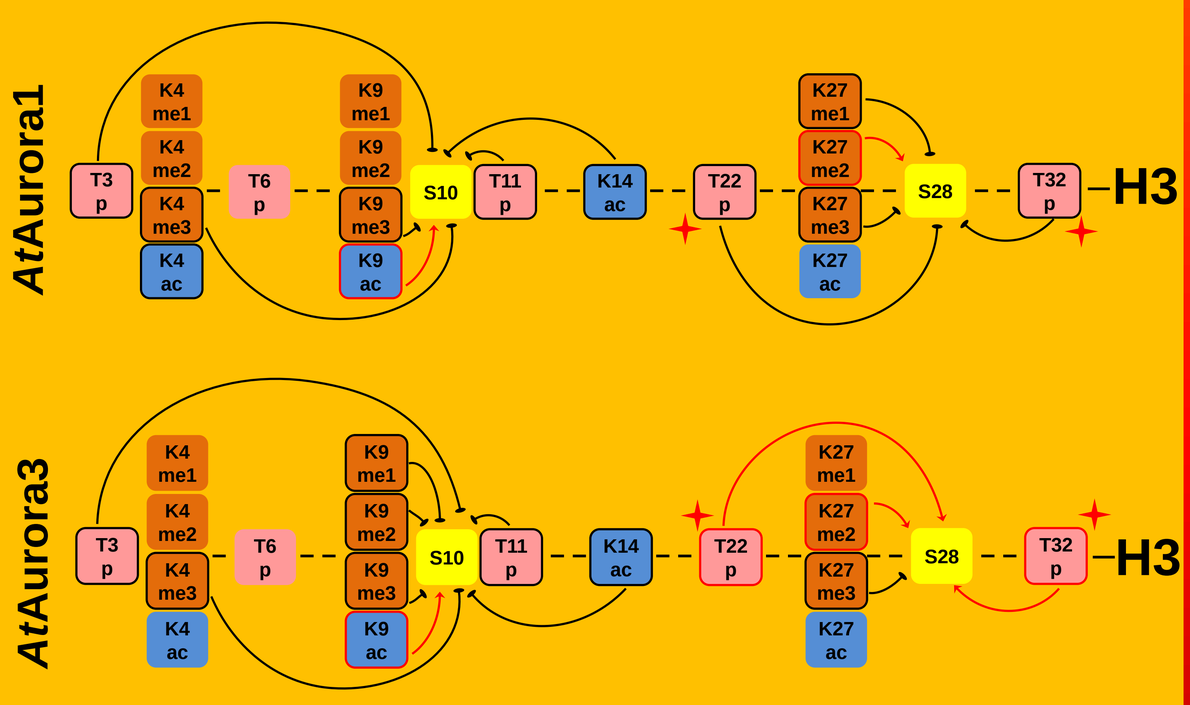


Incorporation of γATP during *in vitro* kinase assay with recombinant *At*Aurora1 and 3

Expression and purification of recombinant *At*Aurora kinase 1 and 3

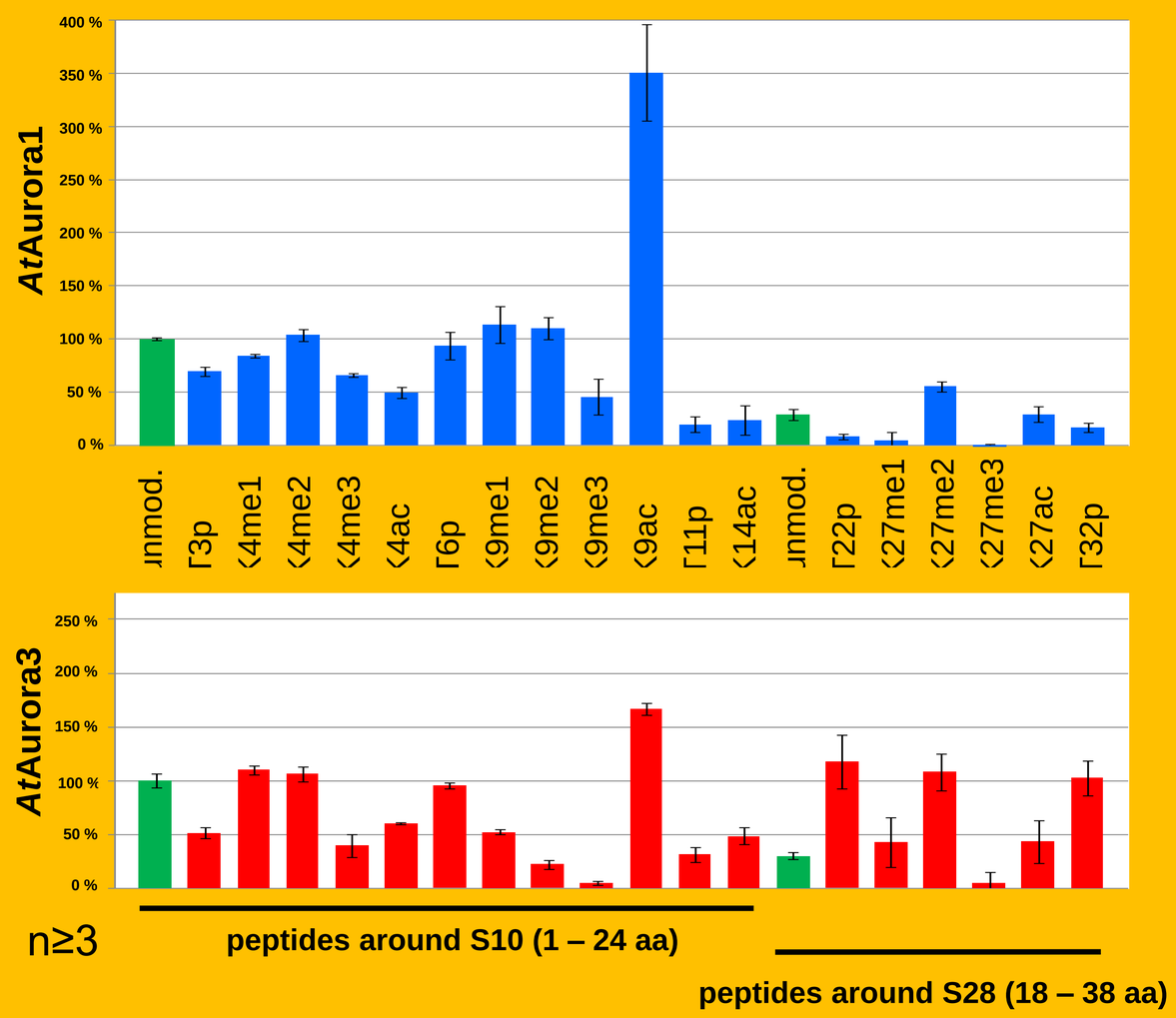


Cross-talk occurs between different posttranslational modifications on histone H3



Similarly to non-plant species, cross-talk occurs between phosphorylation of Ser10 and Ser28 and acetylation or methylation of adjacent residues. *At*Aurora 1 and 3 are either negatively or positively influenced by methylation and/or acetylation of K4, K9, K14 and K27. *At*Aurora activity is also influenced by threonine phosphorylation. Differences occur ( ) between *At*Aurora1 and *At*Aurora3.

Phosphorylation of H3 by *At*Aurora1/3 is influenced by neighboring post-translational modifications



In vitro kinase assays were performed with modified peptides related to H3S10 and H3S28. Activity of both Aurora kinases is influenced by various post-translational modifications on N-terminal part of histone H3 related to Ser10 and Ser28 positions *in vitro.*

Conclusions:

- Cross-talk between different H3 modifications occurs also in plants, although in a similar rather than the same manner as in non-plant species
- Some modifications influence activity of *At*Aurora 1 and 3 similarly, however differences between T22ph and T32ph, indicate different substrate specificity of both kinases
- Influence of Aurora kinase phosphorylation activity by different modifications suggest that the so-called methyl/phos switch might occur also in plants

This work was supported by ED0007/01/01 and IGA UP Prf/2013/003 for E.T. and DFG Germany (SFB 648) for D.D. and A.H.

APPENDIX XII

Formation of ectopic microtubular fibres within nuclei and with nuclear envelope requires overproduction of *Arabidopsis* TPX2 protein

Jeřábková H, Petrovská B, Cenklová V, Pochylová Ž, Gelová Z, Váchová L, Kurejová M, Tomaštíková E, Binarová P

In: Plant Biotechnology: Green for Good 2013, Olomouc, Czech Republic
(poster abstract)

Formation of ectopic microtubular fibres within nuclei and with nuclear envelope requires overproduction of *Arabidopsis* TPX2 protein.

Jeřábková H[1], Petrovská B[1], Cenklová V[2], Pochylová Ž[2], Gelová Z[2], Váchová L[2], Kurejová M[1], Tomaštíková E[1], Binarová P[3]

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The targeting protein for Xklp2 (TPX2) is a microtubule associated coiled-coil protein with multiple functions in microtubule nucleation and spindle organization. The plant TPX2 molecule contains all the functional domains of vertebrate counterpart, TPX2 signature motif is present once in vertebrate sequences but twice in plants. Compared to animals, in plant systems, proteins with coiled-coil signature are less understood. Previously we showed that AtAurora1 kinase and AtTPX2 localizes in plant microtubules in a cell cycle specific manner from preprophase to early telophase. In addition, AtTPX2 protein is intranuclear and although important mitotic functions for plant TPX2 protein have already been well documented, any functional role for its accumulation in interphase nuclei is far from understood.
Here we present data on specific arrays of microtubules decorated with AtTPX2 formed in the vicinity of the nuclear envelope and in nuclei. Microscopic analysis of cells overproducing AtTPX2 showed the "dots" of overexpressed AtTPX2-GFP signal in interphase nuclei. Later AtTPX2-GFP dots were rebuilt into the thick fibrilar structures or new ectopic sites for microtubule formation positive for AtTPX2-GFP. AtTPX2-GFP signal decorated filamentous structures reminiscent of microtubules. Fibrils were arranged into cage-like structures surrounding nuclei. We proved that AtTPX2 fibres were positive for α-tubulin immunolabeling, while they were not recognized by anti-actin antibody. The interconnection between the cage-like structures surrounding the nuclear envelope and intranuclear arrays was visible and fibres often extended to the cell periphery as well. The rearrangement of AtTPX2 from the dots through the fibres might be caused by co-assembly of the AtTPX2-GFP with endogenous microtubule-nucleating units comprised γ-tubulin and TPX2 protein.

Acknowledgements: This work was supported by the Grant Agency of the Czech Republic [204/09/P155, 204/07/1169, P501/12/2333, P501/12/G090] and the Centre of the Region Haná for Biotechnological and Agricultural Research [CZ.1.05/2.1.00/01.0007 to H.J., B.P., and E.T.].

Formation of ectopic microtubular fibres within nuclei and with nuclear envelope requires overproduction of *Arabidopsis* TPX2 protein

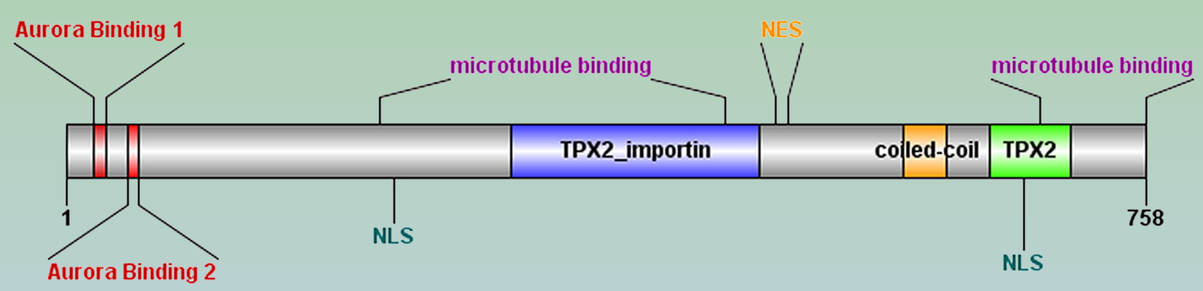
Hana Jeřábková[1], Beáta Petrovská[1], Věra Cenklová[2], Žaneta Pochylová[2], Zuzana Gelová[2], Lenka Váchová[2], Michaela Kurejová[1], Eva Tomaštíková[1], and Pavla Binarová[3]

[1] Centre of the Region Haná for Biotechnological and Agricultural Research, Institute of Experimental Botany AS CR, v.v.i., Šlechtitelů 31, Olomouc, 783 71, Czech Republic.
[2] Institute of Experimental Botany, AS CR, v.v.i., Sokolovská 6, 772 00, Olomouc, Czech Republic.
[3] Institute of Microbiology, AS CR, v.v.i., Vídeňská 1083, 142 20 Prague 4, Czech Republic.

INTRODUCTION: The targeting protein for Xklp2 (TPX2) is a microtubule associated coiled-coil protein with multiple functions in microtubule nucleation and spindle organization. In plant systems, proteins with coiled-coil signature are less understood. Previously we showed that AtAurora1 kinase and AtTPX2 localize in plant microtubules in a cell cycle specific manner from preprophase to early telophase. In addition, AtTPX2 protein is intranuclear and although important mitotic functions for plant TPX2 protein have already been well documented, any functional role for its accumulation in interphase nuclei is far from understood. Here we present data on specific arrays of microtubules decorated with AtTPX2 formed in the vicinity of the nuclear envelope and in nuclei.

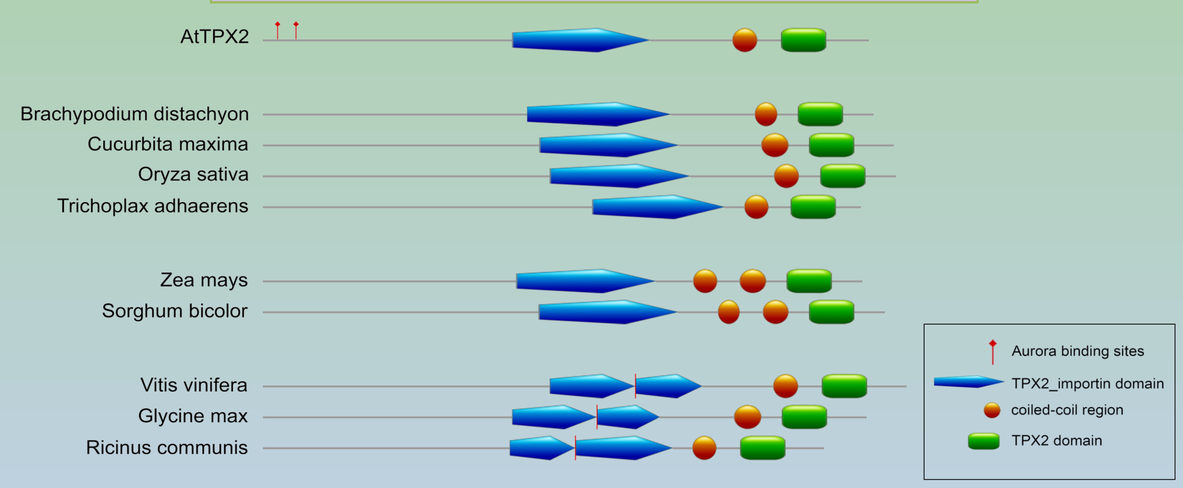
RESULTS:

AtTPX2 domain, putative interaction sites and binding motifs in amino acid sequence



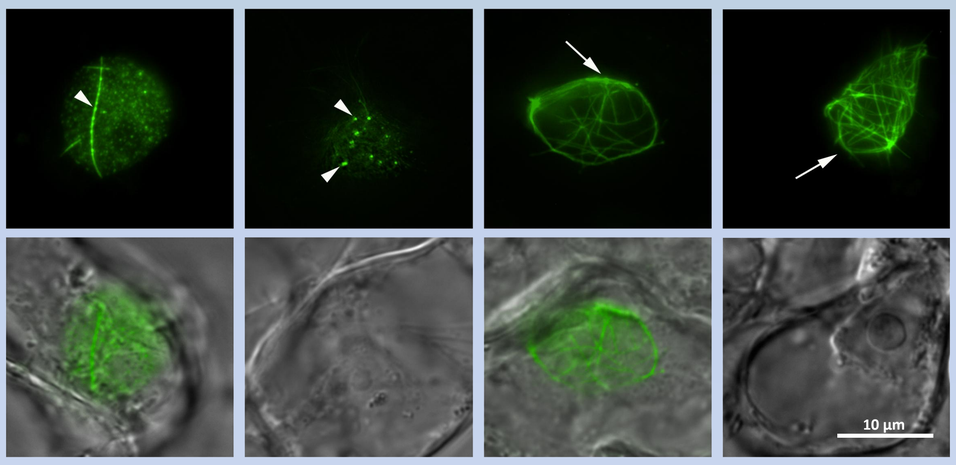
The main features of AtTPX2 protein domains are TPX2_importin and TPX2 domain, two nuclear localisation signals (NLS), nuclear export signal (NES), two Aurora kinase binding sites, coiled-coil domain, microtubule binding domains.

AtTPX2 protein domain organization and its plant homologs



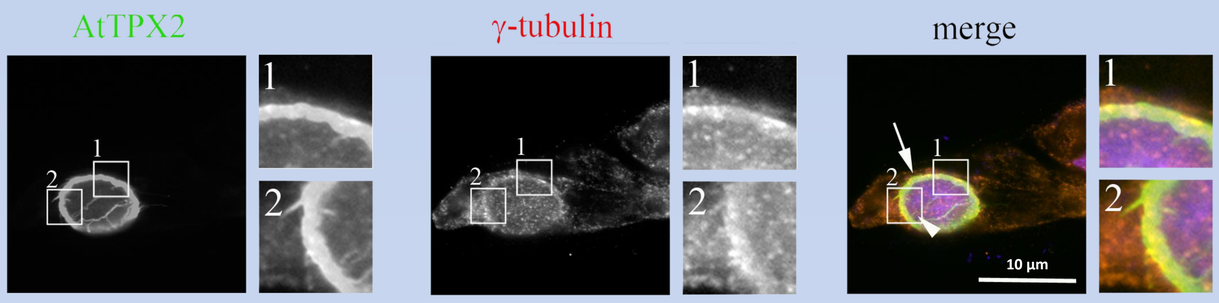
Similarly to the AtTPX2 protein the coiled-coil region together with TPX2_importin and TPX2 motif was found in various plant protein homologs, mostly yet uncharacterised or only hypothetical.

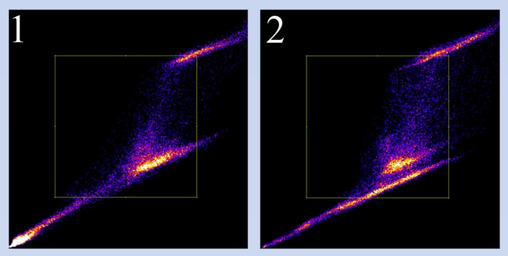
Localization of overproduced AtTPX2-GFP in nuclei



Dots (arrowheads) and fiber-like structures (arrows) decorated with AtTPX2-GFP were formed 72 hours after transformation around and inside the interphase nuclei of cells overexpressing AtTPX2.

γ-tubulin colocalized with overexpressed AtTPX2-GFP



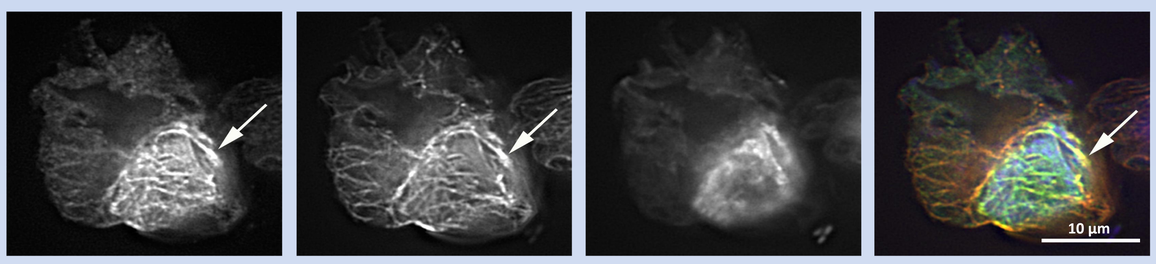


Rr = 0.923
ICQ = 0.458

Rr = 0.820
ICQ = 0.237

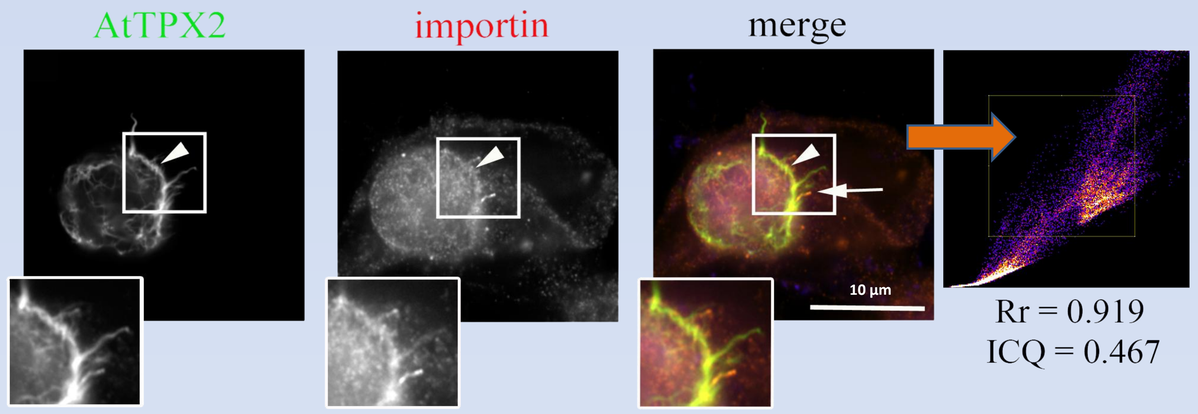
γ-tubulin is localized together with AtTPX2-GFP on ectopic microtubules in the vicinity of nuclear envelope (arrow) and with intranuclear microtubules (arrowhead)
Inset 1 and 2 show complete colocalization of both proteins with Pearson´s coefficients (Rr) 0.923 and 0.820, respectively.

α-tubulin positive fibres colocalized with AtTPX2-GFP



α-tubulin immunolabeling corelated with AtTPX2-GFP signal; thinner AtTPX2 positive fibres were decorated with α-tubulin along the entire length, thicker fibres showed patchy or weak pattern of decoration with α-tubulin (arrows).

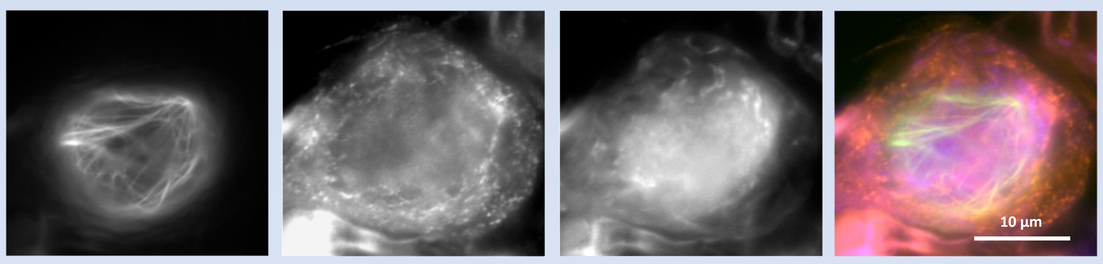
AtTPX2-GFP colocalized with importin



Rr = 0.919
ICQ = 0.467

AtTPX2 and importin colocalized on nuclear envelope (arrowhead) and on the microtubular fibers (arrow). Quantitative analyses showed high degree of colocalisation with Pearson‘s coefficient (Rr) = 0,919.

Immunolocalization of AtTPX2 and actin



To exclude the localisation with actin fibres, an anti-actin antibody was used. AtTPX2 fibers were not positive for actin immunolabeling.

Conclusion: Overproduction of AtTPX2 protein resulted in excessively branched and bundled microtubules and their formation in perinuclear and intranuclear space. The rearrangement of AtTPX2 from the „dots“ to the bundled fibres might be caused by the co-assembly of the AtTPX2-GFP with endogenous microtubule nucleating units comprised with γ-tubulin and TPX2 protein.

This work was supported by the Grant Agency of the Czech Republic [204/09/P155, 204/07/1169, P501/12/2333, P501/12/G090], IGA UP Prf/2013/003 for H.J. and the Centre of the Region Haná for Biotechnological and Agricultural Research [CZ.1.05/2.1.00/01.0007 to H.J., B.P., and E.T.].

APPENDIX XIII

Differential *Arabidopsis* Aurora kinase family members activities on histone H3

Tomaštíková E, Doležel J, Houben A, Demidov D

In: 3rd European Workshop on Plant Chromatin 2013, Madrid, Spain

(oral presentation abstract)

Differential *Arabidopsis* Aurora kinase family members activities on histone H3

Tomaštíková E[1], Doležel J[1], Houben A[2], Demidov D[2]

[1]*Centre of the Region Haná for Biotechnological and Agricultural Research, Institute of Experimental Botany, Šlechtitelů 31, Olomouc 783 71, Czech Republic*
[2]*Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstr. 3, 06466 Gatersleben, Germany*

Histones are targets of various post-translational modifications. Such modifications help to recruit proteins that modulate the chromatin structure. Members of the Aurora kinase family are responsible for cell-cycle dependent phosphorylation of H3 at S10 and S28 in both plant and non-plant species. In animals, cross-talk occurs between phosphorylation and methylation of adjacent residues, resulting in a so-called methyl/phos switch. In plants, data on the cross-regulation between H3 phosphorylation and post-translational modification of neighboring amino acids are limited.

To analyze whether a cross-talk between different histone modifications exists similar to non-plant species, we determined the phosphorylation activity of the *Arabidopsis* Aurora family members (*At*Aurora 1 and 3) on H3 peptides with different modifications related to positions S10 and S28. Our kinase assay confirmed that both serine positions are phosphorylated by both recombinant kinases *in vitro*, although phosphorylation of H3S28 is much weaker than of H3S10. Phosphorylation of H3S28 by both kinases is increased by dimethylation and acetylation of adjacent K27. Pre-phosphorylation of T22 and T32 decrease *At*Aurora1 activity, but increase AtAurora3 activity, respectively. Furthermore, using a peptide microarray, we identified *At*Aurora1 consensus phosphorylation sequence, which could help us to identify additional targets for *Arabidopsis* Aurora kinases.

Taken together, our data suggest that a cross-talk between different H3 modifications occurs also in plants, although in similar rather than the same manner as in non-plant species. Despite the fact that some modifications influence Aurora kinase 1 and 3 activity in a similar way, there are differences between few modifications, indicating different substrate specificity of both kinases. To confirm our data *in vivo*, immunostaining and Western blot analyses of *Arabidopsis* suspension cell cultures treated with inhibitors of different histone-modifying enzymes are in progress.

Supported by ED0007/01/01 and IGA UP Prf/2013/003 for ET, DFG Germany (SFB 648) for DD, AH.

APPENDIX XIV

Arabidopsis TPX2 activates Aurora kinase 1 *in vitro*

Tomaštíková E, Demidov D, Jeřábková H, Houben A, Doležel J, Petrovská B

In: SEB Annual Meeting 2014, Manchester, United Kingdom
(poster abstract)

Arabidopsis TPX2 activates Aurora kinase 1 *in vitro*

Tomaštíková E[1], Demidov D[2], Jeřábková H[1], Houben A[2], Doležel J[1], Petrovská B[1]

[1]*Centre of Plant Structural and Functional Genomics, Institute of Experimental Botany, Czech Republic*
[2]*Leibniz Institute of Plant Genetics and Crop Plant Research, Germany*

Aurora kinases belong to the family of serine/threonine kinases. They play a role in execution a variety of mitotic events, such as chromosome condensation and bipolar mitotic spindle assembly. The function and regulation of Aurora kinases in animal systems have already been described. It has been established that Aurora A is activated by microtubule-associated protein TPX2 and that the kinase is protected from dephosphorylation by TPX2. In contrast, little is known about regulation of Aurora kinases in plants. Here, we characterize *At*TPX2 as *At*Aurora1 regulator *in vitro*. *At*TPX2 acts as a substrate as well as an activator of *At*Aurora1, but not *At*Aurora3. Although the truncated version of *At*TPX2 lacking the Aurora binding domain can phosphorylate the kinase, it is unable to activate it. The activation of *At*Aurora1 results in dramatically increased phosphorylation of downstream targets, histone H3 in particular. The differences in the activation mechanisms of both *At*Aurora1 and *At*Aurora3 point to a specific regulation of both kinases, a fact that might play an important role in cell cycle regulation and signalling cascade transduction.

Our data indicate that similar mechanisms of Aurora kinase activation occur in plants and metazoans. Furthermore, activation of *At*Aurora1 by *At*TPX2 could represent a mechanism for translation of RanGTP signalling to phosphorylation cascade performed by Aurora kinases at kinetochores. Increased *At*Aurora1 activity might also activate various downstream targets such as histones and spindle assembly factors. Such hyperactivation cascade could stimulate a rapid and precise reconstruction of mitotic chromatin and/or mitotic spindle formation.

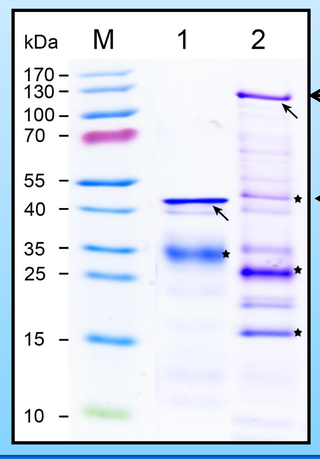
Arabidopsis TPX2 activates Aurora kinase 1 *in vitro*

Tomaštíková Eva[1], Demidov Dmitri[2], Jeřábková Hana[1], Houben Andreas[2], Doležel Jaroslav[1], Petrovská Beáta[1]

[1]Centre of the Region Haná for Biotechnological and Agricultural Research, Institute of Experimental Botany AS CR, Šlechtitelů 31, Olomouc 783 71, Czech Republic
[2]Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstr. 3, 06466 Gatersleben, Germany

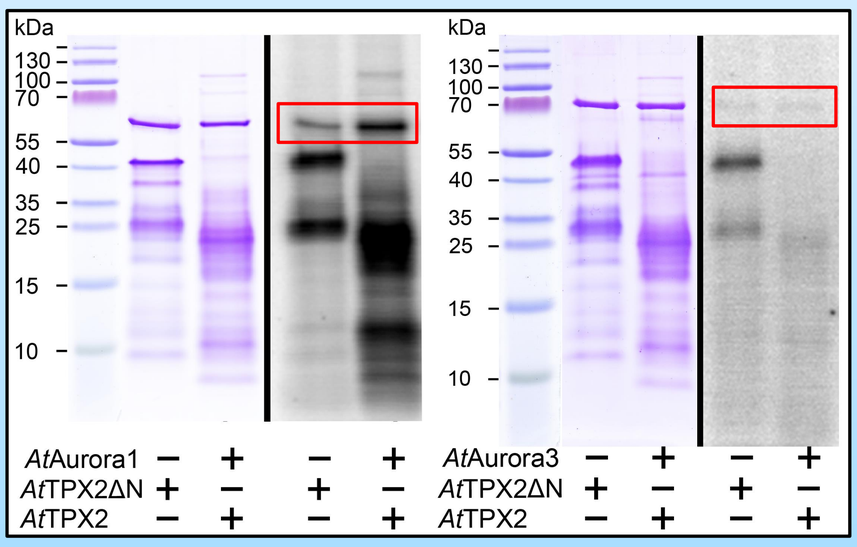
Introduction: Aurora kinases belong to the family of serine/threonine kinases. They play a role in execution a variety of mitotic events, such as chromosome segregation and bipolar mitotic spindle assembly. The function and regulation of Aurora kinases in animal systems have already been described. It has been established that Aurora A is activated by microtubule-associated protein TPX2 and that the kinase is protected from dephosphorylation by TPX2. To analyze whether similar regulation mechanims occurs also in plants, we determined if kinase activity of different Aurora kinase family members (*At*Aurora1, *At*Aurora3) in *Arabidopsis* is influenced by TPX2 protein.

Expression and purification of recombinant *At*TPX2 (lane 2) and *At*TPX2ΔN (lane 1) lacking Aurora-binding domain



Degradation products marked with asterisks. Half time of degradation is about 10 min for humanTPX2 (Stewart *et al*., 2005)

*At*TPX2 increase autophosphorylation activity of *At*Aurora1 but not *At*Aurora3



Incorporation of γATP^{32} during *in vitro* kinase assay with recombinant *At*Aurora1 and 3 and *At*TPX2 or *At*TPX2ΔN (truncated version without Aurora binding domain, Petrovská *et al*., 2012) revealed increased autophosphorylation activity of *At*Aurora1 by *At*TPX2.

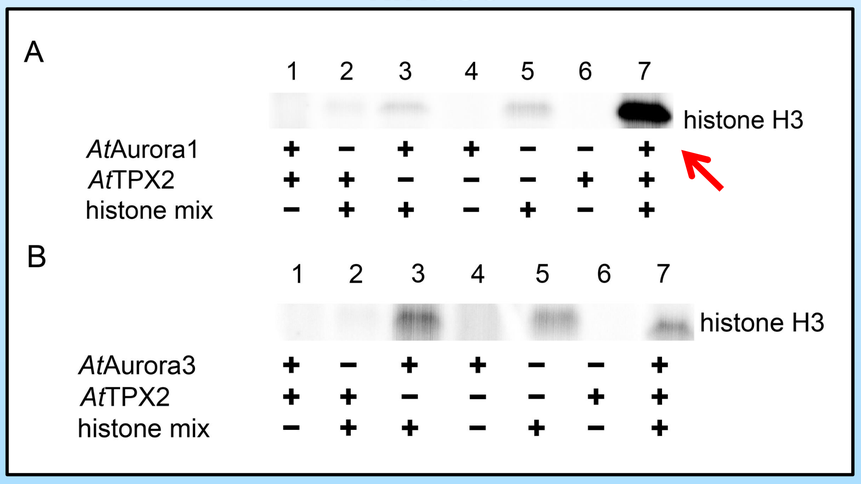
In order to elucidate whether activity of plant Aurora kinases is influenced by *At*TPX2 *in vitro*, kinase assay was performed with recombinant proteins.

We determined:

If autophosphorylation activity of plant Aurora kinases is influenced by *At*TPX2

If increased activity of a kinase also influence phosphorylation of downstream targets

*At*TPX2 increases *At*Aurora1 activity towards histone H3 after coactivation of kinase

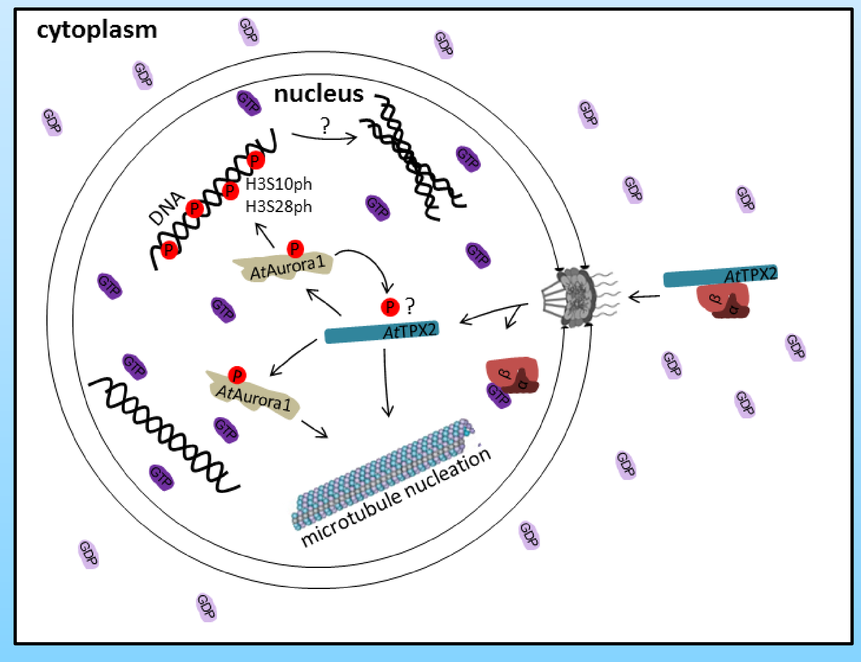


The activation of *At*Aurora1 results in dramatically increased phosphorylation of downstream targets, histone H3 in particular. Hyperphosphorylation of histone H3 by *At*Aurora1 was found in the presence of *At*TPX2 (marked with red arrow) (A). In contrast, no elevated level of H3 phosphorylation was found for *At*Aurora3 (B).

Conclusions:

- Similar mechanisms of Aurora kinase activation occur in plants and metazoans
- *At*TPX2 is a potent activator of *At*Aurora1 *in vitro*
- *At*TPX2 has no effect on *At*Aurora3 activity pointing toward divergent functions for both types of kinases that are probably determined by different regulators
- *At*TPX2 is a regulator of *At*Aurora1 activity not only in targeting the kinase to mitotic spindle but also in increasing its autophosphorylation activity towards histone H3.
- *At*TPX2 could be a mechanism for translation of RanGTP signaling to phosphorylation cascade performed by Aurora kinases at kinetochores

Proposed model of *At*Aurora1 and *At*TPX2 cooperation in mitotic spindle assembly and chromosome segregation



Future perspectives: Determine if *At*TPX2 coactivation of *At*Aurora1 changes the kinase activity towards differently modified histone H3 and therefore influences only subset of substrates, e.g. in particular part of the cell cycle

Supported by Czech Science Foundation (14-28443S) and the National Program of Sustainability I (LO1204) for ET, HJ, BP, the European Social Fund (Operational Program Education for Competitiveness CZ.1.07/2.3.00/20.0165) for BP, Internal Grant Agency of Palacky University, Olomouc (IGA_PrF_2014001, PrF-2013-003) and Interdoc (OPVK-CZ.1.07/2.4.00/17.0008) for ET, HJ. DD and AH are supported by the DFG (SFB 648).

APPENDIX XV

Overexpressed *At*TPX2 reinforces microtubule formation in the nuclei of acentrosomal plant cells

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Overexpressed *At*TPX2 reinforces microtubule formation in the nuclei of acentrosomal plant cells

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The targeting protein for Xklp2 (TPX2) is a microtubule associated coiled-coil protein with multiple functions in microtubule nucleation and spindle organization. The plant TPX2 molecule contains all the functional domains of vertebrate counterpart, TPX2 signature motif is present once in vertebrate sequences but twice in plants. Compared to animals, in plant systems, proteins with coiled-coil signature are less understood. Previously we showed that AtAurora1 kinase and AtTPX2 localizes in plant microtubules in a cell cycle specific manner from preprophase to early telophase. In addition, AtTPX2 protein is intranuclear and although important mitotic functions for plant TPX2 protein have already been well documented, any functional role for its accumulation in interphase nuclei is far from understood.
Here we present data on specific arrays of microtubules decorated with AtTPX2 formed in the vicinity of the nuclear envelope and in nuclei. Microscopic analysis of cells overproducing AtTPX2 showed the "dots" of overexpressed AtTPX2-GFP signal in interphase nuclei. Later AtTPX2-GFP dots were rebuilt into the thick fibrilar structures or new ectopic sites for microtubule formation positive for AtTPX2-GFP. AtTPX2-GFP signal decorated filamentous structures reminiscent of microtubules. Fibrils were arranged into cage-like structures surrounding nuclei. We proved that AtTPX2 fibres were positive for α-tubulin immunolabeling, while they were not recognized by anti-actin antibody. The interconnection between the cage-like structures surrounding the nuclear envelope and intranuclear arrays was visible and fibres often extended to the cell periphery as well. The rearrangement of AtTPX2 from the dots through the fibres might be caused by co-assembly of the AtTPX2-GFP with endogenous microtubule-nucleating units comprised γ-tubulin and TPX2 protein.

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Overexpressed AtTPX2 reinforces microtubule formation in the nuclei of acentrosomal plant cells

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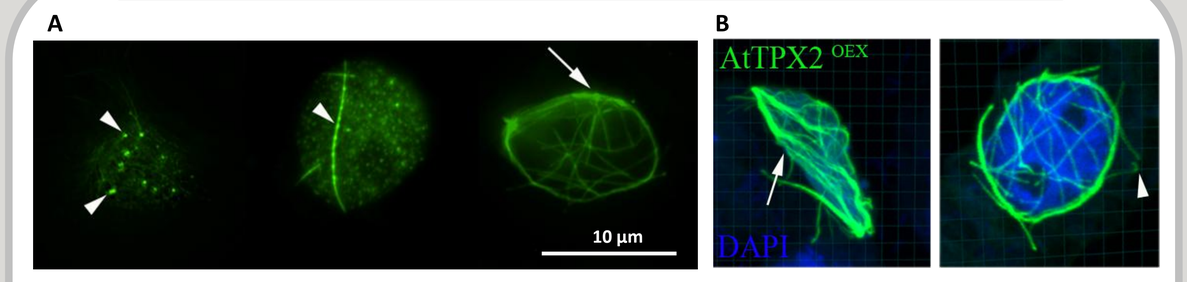
INTRODUCTION

The targeting protein for Xklp2 (TPX2) is a microtubule (MT) associated coiled-coil protein with functions in chromatin-induced MT nucleation and bipolar spindle assembly. It is a component of Ran GTPase pathway when Ran GTP releases complex TPX2-importin and enables TPX2 activation. Overproduction of TPX2 in HeLa cells causes apoptosis.

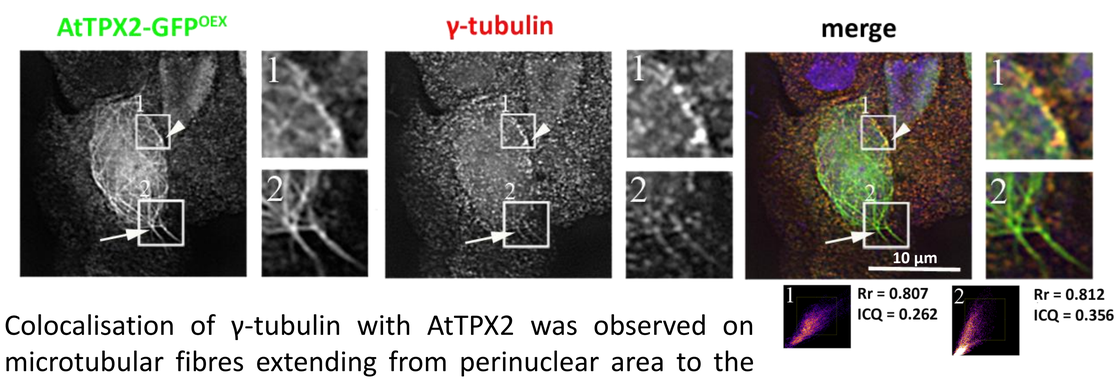
The role of plant TPX2 in MT assembly during mitosis has been suggested, nevertheless, its function during interphase is still unclear. In this study we analysed *Arabidopsis* interphase cells overexpressing AtTPX2-GFP to obtain further insights into TPX2 mediated MT formation in plants. To determine whether the Ran GTPase pathway is involved in AtTPX2 mediated formation of MT arrays, we performed immunolocalisation of importin and Ran in AtTPX2-GFP overproducing cells.

AtTPX2^{OEX} localized around and inside the interphase nuclei



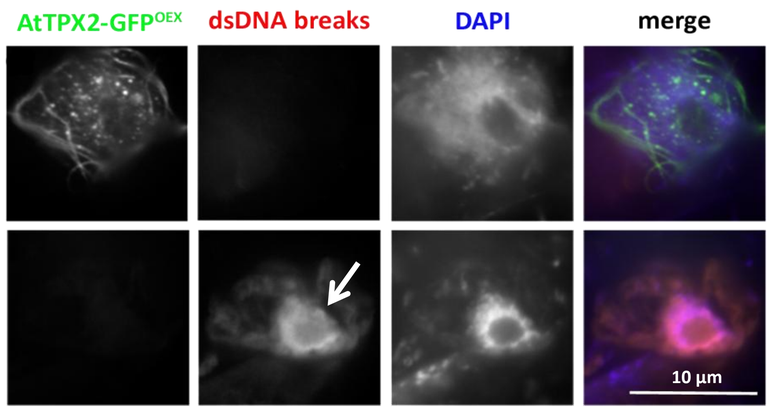
A) Dots (arrowheads) and fiber-like structures (arrows) decorated with AtTPX2-GFP were formed 72 hours after transformation around and inside interphase nuclei of AtTPX2^{OEX} cells.
B) 3D reconstruction of AtTPX2 decorated microtubular arrays twisted in perinuclear area (arrow) and extended to cell periphery (arrowhead).

γ-tubulin (endogenous microtubule nucleating unit) localized together with AtTPX2^{OEX}



Colocalisation of γ-tubulin with AtTPX2 was observed on microtubular fibres extending from perinuclear area to the cytoplasm and membrane (arrow) and on the nuclear envelope (arrowhead). The degree of colocalisation was measured with Pearsons coefficient (R_r).

AtTPX2 induced microtubule formation was not a result of apoptosis

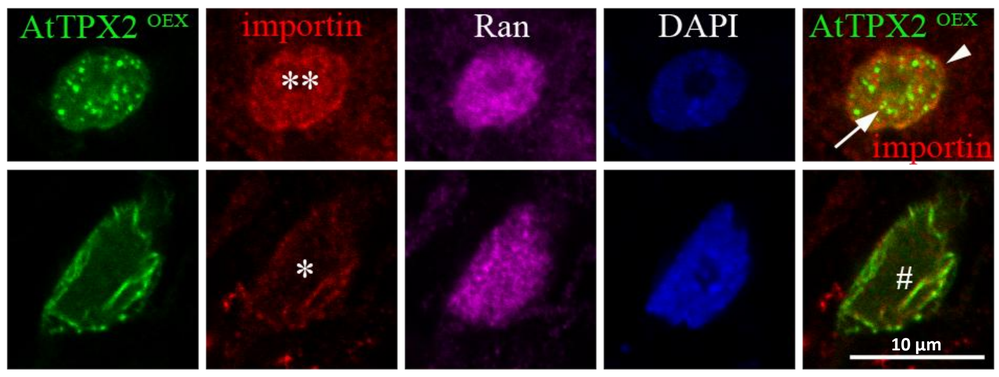


No ectopic AtTPX2–decorated arrays were observed in cells that were positive for TUNEL labelling (arrow), which was used for detection of apoptosis.

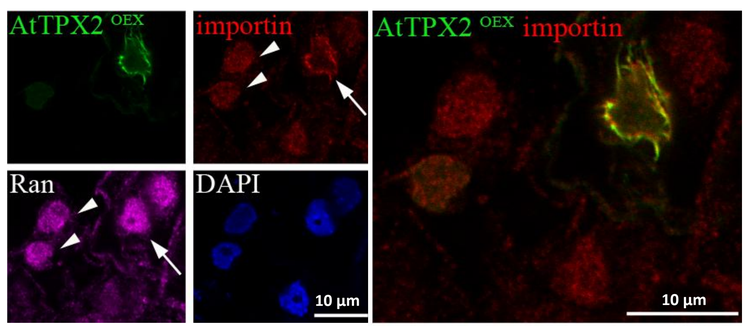
CONCLUSIONS

1) Overproduction of AtTPX2 protein resulted in excessively branched and bundled microtubules in perinuclear and intranuclear space.
2) These features were not related to apoptosis.
3) AtTPX2 overproduction amplifies the ability of nuclear envelope to promote MT nucleation, which is typical for acentrosomal plant cells.
4) RanGTPase pathway is involved in the process of TPX2-induced microtubules formation in plant cells.

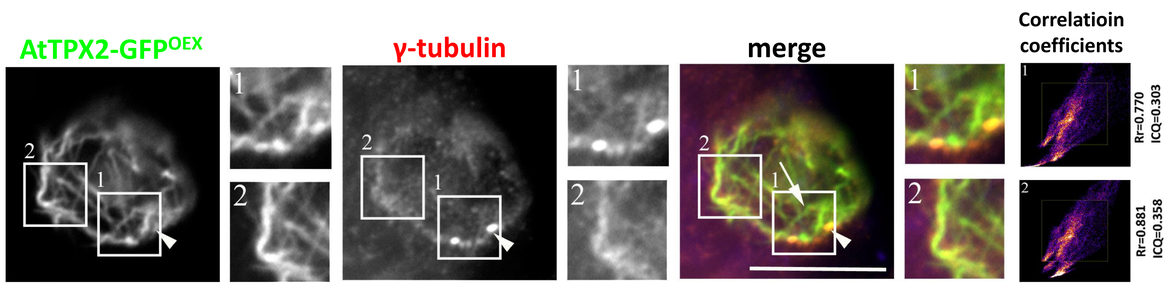
AtTPX2–GFP colocalised with importin in a Ran-GTPase dependent manner



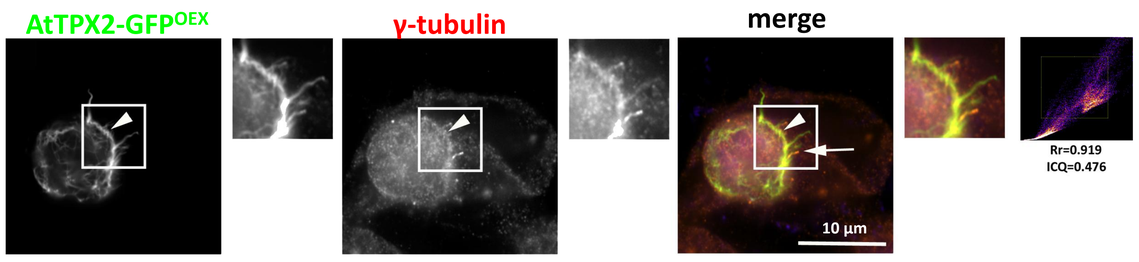
The accumulation of importin and Ran in the nuclei of AtTPX2^{OEX} cells indicates a nuclear import of AtTPX2^{OEX}. Importin signal was present in nuclei (**), around nuclei (arrowhead) and colocalized with some of the AtTPX2 foci (arrow). Nuclear signal for importin was reduced (asterisk) in cells where AtTPX2-GFP fibres were formed and it localized with the fibres around nuclei and in the nuclei (hash mark) indicating the disassociation of AtTPX2 from importin.



The signal for Ran was stronger in nuclei with AtTPX2-GFP nuclear dots and fibres than in untransformed nuclei (arrowheads). Importin signal declined in the nuclei with fibres (arrow).

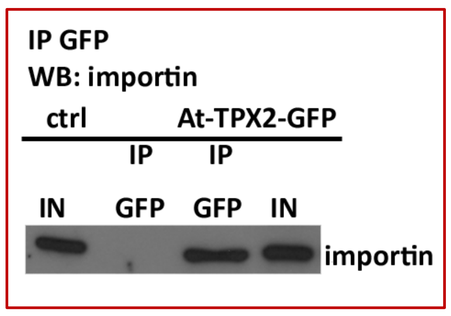


AtTPX2^{OEX} and importin colocalized at microtubular fibres (arrow) and visibly in intranuclear foci (arrowhead). Bar =10 µm.



Colocalisation of AtTPX2^{OEX} and importin with microtubular fibres (arrow) and in the vicinity of nuclei (arrowhead) showing a high degree of colocalisation with Pearson's coefficient (Rr) = 0.919.

The interaction of importin and AtTPX2-GFP was proved by co-purification experiments.



The data based on colocalisation and co-purification experiments suggest the involvement of importin and RanGTPase pathway in overexpressed AtTPX2 reinforced MT formation in acentrosomal plant cells.

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