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Analysis of nuclear 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.

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Abstract:

Cell nucleus is one of the distinctive features of eukaryotic cell. Bi-layer nuclear envelope constitutes the interface between cytoplasm and nucleoplasm comprising chromatin, nucleolus, nuclear bodies and further substructures. The complex of nuclear pores imbedded in the envelope mediates the transport of macromolecules and confers the important regulation function. The nucleus has been the subject of a research for a long time due to the presence of genetic material and apparatus for DNA replication and transcription. The fast progress in sequencing technologies has enabled assembling of complete genome sequences in many species and disclosing of function of genes and non-coding sequences. However, to completely understand the genome function, the knowledge about nuclear proteome is essential. Proteins represent the most abundant molecules in the cell nucleus and provide structural and functional framework for the majority of nuclear processes, whether it is the regulation of gene expression, regulation of the cell cycle and molecular transport, or the structural support for these processes. Although the nuclear proteins perform a large scale of functions, there is a limited knowledge about them. Within plant kingdom, the nuclear proteome has been studied in a few model plants and crop plants with known genome sequences in which the studies were aimed mainly at responses of nuclear proteome to stress factors.

The aim of the Thesis is to study nuclear proteins in plants and to contribute to unraveling the relationship between the structure and function of plant cell nucleus. The intention is to obtain a comprehensive data on nuclear proteome in individual phases of the cell cycle and to characterize the function of selected proteins. In the proteomic analysis, nuclei from root tips of

barley (*Hordeum vulgare* L. cv. Morex) were employed. Exact identification of proteins with the mass spectrometry needs a precise material processing. The previous studies have used predominantly fractionation methods for nuclei isolation. These are time-consuming, but importantly there is a high probability of contamination with non-nuclear proteins. For the isolation of barley nuclear proteins, the new approach based on flow cytometric sorting was employed. Flow cytometry enables high speed analysis of microscopic particles such as intact cell nuclei and mitotic chromosomes. The obtained results proved the feasibility of this approach, which allows achieving a maximum yield and purity of specimen with a minimal contamination with non-nuclear proteins. In order to maximize the number of identified proteins, two mass spectrometry techniques were employed, MALDI-TOF/TOF and ESI-Q/TOF. The uniqueness of each type of spectrometer for protein identification has been showed. The utilization of both methods brings us the coverage of wide spectrum of identified proteins.

Regarding the functional analysis, the aim was to contribute to the characterisation of nuclear protein TPX2 (Targeting Protein for Xklp2), which belongs to the group of microtubule associated proteins. In animal cells, it functions as one of the organisational elements of microtubules in the vicinity of centrosomes and chromosomes. After releasing from the complex with importins α and β, TPX2 activates Aurora kinase A, an important mitotic regulator, which is followed by microtubule nucleation and spindle assembly. In plant acentrosomal cells, the TPX2 protein was found associated with Aurora1, which was proved to organise spindle assembly process in plants as well. The regulation of Aurora1 by plant TPX2 was proposed. To clarify the function of TPX2 further, *Arabidopsis thaliana* cells with overexpressed TPX2 were analysed. The overproduction of AtTPX2 led to ectopic microtubular arrays in the nuclei and at vicinity of the nuclear envelope. This arrangement was not typical for mitotic status of the cell and was independent of Aurora1 presence. Contrary to the animal cells, AtTPX2 mediated microtubule bundling was not a result of apoptosis. The important result is the confirmation of the association/interaction of plant TPX2 homologue with importin β and the participation of RanGTPase pathway in microtubule formation in acentrosomal plant cells.

Further the *in vitro* regulation patterns of AtTPX2 and AtAurora kinase 1 and 3 was evaluated. The results showed AtTPX2 as a substrate and activator of Aurora1, but not Aurora3. Moreover, AtTPX2-induced activation of AtAurora1 enhanced phosphorylation of the downstream targets, particularly histone H3. These results may point to a specific regulation of plant Aurora1 and Aurora3 kinases, which may have its significance in regulation of processes in mitosis and the cell cycle.

In brief, the present Thesis represents a contribution to progress in characterisation and revealing the function of plant nuclear proteins and constitutes the groundwork for further intensive study in this area.

Key words: *Arabidopsis thaliana*, AtTPX2, Aurora kinase, cell cycle, fibres, flow cytometry, importin, mass spectrometry, microtubules, nuclear proteome, nucleus, protein analysis, Ran

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Abstrakt:

Buněčné jádro je jedním z charakteristických organel eukaryotické buňky. Dvouvrstvá jaderná membrána představuje rozhraní mezi cytoplazmou a nukleoplasmou zahrnující chromatin, jadérko, jaderná tělíska a další struktury. Je protkána komplexem jaderných pórů a je tedy hlavním regulátorem importu a exportu molekul. Jádro bylo dlouhou dobu předmětem výzkumu především díky přítomnosti genetického materiálu a jeho replikačního a transkripčního aparátu. Rychlý vývoj sekvenačních technologií umožnil sestavovat úplné sekvence genomů u mnoha eukaryot a odhalovat funkci genů a nekódujících sekvencí. Pro úplné porozumění funkce genomu je však nutné získat znalosti o jaderném proteomu. Proteiny představují nejpočetnější molekuly v buněčném jádře a poskytují strukturní a funkční rámec pro většinu procesů odehrávajících se v jádře, ať už jde o regulaci genové exprese, buněčného cyklu a transportu molekul, nebo o strukturní podporu pro tyto děje. Ačkoli jaderné proteiny plní celou řadu důležitých funkcí, jejich znalost zůstává velmi omezená. U rostlin byl proteom buněčného jádra studován jen u několika modelových rostlin se známými genomovými sekvencemi a u několika hospodářsky významných plodin, u kterých byl sledován vliv stresových faktorů na změny jaderného proteomu.

Cílem předkládané práce je studium jaderných proteinů rostlin a přispění k odhalení vztahů mezi strukturou a funkcí jádra rostlinné buňky. Záměrem je získat ucelený přehled o složení jaderného proteomu v jednotlivých fázích buněčného cyklu a charakterizovat funkci vybraných proteinů. Proteomická analýza byla provedena na jádrech kořenových špiček ječmene (*Hordeum vulgare* L. cv. Morex). Přesná identifikace proteinů pomocí hmotnostní spektrometrie vyžaduje precizní přípravu materiálu. Dřívější práce využívaly pro izolaci jader převážně

frakcionační metody. Ty však s sebou nesou vysoké riziko kontaminace proteiny cytoplasmy a jsou časově náročné. Pro izolaci jaderných proteinů ječmene byl zde využit nový postup založený na třídění pomocí průtokové cytometrie. Průtoková cytometrie umožňuje poměrně rychlou analýzu mikroskopických částic, jako jsou buněčná jádra a mitotické chromozomy. Získané výsledky potvrdily vhodnost nového postupu, který umožňuje přípravu vzorků s minimální kontaminací nejadernými proteiny. S cílem identifikovat maximální počet proteinů byly použity dvě techniky hmotnostní spektrometrie, MALDI-TOF/TOF a ESI-Q/TOF a ukázala se jedinečnost každého typu spektrometru pro identifikaci určitých proteinů. Využití obou metod tak umožňuje pokrytí širokého spektra identifikovaných proteinů.

Funkční analýza byla zaměřena na charakterizaci jaderného proteinu TPX2 (Targeting Protein for Xklp2), který náleží do skupiny proteinů asociovaných s mikrotubuly. V živočišných buňkách působí TPX2 jako jeden z organizačních elementů mikrotubulů v oblasti centrozómu i chromozómů. Protein TPX2 po uvolnění z komplexu s importiny α a β aktivuje Aurora kinázu A, což je významný regulátor mitózy. Následně dochází k nukleaci mikrotubulů a tvorbě dělícího vřeténka. Bylo zjištěno, že TPX2 je asociovaný s kinázou Aurora1 také v rostlinných buňkách a že může působit jako regulátor kinázy Aurora1, která se podílí na organizaci mitotického vřeténka. K dalšímu objasnění funkce TPX2 byly analyzovány buňky *Arabidopsis thaliana* s nadprodukovaným TPX2. Nadprodukce vedla ke vzniku ektopických mikrotubulárních vláken v blízkosti jaderného obalu a uvnitř jádra. Toto uspořádání nebylo specifické pro přechod do mitózy a vyskytovalo se nezávisle na přítomnosti Aurora1 kinázy. Nebyla také prokázána spojitost mezi vytvářením mikrotubulárních svazků vlivem TPX2 a apoptózou, což odlišuje rostlinné buňky od buněk živočišných. Důležitým poznatkem je potvrzení asociace/interakce rostlinného homologu TPX2 s importinem a účasti Ran-GTPasové dráhy při formování mikrotubulů u acentrosomálních rostlinných buněk.

Experimenty zameřené na regulační vztahy AtTPX2 a AtAurora kinázy 1 a 3 potvrdily AtTPX2 jako substrát, ale rovněž i aktivátor pouze u kinázy Aurora1. Aktivace kinázy Aurora1 pomocí AtTPX2 vedla ke zvýšené fosforylaci cílových proteinů, zvláště histonu H3. Odlišná regulace obou Aurora kináz může hrát významnou roli v regulaci procesů mitózy a buněčného cyklu.

Ve svém souhrnu znamená předkládaná disertační práce přispění k charakterizaci jaderných proteinů rostlin a odhalování jejich funkce a vytváří předpoklady pro další intenzivní studium v této oblasti.

Klíčová slova: analýza proteinů, *Arabidopsis thaliana*, AtTPX2, Aurora kináza, buněčný cyklus, hmotnostní spektrometrie, jaderný proteom, jádro, importin, mikrotubuly, průtoková cytometrie, Ran, vlákna

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Contents

1. Introduction

1.1. Revealing the nature of life processes...from genome to proteome

It was in 1995 when the first complete genome sequence of a free living organism was published by Fleischmann and colleagues. It was a nucleotide sequence of bacterium *Haemophilus influenzae* with about two million of base pairs (Fleischmann *et al*., 1995). Since then, along with the development of high-throughput sequencing technologies, the number of completed genome sequences has been growing exponencially. Whole genome sequencing projects were the starting points, which provided platforms for debunking the characterisation of genes encoded within organisms. The information about DNA sequence provided the possibility of generating genetic and physical maps, and comparing gene sequences of particular organisms where differences can be significant for the nature of their life processes. DNA sequence-based techniques from the field of functional genomics enabled the elucidation of gene expression and function. Genomics paved the way for the analysis of gene products – proteins. Proteins are the fundamental building blocks of cells. They are the most structurally complex molecules and execute nearly all cell functions. As structural components of membranes, membrane channels, messengers of signals, enzymes and many others, proteins are embraced in every metabolic pathway. Proteome represents the sum of all proteins coded or expressed within a cell. According to another definition, it is a set of proteins expressed in a defined cell type, tissue or organism in a given time and conditions (Cullis, 2004). The study of proteome at the level of both individual proteins and protein interactions is embraced by ever progressing bioscience field of proteomics. Proteomic approaches enable the mapping of overall distribution of proteins in cells, their identification and characterisation that leads to the elucidation of their function. The organellar proteomics brings more specific information about the processes taking place in individual cellular compartments and the ways of communication among them.

Cell nucleus is a highly dynamic and complex headquarter of the eukaryotic cell. It is functionaly and structurally divided and its main feature is the separation of genetic material from other activities of the cell. Plenty of nuclear proteins provide necessary components for DNA replication and transcription machinery, metabolic reactions and structural and regulatory framework. On a higher organisational level, nuclear proteins influence plant development or defense against environmental stresses. The large scale of functions makes the proteins the most abundant components of cell nuclei (Sutherland *et al*., 2001). They are predicted to compose 10-20% of total cellular proteins (Narula *et al*., 2013). In plants, however, the information about the nuclear protein composition and function still laggs behind. The studies that have been

performed so far were focused mainly on the demarcation of protein groups, which change their level under different stress conditions. Only a limited number of studies aimed to describe the nuclear proteome as a whole. The proteomic identification of nuclear proteins is important for providing a general overview of present proteins and suggesting their functional connections. It is a „spring board“ for the determination of the protein functions.

2. Plant nuclear structure and functional organisation

Nucleus represents the defining morphological and functional feature of Eukaryotes. Enclosing chromatin and the components needed for replication of genetic material makes the nuclear compartment essential for all life processes. Double nuclear membrane enables separation of the replication and transcriptional machinery from the sites of protein synthesis. Communication and connection of nuclear space with cytoplasm is ensured by nuclear pore complex as well as by complex sets of proteins anchored to the nuclear membrane. The intra-nuclear compartmentation then provides the structural framework for organisation of genome at several levels and more effective execution of functions leading to gene expression.

Over the years, the progress in description of particular proteins of individual plant nuclear compartments has been made. The research groups associated with International Plant Nucleus Consortium have brought significant results in the field of plant nuclear envelope proteins description and provided more comprehensive picture of the relation between nuclear envelope components. In the introductory part, a short review of plant nuclear structures is provided with the focus on general overview of studied nuclear proteins involved in structural and functional organisation.

2.1. Nuclear envelope

Nuclear envelope (NE) constitutes the barrier between nuclear genetic content and cytoplasm in eukaryotic cells. It enables a highly regulated transport of proteins and provides a structural framework for the nucleus. The structural organisation of nuclear envelope is homologous in plants and animals. However, there are remarkable differences in protein composition and function. NE is formed by double phospholipid layer which is organized as inner (INM) and outer nuclear membrane (ONM) with about 30 nm perinuclear space between them. Both membranes differ chemically and by the type and number of associated proteins, which are defining for the specificities of nuclear membranes in different cells, tissues and species.

Proteins of nuclear envelope, which are associated with structural proteins of cytoskeleton and nucleoskeleton, form bridging between nuclear interior and cytoplasm (Evans *et al*., 2011; Kiseleva *et al*., 2013). Linker proteins that mediate such connection constitute - LInker of Nucleoskeleton and Cytoskeleton - **LINC complex**. This complex connects

cytoskeleton and cytoplasmic components with the nuclear lamina or lamina-like proteins in plants, which will be mentioned in more detail later, other non-skeletal proteins and chromatin (Tatout *et al*., 2014). In non-plant eukaryotes, there is a deeper knowledge about proteins of LINC complex integrated to nuclear membrane. LINC complex is formed of **Sad1/UNc84 (SUN) domain proteins** at the INM and **Klarsicht/Anc1/Syne1 homology (KASH) domain proteins** at the ONM (Crisp *et al*., 2006).

SUN domain proteins are well conserved among Eukaryotes and are the only known INM-specific transmembrane proteins in plants (Graumann *et al.*, 2010; Tatout *et al*., 2014; Petrovská *et al*., 2015). In *Arabidopsis*, SUN1 and SUN2 were described, both having nuclear localisation signal (NLS), transmembrane domain (TM) anchoring this protein in INM and coiled-coil and SUN domain, which lies within the perinuclear space (Graumann *et al.*, 2010). SUN proteins have been described also in rice, grapevine and maize. In maize, there have been found five different SUN proteins, ZmSUN1-ZmSUN5 (Murphy *et al*., 2010). C-terminal SUN domain interacts with KASH domain. On the cytoplasmatic site, KASH domain proteins associate with cytoskeletal components (Zhou *et al.*, 2012). In plants, candidates for KASH proteins have been identified rather recently, they are **tryptophan-proline-proline (WPP)-Interacting Proteins (WIPs)**. The WIP protein family is conserved in many plant species (grape, tomato, rice, maize, barley sorghum, sugarcane, barrel medic, pine and wheat). In *Arabidopsis*, three WIPs (WIP1, WIP2a and WIP3) were identified (Xu *et al.*, 2007). Although the sequence homology between WIP and KASH proteins has not been proved, it is evidenced that WIPs supply structural and functional role of KASH. WIPs interact with proteins containing WPP domain such as plant **RanGTPase–activating protein 1** (**RanGAP1**). This interaction enables localisation of RanGAP1 to outer nuclear membrane. RanGAP establishes a functional RanGTP/RanGDP gradient across the nuclear envelope, which is important for maintaining the directional interphase nucleocytoplasmic transport (Zhao *et al.*, 2008). In mitotic cells, AtRanGAP1 localises in the place of preprophase band, spindle and phragmoplast (Kiseleva *et al.*, 2013).

WIPs interactors, **WPP domain-interacting tail-anchored (WIT) proteins,** are another group of proteins interacting with WPP domain of plant LINC complex (Zhao *et al*., 2008).

Other group which has the characteristics of KASH domain proteins in plants are the **SINE proteins (SUN-Interacting Nuclear Envelope)**. In *Arabidopsis*, mainly SINE1 has been characterized, which is involved in nucleus positioning in guard cells through connection with actin cytoskeleton and SINE2 contributing to innate immunity against oomycete pathogen (Zhou *et al.*, 2014). Graumann *et al*. (2014) identified a novel nuclear envelope KASH domain protein,

AtTIK, interacting with SUN subfamilies. It functions in nuclear morphology control and has been identified in root cells.

It is also important to mention that components of LINC complex have a certain role during mitosis and cell division. In animal and yeast systems, KASH and SUN proteins are involved in centromeres anchorage and NE reformation. Plant SUN1 and WIPs are dynamically distributed during mitosis and cell division, accumulating around chromatin at the spindle pole, then progressing to the cell plate and surrounding chromatin within the new NE formation (Tatout *et al.*, 2014).

Some proteins of NE mediate the connection with cytoskeletal components. This connection enables the movement of nucleus and maintains its shape. **Myosin XI-i**, a plant specific myosin, localizes to the ONM and was found to interact with WIT1 and WIT2, which anchor Myosin XI-i to the nuclear membrane. Myosin XI-i and WITs are required for nuclear movement as their deficiency changes the reaction of nuclear positioning in different light conditions (Tamura *et al.*, 2013; Griffis *et al.*, 2014).

ONM is also a place with anchored nucleoskeletal proteins. WPPs (WPP1, WPP2 and WPP3) homologous to MFP attachment factor 1 (MAF1) in *Arabidopsis*. WPP1 and WPP2 were shown to localize at ONM during interphase and nuclear pore complexes in undifferentiated cells of root tips (Patel *et al.*, 2004). A novel NE protein **KAKU4**, which locates to the INM, has been characterized by Kimura *et al*. (2010) and Goto *et al*. (2014). It interacts with CRWN1 and four proteins which will be mentioned later in relation to plant nucleoskeleton. It has been suggested that KAKU4 functions in NE size and shape modulation.

Finally, there will be mentioned components of γ-tubulin complex in relation to the NE. γ-tubulin ring complex (γ-TuRC) is a mediator of microtubule nucleation at NE. Recently, several γ-tubulin complex components were identified in plants. They include **γ-tubulin complex protein 2** and **3 (AtGCP2** and **AtGCP3)**, which contain nuclear targeting domains enabling the localisation to the ONM (Graumann and Evans, 2010), and **GCP3-interacting proteins (GIPs)** which beside the role in microtubule nucleation complex adapt the NE-associated proteins and are involved in maintaining of nuclear shape (Seltzer *et al*., 2007; Batzenschlager *et al*., 2013 and 2014). Batzenschlager *et al*. (2015) also recently postulated the function of GIPs in centromeric histone H3 variant (CENH3) deposition and/or maintenance at centromeres. **Augmin** protein complex and **NEDD1** (γ-TuRC-interacting protein) were detected at NE as well, with reported involvement in γ-TuRC-dependent spindle MT assembly (Zeng *et al*., 2009; Hotta *et al*., 2012; Masoud *et al.*, 2013). The anchorage of microtubule organising centre (MTOC) and spindle pole body is facilitated by KASH domain proteins in animal and

yeast cells. However, we still do not have a broader knowledge about proteins facilitating anchorage of cytoskeletal components in plants. An illustrative model of nucleo-cytoplasmic continuum at the NE and perinuclear MT nucleation provides **Fig. 1.**

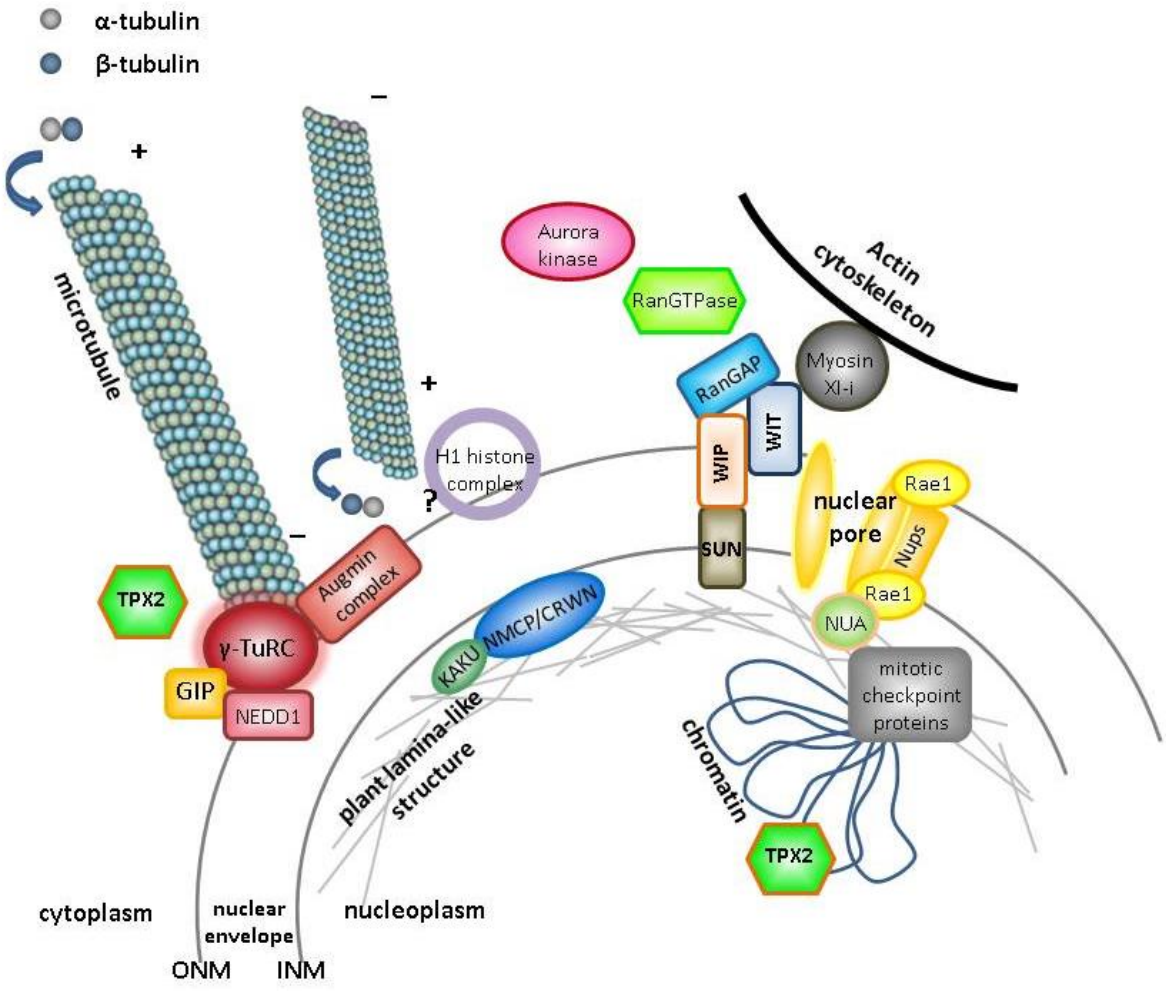


Fig. 1. Model of protein distribution on nuclear envelope with experimentaly confirmed or hypothesized protein interactions and perinuclear microtubule nucleation in late G2 phase of cell cycle. (+) and (–): intrinsic polarity of MTs; γ-TuRC: MT nucleation organisation center, includes GCP2, GCP3 and γ-tubulin; GIPs and NEDD1: γ-TuRC-associated proteins, additional putative MTs assembly factors; Augmin complex: observed at the interphase NE; H1 histone ring complex: hypothetical function in MT nucleation; SUNs, WIPs and WITs: components of plant LINC complex, nucleocytoplasmatic linker; NMCP/CRWN, KAKU: components of „plamina"; Myosin XI-i: mediates the connection of LINC complex to cytoskeleton; Nups: nucleoporins; Rae1: mRNA export factor1, located in outer NP rings; NUA: nuclear pore anchor in nuclear basket; Mitotic checkpoint proteins: often linked to chromocentres and nuclear pores; RanGTPase, Aurora kinase: regulatory enzymes linked to MT nucleation; TPX2: MT associated protein, participates in MT assembly; (?): unknown factor for anchoring the nucleation complex. Adapted from Tamura and Hara-Nishimura, 2013; Masoud *et al*., 2013; Tatout *et al*., 2014 and unpublished figure of Beáta Petrovská.

2.2. Nuclear pore complex

Regulation of bi-directional nucleocytoplasmic transport belongs to the crucial control mechanisms in a cell. Transport of molecules through the nuclear envelope is established by the system of nuclear pore complexes (NPCs), crucial in signalling transduction, transport of transcription and translation components and other macromolecules. The studies in vertebrate cells using field emission scanning electron microscopy (feSEM microscopy) revealed a three layered structure – cytoplasmic ring, spoke ring complex and nuclear ring. NPCs of flowering plants have a conserved structure similar to the vertebrate NPCs structure. The arrangement and density of NPCs varies between species, tissues and nuclear size (Lim *et al.*, 2008; Kiseleva *et al*., 2013). It was found that NPCs are arranged in rows over the nuclear surface and it was suggested that lamina-like structure can play role in such organisation both in animal and in plant cells (Liu *et al*., 2000; Maeshima *et al.*, 2006; Fiserova and Goldberg, 2010). Moreover, in *Xenopus* lamina filaments are attached directly to nuclear pore structures and similar connection was observed in tobacco BY-2 cells as well (Goldberg and Allen, 1996; Fiserova *et al*., 2009).

NPCs are assembled of multiple copies of **nucleoporins** (Nups); yeast and metazoan cells contain approximately 30 nucleoporins and recent studies showed about the same number in plants (Meier and Somers, 2011; Tamura and Hara-Nishimura, 2013). NPC proteins can be classified in groups according to subcomplex formation, or their localisation in different compartments of the pore (Onischenko and Weiss, 2011; Kiseleva *et al*., 2013). In relation to their function in NPC, nucloporins are classified into phenylalanine-glycine repeat (FG)-containing Nups and scaffold Nups. FG Nups play a key role in nucleocytoplasmic transport, since FG domains serve to bind nuclear transport complexes. Scaffold Nups are the main building blocks during NPC biogenesis on the NE (Tamura and Hara-Nishimura, 2014). Several studies were conducted on plant Nups. In general, the function of plant Nups was found important for various processes like chromatin organisation and regulation of gene expression, spindle assembly, linking the NPC to the plant lamina-like structure, hormone signalling pathways, flowering, embryo development, stress tolerance, symbiotic processes and plant disease resistance response (Meier and Brkljacic, 2009; Parry, 2013; Tamura and Hara-Nishimura, 2013 and 2014; MacGregor and Penfield, 2015). Protein composition of plant Nups is not known to such an extent as of the animal Nups. There has been just a few plant homologs identified based on sequence homology database searches. In spite of the low sequence similarity of plant and metazoan Nups, there is a high conservation in shape and predicted fold types (Tamura and Hara-Nishimura, 2013). The basic structure of nuclear pore and Nups identified in

Arabidopsis embedded in particular segments are depicted in **Fig. 2**.

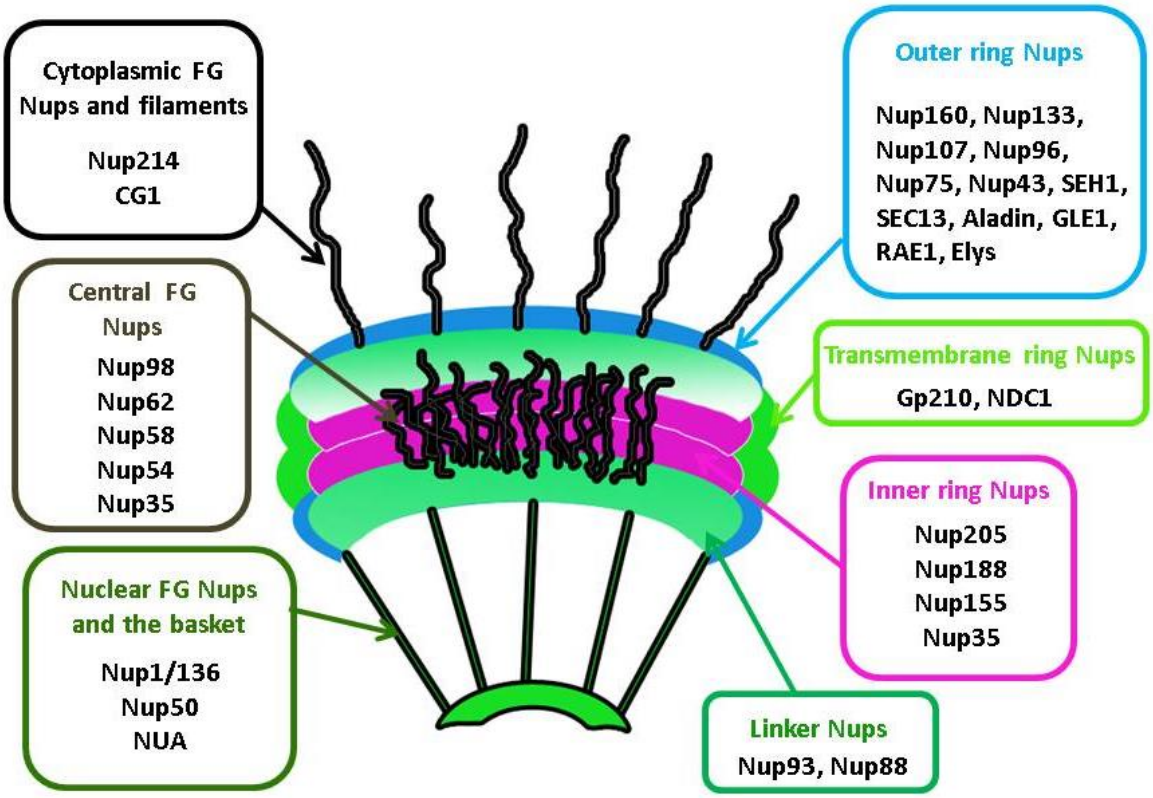


Fig. 2. Overview of nuclear pore complex proteins and their localisation in NPC, according to Tamura and Hara-Nishimura (2013)

Nuclear pore associated proteins in plants are represented by transcription-coupled export 2 (TREX-2) complex, EARLY IN SHORT DAYS4 (ESD4) and RanGAP – WIT – WIP complex (Tamura and Hara-Nishimura, 2013). Components of *Arabidopsis* TREX-2 complex were found at the inner side of the NPC, the nuclear basket (Lu *et al*., 2010), and are supposed to function in mRNA export. ESD4 functions in interaction with Nuclear pore Anchor (NUA) and encodes a Small Ubiquitin-like Modifier (SUMO)-specific protease. WIP and WIT proteins haven't been reported to physically interact with NPC, but are possibly connected with NPC functionally. Their function in anchoring RanGAP to the NE is supposed to replace the function of vertebrate nucleoporin Nup358, which anchors RanGAP to NPC (Tamura and Hara-Nishimura, 2013; Zhou and Meier, 2013).

2.3. Proteins of ion channels

Another form of communication between the cytoplasm and nucleoplasm is represented by ion channels and pumps situated in INM and ONM (Tatout *et al*., 2014). The studies of protein composition in plant ion channels and pumps have been focused predominantly on Ca^{2+} signalling. *Solanum lycopersicum* **LCA** protein, an endoplasmic reticulum type of a calcium pumping ATPase, was one of the first proteins described on plant NE. Perinuclear Ca^{2+} oscilation is triggered by rhizobial Nod factors and was recorded also within arbuscular mycorhisis. This mycorhisal relationship was studied in model legume plant *Medicago truncatula* where the components of Ca^{2+} signalling pathway include a cation channel **Doesn't make infection** (**DMI1**) embedded in nuclear membrane. The homologues of DMI1 localized in the NE - **CASTOR** and **POLLUX,** have been identified also in *Lotus japonicus* in nodulation defect survey (Charpentier *et al*., 2008; Chabaud *et al*., 2011; Huda *et al*., 2013).

2.4. Plant nucleoskeleton and intra-nuclear organisation

Compartmentalisation and dynamic nature of nuclear interior partcipate in controlling gene organisation within the 3D nuclear space and gene expression. Genome is dynamically organised at the level of location of chromosomal domains and chromosomes or even individual genes. This organisation is supported by dynamic intra-nuclear structural framework – nucleoskeleton, where numerous interacting structural proteins provide a scaffold for protein complexes involved in vital processes of the nucleus and cell (Moreno Díaz de la Espina, 2009; Kiseleva *et al*., 2013). The protein composition of plant nucleoskeleton hasn't been fully elucidated. However, in a number of studies, similarities with the animal system were recorded at least at the functional level. Nucleoskeleton in plants consists of peripheral lamina-like structure and intranuclear network. Nuclear lamina is a complex protein network adjoined to the INM, contributing both to mechanical support and rigidity of the nucleus and to chromatin organisation, DNA replication, repair, transcription and communication between nucleus and cytoplasm. As the important component of nucleoskelet-cytoskelet linkage, lamina is reported to play role in maintaining mechanical properties of entire cell (Dechat *et al.*, 2008). Lamina is present and has conserved functions in all eukaryotes, yet the protein composition differs. In metazoans, there are several types of lamins, lamin A and B (both in vertebrates and invertebrates) and lamin C in mammals and belong to the group of type V intermediate filaments

with tripartite structure (Prokocimer *et al.*, 2009). In plants, lamin homologues are lacking but several studies refer to structure and proteins of similar function (Blumenthal *et al*., 2004; Moriguchi *et al*., 2005; Fiserova *et al*., 2009; Fiserova and Goldberg, 2010; Ciska and Moreno Díaz de la Espina, 2014). Candidate lamin-like plant proteins were recently identified. **Nuclear matrix constituent protein 1 (NMCP1)** was first identified in carrot (*Daucus carota*) (Masuda *et al*., 1997) and four related proteins were identified in *Arabidopsis thaliana* named **LITTLE NUCLEI 1-4** after the fenotype of the mutants (Dittmer *et al.*, 2007). Only LINC1 localizes to the nuclear envelope (Dittmer *et al.*, 2007; Kiseleva *et al*., 2013). To prevent the misleading replacement with complex of proteins linking nucleoskeleton and cytoskeleton (LINC), little nuclei were renamed to **crowded nuclei (CRWN)** proteins according to the phenotype of reduced nuclei size (Wang *et al*., 2013). For detailed classification of NMCP proteins see Ciska and Moreno Diaz de la Espina (2014). The functions of NMCPs are still poorly understood. The main function consists in nuclear size and shape control. The requirement for spatial organisation of constitutive heterochromatin has also been proposed (Wang *et al*., 2013). Only quadruple mutants of NMCP/LINC/CRWN were shown not to be viable; viability of single, double or triple mutants may show the complementarity of different NMCP variants (Dittmer *et al*., 2007; Sakamoto and Takagi, 2013; Wang *et al.*, 2013; Ciska and Moreno Diaz de la Espina, 2014). Important result was reported by Graumann (2014), who demonstrated the interaction of nucleoskeletal NMCP protein with AtSUN1 and 2, which is an important indicator of SUN-nucleoskeletal anchorage in plants (Evans *et al*., 2014).

Other putative lamin-like proteins include Filament-like Plant Proteins (**AtFPP1-7)** identified in *Arabidopsis* and its homologues in tomato and rice (Gindullis *et al*., 2002) and other coiled-coil proteins that bind to matrix attachment region on DNA. Matrix/scaffold attachment regions are approximately 200 bp long AT rich sequences that are believed to interact with nuclear matrix/nucleoskeleton (Breyne *et al*., 1992; Wang *et al*., 2010**).** Interaction within these regions is important for higher order chromatin organisation and regulation of gene expression. This is represented by plant specific protein with transmembrane, coiled-coil and DNA binding domain, **Matrix attachment region (MAR)-binding filament-like protein 1 (MFP1),** found in *Arabidopsis*, tomato, tobacco at the nuclear periphery and in chloroplasts. Association of the onion MFP1 with nucleoskeletal filaments was proposed (Meier *et al*., 1996; Harder *et al*., 2000; Samaniego *et al.*, 2006 (a), (b); Samaniego *et al*., 2008).

In plants, MFP1 protein has its binding partner called **MFP1 associated factor (MAF1)** (Gindullis *et al*., 1999). MAF1 contains WPP domain (tryptophan-proline-proline) and it is known that this domain is responsible for location of WIPs and WITs to the nuclear envelope. It

has been speculated that MAF1 may be located by MFP1 to the nucleoskeleton and brought to closer association with certain genome regions (Kiseleva *et al*., 2013).

From the group of proteins identified in plant nuclear matrix, only Nuclear Mitotic Apparatus **NuMA** protein is mentioned here. NuMA homologs were identified in onion meristematic root cells by their cross-reactivity with human and *Xenopus* NuMA. Plant NuMA homologs were distributed in an intra-nuclear network and in spindle matrix during mitosis. In mammals, NuMA is associated with nuclear matrix in the interphase and also functions as the assembly factor for microtubules. Overexpressed NuMA influenced the assembly of intra-nuclear microtubule bundles which may point to the structural role in the nucleoskeleton (Yu and Moreno Díaz de la Espina, 1999).

2.5. Proteins of nuclear bodies

Nuclear bodies are dynamic and regular intra-nuclear organelles comprising nucleolus, Cajal bodies, nuclear speckles, cyclophilin-containing speckles, dicing bodies, AKIP1-containing bodies and photobodies (Petrovská *et al*., 2015; **Fig. 3**). These sub-nuclear organelles spatially compartmentalize inner space of nucleus and help in creating defined areas to enable efficiency and specificity of various intranuclear processes. Although the general function may resemble cytoplasmic organelles, nuclear bodies differ fundamentally in the absence of membrane, which may lead to faster and free exchange of nuclear bodies' components in the nucleoplasm.

Nucleolus is the most distinctive of nuclear bodies in interphase cell. Its position, which is stable from the telophase to the next cell cycle prophase, is determined by nucleolus organising region (NOR) (Kalmárová *et al*., 2007) and also cytoskeleton has been reported to play a role (Stępiński, 2014). Nucleolus is structured in fibrillar centers, dense fibrillar components and granular components, which are the areas where rDNA transcription, pre-rRNA processing and assembly of ribosomal subunits take place. Additional components distinguished in nucleolus are nucleolema and nucleolar chromatin. Detailed information is provided by Stepinski (2014). Although proteins represent the highest proportion of molecules in the nucleolus, only a few have been characterized in plants so far. Homologues of **nucleolin**, which is a major nucleolar protein involved in the processes of ribosomal biogenesis (Petricka and Nelson, 2007; Medina *et al*., 2010), and **fibrillarin** involved in processing rRNA precursors (Pih *et al*., 2000), have been characterized in plants. Proteomic studies of Pendle *et al*. (2005) on nucleolar protein composition in *Arabidopsis* identified 217 proteins. Additionally to ribosomal

and nucleolar proteins, splicing and translation factors such as exon junction complex and other non-ribosomal and non-nucleolar proteins or proteins with unknown function were identified (Pendle *et al*., 2005).

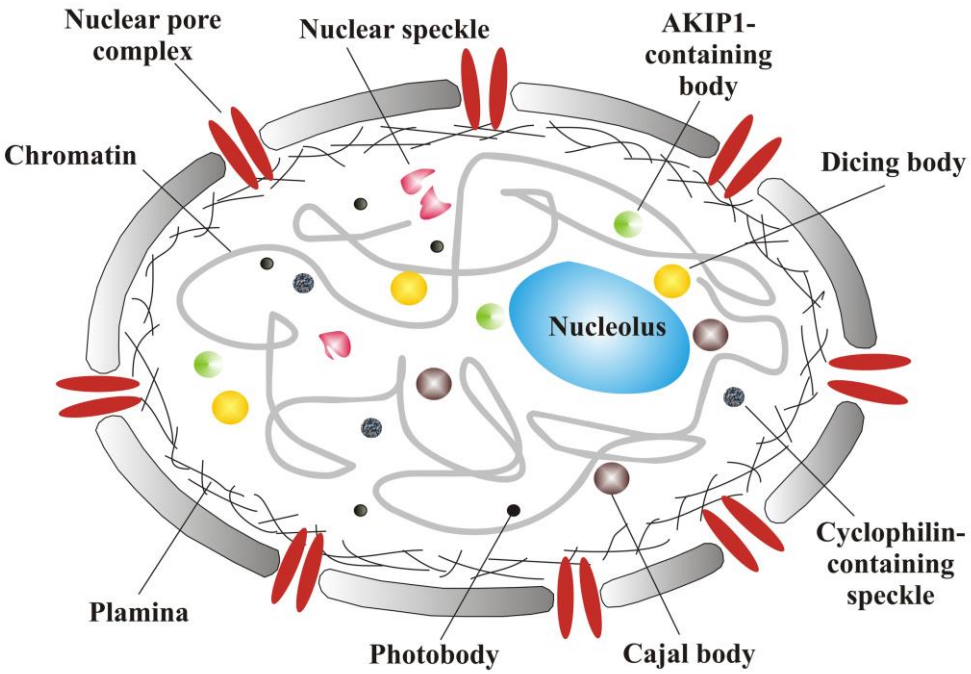


Fig. 3. Illustrative scheme of the plant cell nucleus, adopted from Petrovská *et al*., 2015.

Cajal bodies (CBs) were first identified more than 100 years ago by Ramon y Cajal in neuronal cells (Shaw and Brown, 2012). They are physically and functionally associated with nucleoli and their role consists in maturation of RNA complexes (spliceosomal subcomplexes or small RNAs), but the detailed function of these highly dynamic structures still needs to be fully disclosed (Stanek and Neugebauer, 2006). The most determining protein of CBs is **coilin**, reffered as indespensable for CBs formation and activity. *Arabidopsis* homologue of coilin, **AtCoilin**, interacts with RNA and may function as a scaffolding protein (Makarov *et al*., 2013). Coilin interacts with the **survivor motor neuron protein (SMN),** CBs protein playing role in biogenesis of snRNPs, but its homologue in plants hasn't been identified. Also ARGONAUTE4 - AGO4 protein, a member of transcriptional gene silencing pathway was found co-localized with CBs (Pontes *et al*., 2006).

Nuclear speckles are dynamic structures disassembled during mitosis and reforming in daughter nuclei where they are situated in the interchromatin space often observed near active site of transcription and are a storage place for splicing factors (Lamond and Sleeman, 2003;

Fang *et al*., 2004; Reddy *et al*., 2012). Nuclear speckles contain snRNPs, non-snRNP splicing proteins and transcription and 3' end processing factors (Reddy *et al*., 2012). Proteomic analysis has been performed in metazoans (Saitoh *et al*., 2004), but information about protein composition of nuclear speckles in plants is still lacking.

Cyclophilin-containing speckles are plant-specific nuclear bodies where **arginine/serine-rich (RS) domain-containing cyclophilin (CypRS64)** is present and stored. It is reported to interact with SR proteins and U1 and U11 snRNP-specific proteins suggesting the role in spliceosome assembly (Lorkovic *et al*., 2004; Shaw and Brown, 2004).

Dicing bodies, as the name evokes, consist mainly of **dicer-like1 (DCL1)** and **hyponastic leaves (HYL1)** proteins, which is a dsRNA binding protein. Both are involved in microRNA metabolism (Shaw and Brown, 2004; Song *et al*., 2007).

AKIP1-containing bodies were found in the nuclei of guard cells. **AKIP1** is involved in stress response pathway. Although a detailed function of AKIP1-containing bodies is unknown, it may serve as storage of downstream targets that are accumulated as protection against cellular stress. This idea is supported by observation of AKIP1 that re-localized to speckle-like domains after abscisic acid treatment (Li *et al*., 2002).

Nuclear photobodies in plants were described rather recently and are regulated by an external light signal (van Buskirk *et al*., 2014).

3. Tools to study nuclear proteome

The cell nucleus is a highly dynamic and complex structure with proteins of many diverse functions mutually interconnected. How to investigate such a complex environment? The nuclear protein research can be approached from two directions. The first option is to characterise unknown nuclear proteins (NPs) on the basis of homology with already known NPs of other (non-plant) species. This approach, however, can encounter barriers in the absence of relevant homologs. Moreover, with the anticipated number of nuclear proteins, the progress in NPs characterisation has been rather slow (Petrovská *et al*., 2015). The second option is a direct extraction and identification of unknown or uncharacterised proteins directly from the nucleus. In this respect, proteomics aplications enable large-scale analysis not only of general protein composition of a particular organelle, but also the analysis of properties of individual proteins - structure, post-translational modifications, intracellular distribution or protein-protein interactions (Erhardt *et al*., 2010).

Various tools for proteomic analysis have been developed in the past few decades. Most of them were developed for studying animal proteome and later were adopted for plant proteomics (Anguraj Vadivel, 2015). Contemporary proteomics utilizes high-throughput techniques. High-resolution separation methods (two-dimensional gel electrophoresis) and liquid chromatography provide a material for protein identification with mass spectrometry. Modern mass spectrometers utilize different sources of ionisation and mass analyzers and represent a technique with a high sensitivity, accuracy and resolution (Yates, 2004). Mass spectrometry can be the then employed not only in protein identification, but also protein quantification (Bantscheff *et al*., 2012).

Proteomics utilizes a broad range of strategies. Two common approaches for the analysis of protein sample are "bottom-up" and "top-down" (Chait, 2006). In "bottom-up" strategy, proteins are first separated, then digested to peptides and analyzed by mass spectrometer. Or proteins are digested in a complex mixture without previous separation following the separation via liquid chromatography coupled to mass spectrometry. In the "top-down" strategy, the isolated proteins are fragmented directly in mass spectrometer without any previous digestion. For a general review of different concepts in plant proteomics see Carpentier *et al*., 2008; Ruan *et al*., 2008 or Anguraj Vadivel, 2015. In this chapter, I will mention only the approaches relating to the characterisation of plant nuclear proteomes, on line with the main topic of this Thesis.

3.1. Methodology in nuclear proteomics

In comparison with animal proteome research, the methodology for plant proteomics may be more demanding. This can be due to the distinctive features of plant cells, such as rigid cell wall, the complex extracellular matrix joining cells together, and the presence of a variey of interferring compounds. The protocols may be derived from the methods used for animal proteins analysis, but modified with the respect to the character of plant cells. Proteomic experiment consists of several basic steps: a) sample preparation; b) protein extraction; c) protein or peptide separation; d) protein identification and interpretation of data. The methodological approach is based on physicochemical and structural characteristics of proteins (e.g. solubility, hydrophobicity, molecular weight) thus protocols must be optimised.

3.1.1. Isolation of organeles

The first step of sample preparation from any biological material consists in selection of an appropriate method for isolation of pure organelles, cells or tissue of interest in which protein fraction is being analyzed. A majority of published protocols is based on homogenisation of plant material, filtration of the homogenate and removal of debris, pelleting, and elimination of contaminating organelles by separation techniques such as differential or density gradient centrifugation. Cell or tissue disruption can be performed by several means: pulverizing with liquid nitrogen, which can minimize proteolysis and is being commonly used for leaf tissues, fruits and seeds (Wang *et al*., 2008). Mechanical homogenisation with different types of homogenizers was reported to be very useful tool in plant tissues with rigid cell wall (Martínez-Maqueda, 2013).

Centrifugation has been so far the most exploited method for organeles isolation in nuclear proteomic studies (see Table 1). Multiple centrifugation steps result in separation of the cellular organelles in homogenate into different layers based on size, shape and density of individual organelles. Differential centrifugation is performed by successive centrifugations with increasing relative centrifugal force, where large and heavy particles sediment faster than small and lighter particles. Density gradient centrifugation is used to achieve higher resolution of isolated particles with the use of sucrose gradients. Layers of isolated particles with different densities are present at the interfaces between sucrose layers of different concentration (Mikkelsen and Cortón, 2004).

Another highly efficient method for isolation of cell substructures is the flow cytometry. Flow cytometry (FCM) represents one of the high-throughput modern techniques with a broad use in basic and applied research. Its main advantages are high speed of analysis providing statistically relevant data, high accuracy and resolution, and negligible destructiveness (Vrána *et al*., 2014). In principle, flow cytometry is based on the analysis of optical parametres (fluorescence intensity and light scatter) of microscopic particles moving in a narrow stream of liquid. Due to the limitations which plant cells and protoplasts pose for their use in FCM, the most frequent application of FCM in plant science has been the analysis of subcellular organelles such as plastids, nuclei or chromosomes (Vrána *et al*., 2014). The analysis of plant nuclei with FCM is based on the assesment of fluorescence. The result of processed and digitalized fluorescent signal is presented as a single parameter histogram with relative fluorescence intensity (relative nuclear DNA content) at X axis and a number of particles at Y axis, or as two patrameter correlated plot (dot plot, density, contour plot). Importantly, some flow cytometers can be used for sorting the particles of interest. In this process, the liquid stream is broken to droplets and the droplets carrying particles of interests are charged and deflected during a passage through electrostatic field and collected in suitable vessels (Suda, 2011). This permits the separation of particles on the basis of size or DNA content, such as nuclei in individual cell cycle phases. The exploitation of this method for nuclei purification prior to proteomic analysis is rather new and so far it hasn't been employed in plant cells for this purpose.

3.1.2. Protein extraction

Plants are among highly differentiated organisms where different parts of tissue, organs or organeles are specialized for different functions. Not only the function, but also the age and stress factors can influence the presence of a specific set of proteins in individual samples. Preparation of the sample belongs to the most crucial steps in proteomic analysis for obtaining high quality resolution of proteins. Sample preparation includes protein extraction from the source material, removal of interfering compounds and solubilisation of proteins. Preparation of plant protein samples comprises several obstacles. In general, the protein content and concentration in plant samples is low as compared to animal cells or microorganisms (Jacobs *et al*., 2000; Isaacson *et al*., 2006). Plant cells are rich in inhibiting/interferring compounds. Lipids, nucleic acids, salts, secondary metabolites, such as phenolics compounds, proteases and others may interfere with protein extraction. Modification of proteins with proteases and phenolic

compounds may result in changes in molecular weight or isoelectric point which may bring altered results of the two-dimensional electrophoresis 2-DE process.

To reproducibly obtain all the proteins from cell compartment or tissue of interest of different species, optimisation of extraction procedure is necessary. Methods widely used for protein extraction in plants include extraxtion of proteins in an aqueous buffer (Tris-HCl), followed by desalting and concentration (Jacobs *et al.*, 2000). Disadvantage of this method is the insolubility of hydrophobic proteins, only water-soluble proteins can be analysed. The widely used method for the plant protein extraction is the precipitation of proteins by trichloroacetic acid (TCA) or acetone or both (Damerval *et al*., 1986; Jacobs *et al*., 2000, Isaacson *et al*., 2006). Many interferring compounds are soluble in acetone and thus can be removed from the sample. Combination of TCA/acetone extraction is reffered to be effective for inhibition of proteases, phenoloxidases and peroxidases. The likely loss of proteins which are precipitated but not resolubilized in later phases poses a disadvantage of this method (Wang *et al*., 2006 and 2008; Pavoković *et al*., 2012). Protein extraction with the use of phenol is reffered to be effective for proteins of recalcitrant tissues (sensitive for desiccation and high and low temperatures). Phenol dissolves lipids and proteins, which are subsequently concentrated by acetone or methanol precipitation and substances soluble in water (e.g. polysaccharides) are left in aqueous phase (Isaacson *et al*., 2006). Phenol-based extraction has been succesfully used in many types of plant tissues and species rich in components interferring with electrophoresis and is considered more efficient then TCA/acetone extraction (Wang *et al*. 2008). However, its toxicity and time-consuming character may be the disadvantage. Further, both in animal and plant cells, Radio-Immunoprecipitation Assay (RIPA) lysis buffer, was succesfully used in isolation of membrane, cytoplasmic or nuclear proteins (Ngoka, 2008; Petrovská *et al*., 2014).

3.1.3. Proteins or peptides separation

The essential steps in proteome research workflow are the separation techniques. Electrophoretic separation of protein mixtures based on charge and size is the commonly used preparative and analytical tool (Martínez-Maqueda *et al*., 2013). The core and the most widely used methods for separation complex protein mixtures are one dimensional (1-DE) and two dimensional (2-DE) electrophoresis (Anguraj Vadivel, 2015). 1D-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a well established and highly reproducible method, which provides the resolution in a broad molecular mass range. It can be used prior to

the mass spectrometry analysis when the stained bands in gels are sliced according to molecular mass markers and subdued to enzymatic digestion. 2-DE provides even higher resolution due to the combination of independent protein parametres and establishes a protein map. The first dimension represents the separation of proteins based on their isoelecric point (pI) in a pH gradient (isoelectric focusation - IEF). Today the most exploited method for generating pH gradient is IPG (immobilized pH gradient) gel/strips, when pH gradient is made by a range of well defined chemicals co-polymerized on matrix. The second dimension then separates proteins according to their molecular weight in SDS-PAGE (Jacobs *et al*., 2000). 2-DE electrophoresis is routinely and widely used method. However, commercially available components are rather expensive.

3.1.4. Protein identification

In the bottom-up strategy, prior to protein identification by mass spectrometry (MS), proteins must be digested to obtain peptide mixtures (Martínez-Maqueda *et al*., 2013). The specific cleavage of peptide bonds is mediated by different proteolytic agents (trypsin, endoproteases, chemical reagents and others). Basically there are two types of digestion: (i) "in-gel", which follows protein separation, or (ii) "in-solution"- digestion of complex protein mixture without previous separation. With the in-solution digestion, unwanted loss of less abundant proteins can be avoided. On the other hand, with the in-gel digestion low molecular weight impurities can be removed after the separation. Prior to analysis, the samples are usually desalted to prevent the interference with MS techniques (Martínez-Maqueda *et al*., 2013).

The key proteomic technique for proteins identification is the mass spectrometry. Mass spectrometry analysis is based on measuring the mass to charge ratios (m/z) of ions in a vacuum (Westermeier and Naven, 2002). Mass spectrometers consist of three basic components: a source of ionisation, a mass analyzer and a detector (**Fig. 4**). During the ionisation, negatively or positively charged sample ions are created; mass analyser then separates or/and resolves ions created in the ionisation source based on their m/z ratios and detector monitors the ion flow and transmits the signal to data analysis system, which records the mass spectra and processes the properties of the analyte.

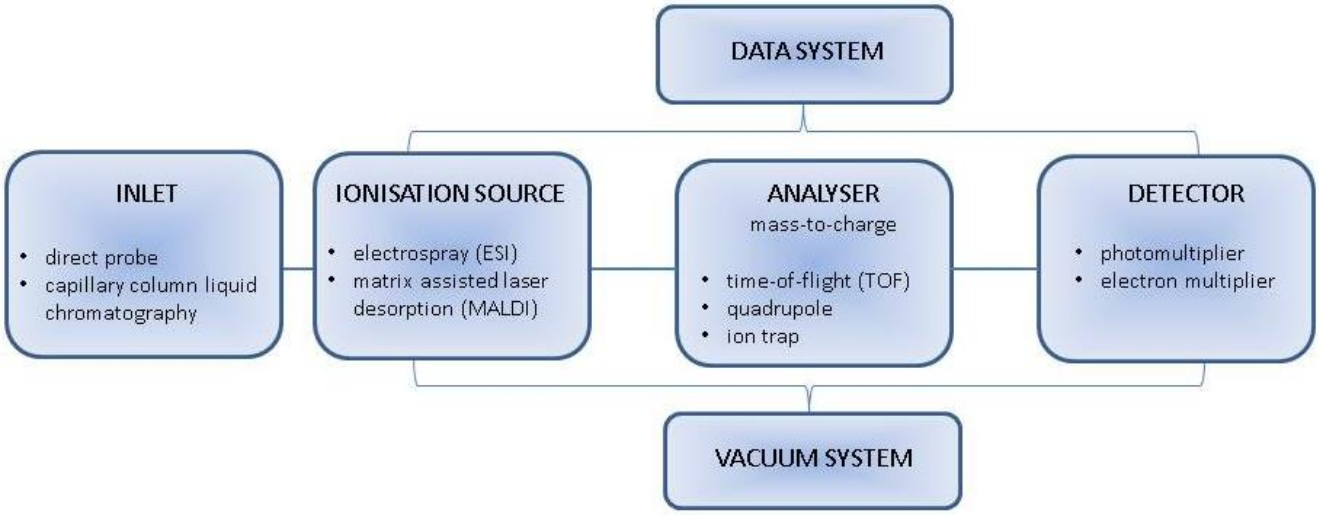


Fig. 4. Simplified scheme of a mass spectrometer with the examples of types in particular components. According to Kinter and Sherman, 2000.

With the advancing technical improvements, various sources of ionisation, analysers and detectors have been developed and there is a variety of MS instruments and their combination available (Ruan *et al.*, 2008). A brief description two types of soft ionisation techniques is provided below, which are used in the majority of biochemical analyses: matrix assisted laser desorption/ionisation mass spectrrometry (MALDI) and electrospray ionisation mass spectrometry (ESI) (**Fig. 5**). These techniques provide efficient analytical tools for obtaining intact molecules with even subpicomole sensitivity (Lewis *et al.*, 2000; Ruan *et al.*, 2008).

ESI method is based on the transfer of ions from volatile solution in a capillary into gaseous phase after a high voltage application. It includes several steps: dispersion of fine spray of charged droplets, solvent evaporation, and ion ejection from charged droplets. Ejected ions are then accelerated into the mass analyser for the analysis of molecular mass and ion intensity (Ho, *et al*., 2003).

In MALDI, the analyte of interest is anchored to an excess of matrix compound (co-crystalization). The laser targets the mixture of analyte and sample. The matrix transforms laser energy to the excitation energy of the analyte causing its vaporisation and, as a proton donor and receptor, also its ionisation (Lewis *et al*., 2000). Vaporizing ions enter the flight tube for mass separation in eletric field. Then, through ion detector and data processing, the mass spectrum is obtained. Mass spectra generated by MALDI are single charged ions. Ions generated by ESI have multiple charges (Ruan, *et al*., 2008).

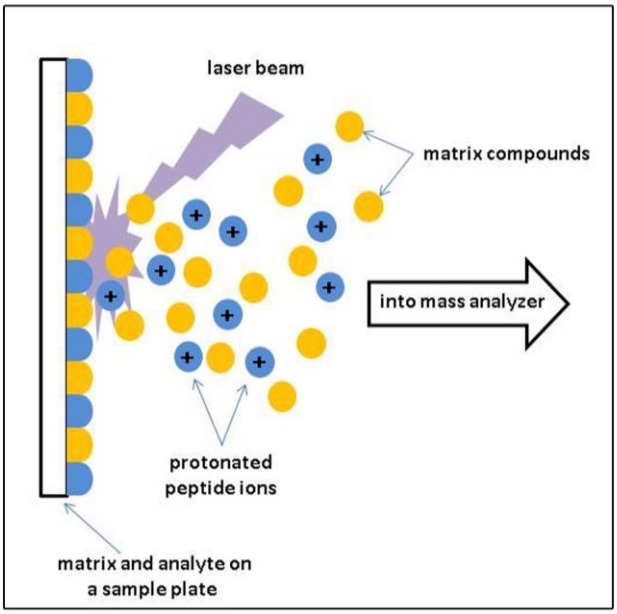
There are several mass analysers available, for example time-of-flight, TOF;

quadrupoles and others, with different sensitivity, scale of resolution or compatibility with ionisation sources (Dass, 2002).

The MS is often conjuncted with other analytical techniques. Thus, with liquid chromatography, the protein sample is fractionated prior to MS. Tandem mass spectrometers (MS-MS) then have more than one analyser and can be used to determine protein structure and sequence, based on the fragmentation of selected sample ions inside the mass spectrometer and identification of resulting fragment ions (Ashcroft, 1997).

The identification of proteins by MS is based on correlation with available sequence databases. A prerequisite for database searching are measured m/z ratios. The protein is then identified on the base of statistical matching of analyzed and predicted peptides, or peptide sequence information (Ruan *et al.*, 2008)

a)



b)

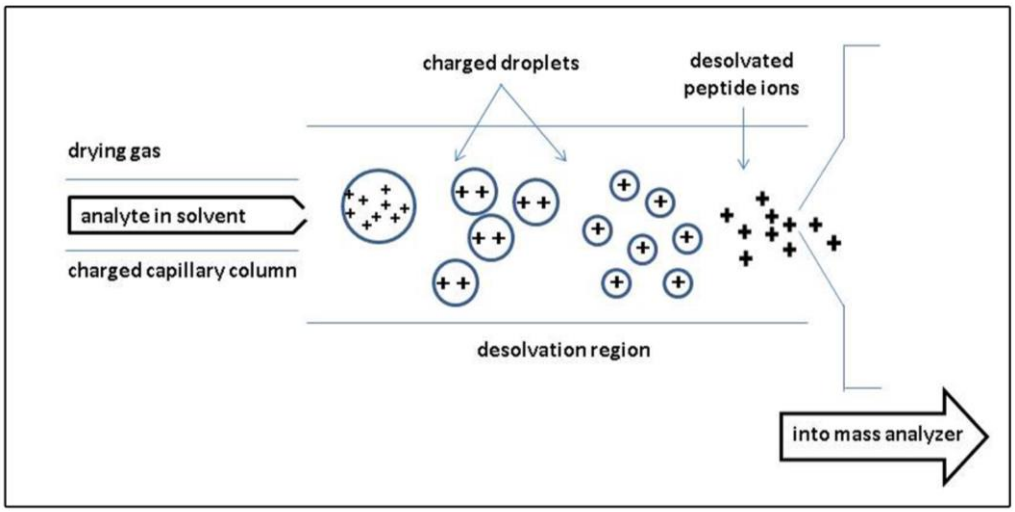


Fig. 5. Illustrative scheme of (a) MALDI and (b) ESI, according to Kinter and Sherman, 2000.

The availability of advanced instrumentation is, to a great extent, helpful in comprehensive identification of proteins and their expression patterns within a broad range of different species, tissues or single cells. The recognition of an entire proteome in one organism is nevertheles a difficult task. The analysis of proteomes in individual types of cells or cell sub-compartments may simplify this process and provide information about the speciffic expression and function of proteins at the lower organisational levels. Within the concept of functional proteomics, we can identify groups of functionaly related proteins. The identification of their differential expression in various conditions can help us to discover the proteins as specific markers for specific conditions. The analysis of plant nuclear proteome may be the example of such an approach.

4. Plant nuclear proteomics

Studies of nuclear proteome in plants have been performed in just a few species. They are represented by model plant *Arabidopsis*; *Xerophyta* and crop plants such as rice, soybean, hot pepper, chickpea and barley (Bae *et al*., 2003; Khan and Komatsu, 2004; Lee *et al*., 2006; Pandey *et al*., 2006; Casati *et al*., 2008; Li *et al*., 2008; Repetto *et al*., 2008; Choudhary *et al*., 2009; Abdalla *et al*., 2010; Cooper *et al*., 2011; Petrovská *et al*., 2014). Moreover, mapping of plant nuclear proteome focused so far mostly on stress-condition related protein composition (reviewed in Petrovská *et al*., 2015). Stress proteomic studies, in general, can significantly contribute to the revealing of key proteins functioning in response to stress factors. The proteins, which prove to be responsible for tolerance to abiotic or biotic stress can then serve as a markers for a given stress condition, which can be utilized in plant breeding and improvement (Gong *et al*., 2015).

In this part, I will bring the short overview of the plant nuclear proteomes that have been analysed to date.

4.1. Analysis of *Arabidopsis thaliana* nuclear proteome

The first insight which brought more comprehensive results in plant nuclear proteomics was obtained nearly 10 years ago in a model plant *Arabidopsis thaliana*. Using density gradient approach for purification of nuclei and 2-DE and MALDI-TOF-MS, there were 158 nuclear proteins of different functions identified of which nearly one third was associated with cold stress. On the basis of measured intensity of protein spots in stress treated and control specimen, 14 proteins repressed and 40 proteins induced by cold stress were identified. Six cold stress-induced proteins were then selected for detailed characterisation, after which only Hsc70-1 protein has been proposed to function in the stress response (Bae *et al*., 2003).

The completion of *Arabidopsis* genome sequencing enabled to perform also the first proteomic study of plant nuclear matrix (Calikowski *et al.*, 2003). Using ESI coupled to tandem MS, 36 proteins were identified. They comprised a number of *Arabidopsis* homologs of nucleolar proteins such as IMP4, Nop56, Nop58, fibrillarins, nucleolin, as well as ribosomal components. Also homologs of proteins present in nucleolus or nuclear matrix of human cells (eEF-1, HSP/HSC70, and DnaJ) were identified.

First phosphoproteomic study performed in *Arabidopsis* by Jones *et al*. (2009) identified over 300 phosphoproteins. In this work, novel phosphorylation sites on transcription factors, chromatin remodelling proteins, RNA silencing components and the spliceosome were identified. Moreover, identification of phosphorylation sites on proteins associated with Golgi vesicle trafficking, such as the exocyst complex, lead to the speculation about their contribution to cell plate formation during cytokinesis. Another phosphoproteomic analysis of chromatin associated proteins of *Arabidopsis* nuclei revealed up to 879 proteins with almost 200 phosphoproteins with a role in chromatin remodelling, regulation of transcription and RNA processing (Bigeard *et al*., 2014).

4.2. Analysis of rice nuclear proteome

Among the crop plants, rather extensive studies have been conducted in rice (*Oryza sativa*). The first comprehensive studies were performed by Khan and Komatsu, 2004. Over five hundred proteins were detected and approximatelly half of them was analyzed. The highest percentage made up proteins involved in signal transduction and gene regulation, but similarly high percentage was represented by proteins with no assigned function (Khan and Komatsu, 2004).

The study of chromatin associated proteins from rice suspension cells was conducted by Tan *et al*. (2007). Nearly 200 nuclear protein associated with chromatin were identified, mostly histone variants, transcription factors and nucleosome assembly proteins.

Li and co-workers developed an efficient method for extraction of rice endosperm nuclear proteins (Li *et al*., 2008). The authors identified 468 nuclear proteins, which included transcription factors, histone modification proteins, microtubule binding proteins and transposon proteins and more than one third of unidentified proteins.

The influence of stress conditions on proteomic profile of rice nuclei has been examined mainly in plants exposed to watter deficit condition. Proteomic analysis of a drought tolerant rice variety revealed 109 proteins, which changed their levels in response to the stress. Novel regulatory proteins were discovered, including RF2b transcription factor, WIP1, von Willebrand factor type A and Alba domain-containing protein, suggesting their role in plant dehydration response (Choudhary *et al*., 2009).

A more recent comparative study of proteome from dehydration-sensitive and tolerant rice cultivars identified 78 dehydration responsive nuclear proteins. In dehydration-sensitive cultivar, the highest percentage of proteins belonged to the class of signaling and gene

regulation, in dehydration-tolerant the most abundant were cell defense and rescue proteins. The differentially expressed proteins were mapped into a hypothetical model depicting their roles in nucleus during dehydration (Jaiswal *et al*., 2013).

4.3. Analysis of chickpea nuclear proteome

Proteomic analysis in another important crop species – chickpea (*Cicer arietinum*) revelaed about 150 proteins out of 600 2-DE resolved spots. Besides the higher abundance of proteins involved in signaling and gene regulation (36%) and in DNA replication and transcription (17%), the study identified structural proteins such as actin, kinesin, MFP1, histone H3, metabolism-related proteins, translation and degradation machinery proteins or chaperones (Pandey *et al*., 2006).

Proteomic analysis in chickpea in relation to water stress contributed to characterisation of regulatory and functional network activated under dehydration. A total of 147 differentialy expressed proteins were identified under dehydration stress. They were assigned to various functions including gene transcription and replication, molecular chaperones, cell signalling and chromatin remodelling (for detailed information see Pandey *et al*. 2007).

The proteome comparison of dehydration sensitive and tolerant chickpea cultivars showed cultivar-specific expression of proteins involved in different cellular functions (Subba *et al*., 2013). A study of chickpea phosphoproteome identified 107 putative nucleus specific phosphoproteins of which the highest part is represented by stress-responsive and nucleotide-binding proteins (Kumar *et al*., 2014).

4.4. Analysis of maize nuclear proteome

Another study following changes in protein composition during different abiotic stress conditions was performed in maize (*Zea mays*) first by Casati and co-workers in 2008. Nuclear proteome before and after the exposure to UV-B light was compared, with the result of 98 identified proteins, differently abundant in each of condition, mostly classified as DNA binding and chromatin factors (Casati *et al*., 2008).

Recently, Guo *et al*. (2014) constructed a reference map of the maize nuclear proteome in the basal region of the seadling leaf. Among other, 10% of identified proteins were involved in cell division and growth. Interesting results were obtained after the comparison of two maize

inbred lines and their hybrid, where almost 12% of proteins were differentialy expressed. Such a comparison may be important for monitoring of proteins which are responsible for specific behaviour of hybrids.

4.5. Analysis of soybean nuclear proteome

Except of studies analysing plant nuclear proteomes under abiotic stress conditions, studies of proteome changes in response to biotic factors such as fungal or viral pathogens were conducted. Soybean (*Glycine max*) nuclear proteome was screened after rust infection. The comparison of susceptible and resistant cultivars revealed sets of proteins with differential accumulation (Cooper *et al*., 2011). The authors discovered post-translational modification in a number of proteins after the infection and proposed regulation of protein accumulation in nucleus under control of disease resistance gene *RPP1*.

4.6. Analysis of hot pepper nuclear proteome

The analysis of nuclear proteins expressed during hypersensitive reaction in hot pepper (*Capsicum annuum* cv. Bugang) after the infection with *Tobacco mosaic virus* (TMV) revealed six nuclear proteins. Among other they included 14-3-3 regulation protein involved in responses to abiotic and biotic stresses. The nuclear protein 14-3-3 was confirmed also in chickpea in the above mentioned study of Pandey *et al*. (2006). This protein is rather well-known for its function in plant defense responses to pathogen and different kinds of abiotic stresses (Roberts *et al*., 2002; Lozano-Durán and Robatzek, 2015).

Of the identified hot pepper nuclear proteins, a functional study was performed on 26S proteasome subunit RPN7 (CaRPN7) and the results showed a possible involvement of TMV-induced CaRPN7 in programmed cell death (Lee *et al*., 2006).

4.7. Analysis of barrel clover nuclear proteome

In order to understand better the regulation of developmental processes within legume seeds, Repetto *et al*. (2008) analysed nuclear protein composition in barrel clover (*Medicago truncatula*) seeds in a transition stage between embryogenesis and seed filling. Using nanoLC-MS/MS, they identified 143 proteins and described novel proteins possibly involved

in biogenesis of the ribosomal subunit (PESCADILLO-like) or in nucleo-cytoplasmic trafficking (dynamin-like GTPase). The analysis revealed groups of proteins related to ribosome biogenesis, RNA maturation and processing, and transport which may play role in seed development (Repetto *et al*., 2008).

4.8. Analysis of recalcitrant plant black-stick lily nuclear proteome

Changes of proteome in response to dehydration stress were studied also in black-stick lily (*Xerophyta viscosa*), which is a monocot resurrection plant able to withstand long periods of drought (Abdalla *et al*., 2010). Out of 438 2-DE resolved protein spots, 18 was up-regulated in response to dehydration and are believed to play a role in gaining desiccation tolerance. Besides the five novel proteins, the study identified proteins with gene regulation, molecular chaperone, translation and metabolic activities (Abdalla *et al*., 2010).

As it is apparent from this general overview, nuclear protein composition has been studied in only a few representatives of the plant kingdom. The overview of analysed plant proteomes is summarized in **Table 1**. Comparative *in silico* analysis of studied plant nuclear proteomes revealed a high percentage of unique proteins in each proteome (reviewed in Narula *et al*., 2013), which may reflect high generic and condition related specificity. Further analysis of complete protein composition of cell nuclei and their functional characterisation in different plants could bring a more comprehensive picture on the molecular processes not only at the level of nucleus but also at the level of the whole cell and organism. Nuclear proteomics seems an adequate initial step to tackle this daunting task.

Table 1. List of plant nuclear proteomes characterised in standard and altered conditions. *) 1-DE, one dimensional electrophoretic separation; 2-DE, two dimensional electrophoretic separation; ESI, electrospray ionisation; LC, liquid chromatography; MALDI, matrix-assisted laser desorption/ionisation; MS, mass spectrometry; •) Studies aiming to characterize complete nuclear proteome; adapted from Petrovská *et al*., (2015) and Petrovská *et al*., (manuscript in preparation).

	Species	Method for nuclei purification	Proteomic approach*	No. of nuclear proteins identified	Monitored influence
2003	***Arabidopsis***	density gradient	2-DE, MALDI-TOF-MS	**158**	**cold stress**
			1-DE, 2-DE, MALDI-TOF-MS NanoLC-MS, MS/MS	**36•**	**-**
2004	**rice**	density gradient	2-DE, MALDI-TOF-MS	**190•**	**-**
2006	**hot pepper**	density gradient	2-DE, MALDI-TOF-MS	**6**	**response to TMV infection**
	chickpea		2-DE, LC-MS, MS/MS	**150•**	**-**
2007	**rice**	density gradient	2-DE, MALDI-TOF/TOF-MS, MS/MS	**269•**	**-**
2008	**rice**	density gradient	2-DE, LC-MS, MS/MS MALDI-TOF/TOF-MS, MS/MS	**468•**	**-**
	barrel clover		1-DE, NanoLC-MS, MS/MS	**143**	**seed filling**
	maize		2-DE, LC-MS, MS/MS	**98**	**UV-B light treatment**
	chickpea			**147**	**dehydration response**
2009	**rice**	density gradient	2-DE, LC-MS, MS/MS	**109**	**drought stress**
			1-DE, NanoLC-MS, MS/MS	**657**	**sugar response**
	Arabidopsis		LC-MS, MS/MS	**345•**	**-**
2010	**black-stick lily**	density gradient	2-DE, MALDI-TOF-MS	**18**	**dehydration response**
2011	**soybean**	density gradient	LC-MS, MS/MS	**4975**	**rust infection**
2012	**black-stick lily**	density gradient	2-DE, LC-MS, MS/MS	**122**	**dehydration response**
2013	**rice**	density gradient	1-DE, LC-MS, MS/MS	**382**	**cell wall removal response**
			2-DE, MALDI-TOF/TOF-MS, MS/MS LC-MS, MS/MS	**78**	**dehydration response**
	chickpea		2-DE, LC-MS, MS/MS	**75**	**dehydration response**
2014	***Arabidopsis***	density gradient	1-DE, NanoLC-MS, MS/MS	**879•**	**-**
	chickpea		1-DE, 2-DE, MALDI-TOF/TOF-MS, MS/MS NanoLC-MS, MS/MS	**107•**	**-**
	maize		2-DE, MALDI-TOF-MS	**163•**	**heterosis**
	barley	flow cytometric sorting (nuclei at G1, S, and G2 phase)	1-DE NanoLC-MS, MS/MS NanoLC-MALDI-MS, MS/MS	**803•** (G1 nuclei)	**-**

5. Nuclear protein TPX2

TPX2 (targeting protein for *Xenopus* kinesin-like protein 2) protein has been a subject of research for rather long time. First TPX2 was identified in mammalian cells by Heidebrecht *et al*. (1997) as p100 protein, which was enriched in the interphase nuclei and even more during mitosis with spindle apparatus. Cell cycle dependent manner of TPX2 accumulation and its high abundancy in dividing cells designated TPX2 as a potential marker in cancer cells. Its existence was simultaneously confirmed in *Xenopus* egg extract by Wittmann *et al*. (1998), where the role of TPX2 in spindle pole organisation was proposed.

5.1 TPX2 in the spindle assembly process

Transmission of genetic material during mitosis and cell division is a fundamental biological process. Correct distribution of chromosomes to daughter cells is a complex process especially in organisms containing large genomes and high number of chromosomes. To manage the difficult task of governing the proper progression of mitosis and cell division, eukaryotic cells have developed a complex machinery of spindle apparatus and cell cycle regulators (Fant *et al*., 2004).

The spindle apparatus is a polar network of microtubules (MTs) and associated proteins. Microtubules are dynamic heteropolymers composed of α and β-tubulin with polar orientation - faster growing "plus end" and slower growing "minus end", which enables a directional movement of motor proteins along the MTs. This structure is common to fungi, plant and animal kingdom. The indispensable parts of microtubular apparatus are microtubule associated proteins (MAPs) that bind MTs and control their polymerisation and organisation. Microtubules can be organized by several different ways. Microtubules can be nucleated from centrosomes, which are the dominant microtubule organizing center (MTOC) and are typical for metazoan cells. Minus ends of MTs are attached to MTOC and plus ends are oriented outwards, create aster-like arrays and undergo rapid cycles of growth and shrinkage (Fant *et al.*, 2004; Walczak and Heald, 2008; Zhang and Dawe, 2011). Kinetochores on chromosomes can anchor the MT plus ends and connecting with centrosome the stable spindles are formed. This structure is reffered as "search and capture" model (Kirschner and Mitchison, 1986). Similarly in yeast and fungal organisms, the spindle pole body functions as a nucleation center. Components of MTOCs are γ-tubulin and associated proteins assembling into γ-tubulin ring complexes, which serve as nucleation units. In

cells that lack centrosomes such as animal oocytes and plant cells, the self-organisation of spindle microtubules proceedes from dispersed MTOCs in cytoplasm but also from the vicinity of chromatin mass (Gueth-Halonet *et al*., 1993; Heald *et al*., 1996; Binarová *et al*., 2000; Fant *et al*., 2004; Binarová *et al*., 2006). Microtubules growing distally out from the chromatin area were described first in *Drosophila* oocytes (Matthies *et al*., 1996) and confirmed by *in-vitro* experiments in *Xenopus* egg extract, when DNA-coated beads induced formation of MTs around the DNA surface followed by microtubule bundling and forming a bipolar spindle apparatus (Heald *et al*., 1996). This means that the chromatin induced spindle formation is performed in an opposite way, from inside to outside, and needs the components which stabilize the spindle at spindle poles.

Motor proteins including kinesin-like proteins play an important role of MTs organisation and chromosome segregation. Here, I will mention particularly one, related to the nuclear protein of interest, which is one of a pivotal topic of this Thesis. Motor protein *Xenopus* kinesin-like protein 2 (Xklp2) in *Xenopus* and KLP2 homologs move towards plus ends of microtubules and during mitosis they accumulate at the poles of spindle MTs. Xklp2 binding to microtubules is mediated by **TPX2 (targeting protein for Xklp2)** and its minus end localisation is related to the activity of dynein-dynactin complex (Wittmann *et al*., 1998; Wittmann *et al*., 2000, Fant *et al*., 2004).

TPX2 belongs to the group of microtubule associated proteins (MAPs). During mitosis, TPX2 is enriched near spindle poles and is also found near kinetochores. It functions as the main factor for centrosome-directed assembly of microtubules. When TPX2 was depleted, the multipolar spindles and defects at spidle poles were observed (Wittmann *et al*., 2000; Garrett *et al*., 2002). The question of exact function of TPX2 in this process has not been fully resolved. The study of Garrett *et al*. (2002), where the multipolar spindle formation was observed in siRNA knocked-down expression of TPX2 in human cells, brought indications that hTPX2 is not necessary for focusing the MT minus ends at spindle pole. Further, they speculated the hTPX2 function antagonistic to motor proteins which separate the spindle poles.

Metazoan TPX2 is a key factor that promotes microtubule nucleation process regulated by RanGTPase (Gruss *et al*., 2001; Kufer *et al*., 2002). Here a cascade of events begins with RanGTP (Ran bound to guanosine tri-phosphate) gradient around chromosomes. During interphase, presence of RanGDP in cytoplasm and excess of RanGTP in the nucleus makes a gradient which functions in the directional nucleocytoplasmic transport. RanGDP moves into nucleus where it is converted into RanGTP by Ran guanine nucleotide exchange factor, RCC1. In the nucleus, RanGTP binds importin-β thus regulating its binding properties and dissociation

from cargo proteins (Zhang and Dawe, 2011). In animals, after nuclear envelope breakdown at the onset of mitosis when RanGTP diffuses into cytoplasm, RCC1 maintains higher concentration of RanGTP around chromosomes. RanGTP then binds importin-β which causes release of importin-α and its dissociation with bound TPX2. Afterwards, TPX2 can function in downstream processes (Gruss *et al.*, 2001; Wiese *et al.*, 2001; Schatz *et al*., 2003; Ciciarello *et al*., 2004; Giesecke and Stewart, 2010). The requirement of TPX2 for MT nucleation and branching in *Xenopus* was confirmed recently by Petry *et al*. (2013). There are number of studies describing the impact of knock-downed or overexpressed TPX2 in metazoans confirming TPX2 function of spindle formation via Ran/importin-dependent and independent pathways, which regulate nucleation, branching and organisation of MTs (Gruss *et al*., 2002; Tulu *et al*., 2006; Neumayer *et al*., 2014).

5.2 TPX2 in the regulation of animal Aurora kinase

The role of TPX2 in regulation of human Aurora A kinase, which belongs to the group of conserved serine/threonine kinases, also contributed to its more profound functional studies. Aurora A has been established as a one of the key regulators of mitosis and cell cycle progression, and being detected in many types of cancer cells, its research earns a big importance (Kufer *et al*., 2002; Bayliss *et al*., 2003). TPX2 has several functions in relation to Aurora A. Binding to TPX2 increases Aurora A's *in vitro* autophosphorylation activity and, at the same time, prevents Aurora A from dephosphorylation by protein phosphatase1 (PP1) (Bayliss *et al*., 2003; Eyers and Maller, 2004). TPX2 prevents premature proteasomal degradation of Aurora A (Giubettini *et al*., 2011) and activation of TPX2 enables targetting of the kinase to spindle microtubules in RanGTP-dependent manner (Trieselmann *et al*., 2003). Recently, TPX2 was identified as a new scaffolding protein and co-activator of Aurora B in chromosomal passenger complex (Iyer and Tsai, 2012).

5.3 Plant TPX2

It is obvious that most functions assigned to TPX2 have been well-characterized in animal cells. The research of plant TPX2 ortholog has begun lately with the work of Vos *et al*. (2008). The amino acid sequence structure analysis of *Arabidopsis* TPX2 showed similarities in the functional domains between AtTPX2 and its vertebrate counterparts (**Fig. 6**). Besides the TPX2

signature sequence, AtTPX2 contains two nuclear localisation signals (NLS), nuclear export signals (NES) and two microtubule binding domains (MBDs). It is known that plant proteins sharing the same MBD domain as TPX2 function in organisation of MTs, cell growth and cell division control (Hamada, 2007; Petrovská *et al*., 2013). Plant TPX2 sequence also contains N-terminal Aurora binding domains and coiled-coil signature which is common for plant proteins containing TPX2_importin domain (Vos *et al*., 2008; Evrard *et al*., 2009; Petrovská *et al*., 2013). For detailed information about domains, binding motifs and putative interaction sites of AtTPX2 sequence see Vos *et al*. (2008) and Petrovská *et al*. (2013).

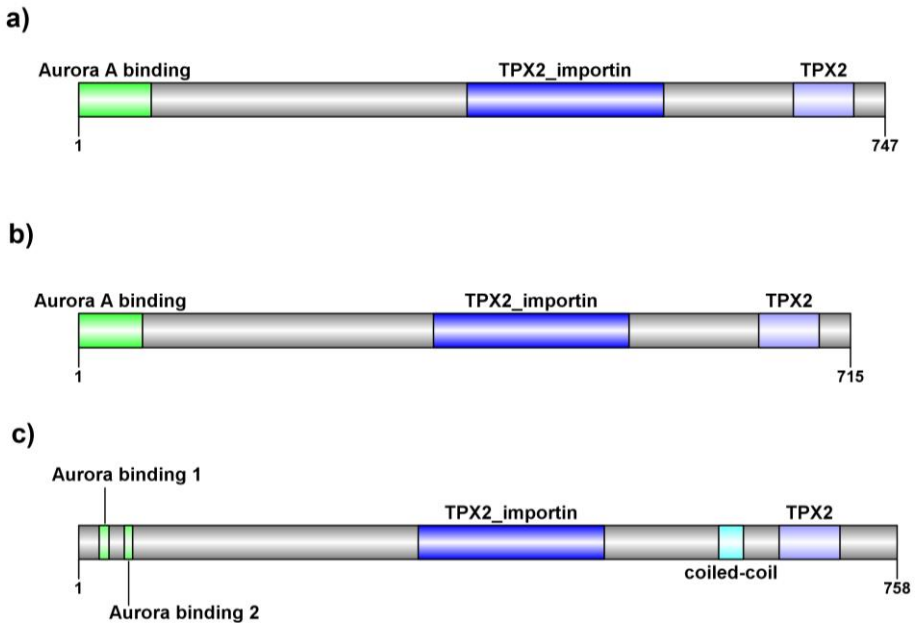


Fig. 6: Domain composition of TPX2 proteins in (a) human (acc.nr. NP_036244), (b) *Xenopus* (acc. nr. AAF81694), (c) *Arabidopsis thaliana* (acc. nr. NP_973754); source for *in silico* analyse of domains: CCD-NCBI (Marchler-Bauer *et al*., 2015), Pfam (Punte *et al*., 2012), SUMOsp2.0 (Ren *et al*., 2009a), graphics constructed in DOG (Domain Graph; Ren *et al*., 2009b)

Plant TPX2 as the cell cycle regulated protein shares similar localisation patterns as its animal counterparts. It is accumulated in the cell nuclei during interphase and actively transported out of the nuclei before the nuclear envelope breakdown. During mitosis it localises with the microtubular arrays from prophase to early telophase and is degraded at the end of mitosis (Vos *et al*., 2008; Petrovská *et al*., 2013). The interesting exception was observed in fern *Asplenium nidus* dividing leaf cells, where the signal of homologous TPX2 is not degraded at telophase but persists and colocalises with phragmoplast (Panteris *et al*., 2012).

With the absence of centrosomes, plants have to employ rather different mechanism of

spindle formation, which is considered similar to the spindle self-organisation in animal cells. Also in plants, kinetochores can next to the capturing of kinetochore fibres, form kinetochore fibres by direct nucleation of MTs or promote growth of MTs in their vicinity (Zhang and Dawe, 2011). It is known that nucleation of MTs occurs from the sites of γ-tubulin complexes dispersed as a MT nucleating units on pre-existing microtubules and at the nuclear envelope (Binarová *et al*., 2000 and 2006; Nakamura *et al*., 2010). Also the promotion of aster-like arrays formed out of complexes of histone H1 and α/β tubulin at the nucleo-cytoplasmic interface reffered in tobbaco cells points to the role of such MTOCs in plant nuclear envelope (Hotta *et al*., 2007). Similarly to animals, the Ran pathway is supposed to be a part of spindle formation process in plant cells (Zhang and Dawe, 2011). The increased concentration of Ran which was observed at the area of nuclear envelope (Ma *et al*., 2007) implies Ran pathway involvement in MT assembly from the MTOCs in nuclear envelope. RanGTP gradient, after nuclear envelope breakdown, may also enable the MT formation around chromosomes. However the precise mechanism of Ran pathway engagement in plants is still unknown.

The role of AtTPX2 in spindle assembly and mitosis promotion in plant cells was proposed by Vos *et al*. (2008) who observed delay or even arrest of the cell cycle in late prophase or prometaphase after the application of anti-human and anti-AtTPX2 antibodies. AtTPX2 association with importin-α (Vos *et al.*, 2008; Petrovská *et al.*, 2013) and its ability to promote spindle assembly *in vitro* also indicates that AtTPX2 is involved in RanGTP pathway regulating MT nucleation in plant cells. Moreover, the observed moderately elevated concentration of AtTPX2 at the nuclear envelope during interphase may point to the activity of AtTPX2 in MT nucleation at this area.

The MT assembly patterns were examined in interphase cells with overexpressed AtTPX2 in Petrovská *et al*., 2013. Signal of overexpressed AtTPX2 in the interphase cells was initially observed in the form of dots and patches from which MTs could grow and organise. Later, the arrays of intranuclear and perinuclear AtTPX2-decorated thick fibrils were formed, extending out to the cell periphery. It is likely that overexpressed AtTPX2 may amplify the ability of nuclear envelope and chromatin to promote microtubule nucleation, which is typical for acentrosomal cells.

We may just speculate if AtTPX2 plays a role in nuclear assembly similar to that of *Xenopus* TPX2 reffered in O'Brien and Wiese (2006), who suggest that *Xenopus* TPX2 may function in targeting or anchoring of LAP2 (lamin and chromatin binding nuclear protein playing role in nuclear assembly and DNA replication) and point that TPX2 may be important for post-mitotic nuclear assembly. Potential function of AtTPX2 in plant equivalent of LINC (linker of

cytoskeleton and nucleoskeleton) complex as a connecting element of cytoskeleton with nuclear interior and chromatin needs to be revealed (Petrovská *et al*., 2013).

5.4 Interplay between plant TPX2 and Aurora kinases

In *Arabidopsis thaliana*, there were three Aurora kinase orthologs identified, AtAurora1, 2 and 3. These phosphorylate serine 10 in histone H3 *in vitro*. AtAurora kinases share similar localisation as their animal orthologs. AtAurora1 and 2 localize to the nuclei, especially to the nuclear membrane, during interphase and to the mitotic spindles during cell division (Kawabe *et al*., 2005). AtAurora3 localizes with chromosomes, which seems to indicate its chromosomal-passenger-like role similar to animal AuroraB (Kawabe *et al*., 2005; Demidov *et al*., 2005). Plant Aurora kinases are designated as one of the main coordinators of spatio-temporal events of mitosis and cytokinesis and also regulation factors for acentrosomal microtubule nucleation at dispersed sites within nuclei and on membrane. Their influence in control of cell division and differentiation was also proposed (Petrovská *et al*., 2012). The work of Petrovská *et al*. (2012) contributed to the elucidation of the AtAurora kinase-AtTPX2 association. Based on colocalisation data it was suggested that AtTPX2 may guide AtAurora kinase1 to microtubules and contribute to spatio-temporal modulation of kinase function. Also the presence of Aurora1, its regulator AtTPX2 and γ-tubulin on microtubules suggests the linkage towards γ-tubulin mediated MT formation in acentrosomal plant cells (Petrovská *et al*., 2012).

As the regulator of AtAurora kinases activity, AtTPX2 influences the phosphorylation of kinase downstream targets. The ability of AtTPX2 to regulate AtAurora kinases *in vitro* were examined recently by Tomaštíková *et al*. (2015). Clearly, the nuclear TPX2 protein has an indispensable role in both animal and plant cells. Its mitotic function has been rather well studied, albeit predominantly in animal cells. The role of interphase TPX2 in DNA damage response was suggested (Neumayer *et al.*, 2012; Neumayer and Nguyen, 2014). Nevertheless, what is the function of TPX2 in plant nucleus is still not fully elucidated.

6. Aims

This Thesis aimed at contributing to the characterisation of so far poorly explored plant nuclear proteomes. It provides a general overview of the organisation of plant cell nuclei and their protein composition, plant nuclear proteome research and fundamental methods of proteomics. The experimental part of the Thesis covered the two complementary research topics. The first of them focused on development and application of a new approach to characterise plant nuclear proteome, which is based on nuclei purification by flow sorting and protein identification using soft ionisation MS techniques. The second part of the Thesis focused on functional analysis of plant nuclear proteins, namely on AtTPX2 protein and its relationship with plant Aurora kinase 1 and 3.

Particular aims of the Thesis were:

1) Development of an alternative strategy for purification of plant cell nuclei for proteomic analysis addressing the efficiency, sensitivity and speed, while paying particular attention to maintaining protein integrity and avoiding contamination by other cellular components.
2) Sub-proteomic analysis of barley cell nuclei using the state of the art proteomic approaches.
3) Functional characterisation of plant nuclear protein TPX2.

7. Conclusions

7.1 Proteomic analysis of barley cell nuclei

Although barley belongs to the important crop species, no specific analysis of its nuclear proteome has been performed until the recent study of Petrovská *et al*. (2014). For the characterisation of barley (*Hordeum vulgare* L. cv. Morex) nuclear proteome, we developed a novel approach for nuclei purification. We replaced the conventional multi-step fractionation procedure, which is laborious and prone to contamination by cytoplasmic proteins, with the method based on flow cytometric sorting. Using this method, the contamination by non-nuclear elements and proteins was negligible. At the same time, flow cytometry enables isolation of nuclei in G1, S and G2 phases of the cell cycle, which provides opportunities for characterisation of nuclear proteome changes during cell cycle progression.

Samples of intact nuclei of G1, S and G2 phases of the cell cycle were used for subsequent proteomic analysis performed simultaneously on different hybrid mass spectrometers, ESI and MALDI. Altogether 803 distinct proteins were identified in G1 phase. Analogously, G2 phase nuclei analysis resulted in 2003 identified proteins (unpublished data). An overlap between ESI and MALDI made 147 proteins in G1 and 113 proteins in G2, which points to the uniqueness of each MS technique. The highest proportion of G1 proteins belonged to the class of translation and ribosome and ribonucleoprotein complex biogenesis. In contrast, functions of G2 nuclear proteins were predominantly linked to chromatin assembly, nucleosome assembly and organisation, and DNA packaging (data not published). These results are in accordance with the processes that are known from the respective cell cycle phase. Currently, the work continues on analysis of S-phase proteins to complete the dataset.

This work proved that flow cytometric sorting of barley nuclei is a rapid and precise method that can be coupled with proteomic analysis. This method should be feasible for sorting subcellular particles from a whole range of plant material. Thus the future prospect is an exploitation of the approach not only in nuclear proteome research within broad spectrum of plants but also in studying the chromosomal proteome, which has been analysed so far in human only (Uchiyama *et. al*., 2005; Ohta *et al*., 2010). Also coupling the two different MS soft ionisation techniques, ESI and MALDI, proved to extend the number of identified proteins and becomes therefore a preferred strategy. (Supplement I)

7.2 Functional characterisation of nuclear protein TPX2 in *Arabidopsis thaliana*

TPX2 protein has been rather well examined in metazoan cells in relation to its function in microtubule assembly processes and Aurora kinase A activation. The similar role of plant TPX2 has also been suggested (Vos *et al*., 2008). To provide more information about the structure and function of plant TPX2, I have studied *Arabidopsis* cells overexpressing AtTPX2.

In the interphase cells overexpressing AtTPX2, formation of nuclear dots and patches appeared within three days from transformation. These dots were gradually assembled into fibrillar structures in and around cell nuclei. AtTPX2 foci and fibers in nuclei were positive for α-tubulin signal which implicated that AtTPX2 decorated structures represented cytoskeletal polymers. The fibrillar structures decorated with AtTPX2 were resistant to drug-induced depolymerisation and were not proved to be a manifestation of apoptosis as it was observed by Moss *et al.* (2009). Further, MT bundling was not related to the onset of mitosis and immunofluorescence of AtAurora1 showed only a weak signal at the fibres decorated with overexpressed AtTPX2. Moreover, using Aurora kinase inhibitor treatment, the arrangement of nuclear and perinuclear fibres was not affected.

The immunolocalisation of overexpressed AtTPX2 with Ran and importin respectively showed Ran and importin signal stronger in dots and patches than in cells with MT bundles. This suggested weakening of Ran and importin signal during assembly of microtubular arrays and indicated that MT formation was triggered by sequestration of importin from AtTPX2. The interaction of Ran as well as importin with AtTPX2 respectively was further confirmed by co-immunopurification. Next, AtTPX2 colocalized with γ-tubulin, which may point to the role of AtTPX2 in the mechanism of MT assembly from MTOCs comprising γ-tubulin, nevertheless immunoprecipitation of these two proteins couldn’t demonstrate any interaction.

This part of Thesis contributed to the characterisation of plant TPX2 with the confirmation of AtTPX2 and importin interaction, which indicates the involvement of Ran pathway in the process of microtubule assembly in the cell nuclei with overexpressed AtTPX2. (Supplement II)

7.3 AtTPX2 *in vitro* regulation of Aurora kinase family members

For further characterisation of AtTPX2 protein and plant Aurora kinases relationship, the subsequent study aimed to assess the ability of AtTPX2 to regulate members of Aurora kinase family. *In silico* analyses revealed that protein sequences of human TPX2 and plant TPX2 homologues comprised Aurora binding domain and TPX2 motif, which have high sequence conservation in plant homologs. It is known that human TPX2 is phosphorylated by Aurora A (Kufer *et al*., 2002) and binding of TPX2 at the same time increases autophosphorylation activity of Aurora A (Bayliss *et al*., 2003). TPX2 is also necessary for activation of Aurora B (Iyer and Tsai, 2012).

To find if AtTPX2 is phosphorylated in the same manner as its human counterpart, phosphorylation of AtTPX2 by AtAturora1 and AtAurora3 (analog of mammalian Aurora B) was tested *in vitro*. The results showed that only AtAurora1and not AtAurora3 reproducibly phosphorylates AtTPX2 *in vitro*. Examination of the autophosphorylation activity of AtAurora1 after AtTPX2 binding revealed that similarly to human homologues, AtTPX2, comprising Aurora binding domain, increased autophosphorylation activity of AtAurora1. AtAurora3, by contrast, was not activated by AtTPX2. Co-activation of AtAurora1 by AtTPX2 then enhanced the activity of the kinase towards one of its most important downstream target, histone H3. Further it was detected that truncated version of AtTPX2 without Aurora binding domain is unable to activate the kinases, however, it can still be phosphorylated. This suggests a responsibility of Aurora binding domain in AtTPX2 molecule for kinase activation.

This study confirms the importance of AtTPX2 having function not only in microtubule assembly machinery. AtTPX2 – induced activation of AtAurora 1 resulted in increased phosphorylation level of histone H3 and might also activate other downstream targets such as spindle assembly factors. Thus AtTPX2 may have a role in mitotic regulation, mitotic chromatin organisation and mitotic spindle formation. (Supplement III)

8. Publications (ISI Web of Knowledge)

I. Petrovská, B., Jeřábková, H., Chamrád, I., Vrána, J., Lenobel, R., Uřinovská, J., Šebela, M., and Doležel, J. (2014). Proteomic analysis of barley cell nuclei purified by flow sorting. Cytogenetic and Genome Research, 143, 78-86. **(Supplement I)**

II. Petrovská B., Jeřábková, H., Kohoutová, L., Cenklová, V., Pochylová, Ž., Gelová, Z., Kočárová, G., Váchová, L., Kurejová, M., Tomaštíková, E., and Binarová, P. (2013). Overexpressed TPX2 causes ectopic formation of microtubular arrays in the nuclei of acentrosomal plant cells. Journal of Experimental Botany, 64(14), 4575-4587. **(Supplement II)**

III. Tomaštíková, E., Demidov, D., Jeřábková, H., Binarová, P., Houben, A., Doležel, J., and Petrovská, B. (2015). TPX2 protein of *Arabidopsis* activates Aurora kinase 1, but not Aurora kinase 3 *in vitro*. Plant Molecular Biology Reporter, 890, DOI: 10.1007/s11105-015-0890-x. **(Supplement III)**

9. List of abstracts

9.1. Oral presentations

Dvořák Tomaštíková, E., Demidov, D., Jeřábková H., Binarová, P., Houben A., Doležel, J., Petrovská, B. (2015). *Arabidopsis* Aurora kinase 1 activation by TPX2 is essential for increased histone H3 phosphorylation *in vitro*. In: International Plant Nucleus Consortium meeting, 3-5 July 2015, Olomouc, Czech Republic, p. 15.

Jeřábková, H., Uřinovská, J., Chamrád, I., Rutten, T., Lermontova, I. (2015). AtSim3-like protein as a putative CENH3/histone assembly factor. In: International Plant Nucleus Consortium meeting, 3-5 July 2015, Olomouc, Czech Republic, p. 16.

Petrovská, B., Jeřábková, H., Chamrád, I., Vrána, J., Lenobel, R., Uřinovská, J., Šebela, M., Doležel, J. (2015). Update on plant nuclear proteins. In: International Plant Nucleus Consortium meeting, 3-5 July 2015, Olomouc, Czech Republic, p. 14.

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11. Abbreviations

BY-2 – bright yellow 2, tobacco

CBs – Cajal bodies

CENH3 – centromeric histone H3 variant

CRWN – crowded nuclei

DCL1 – dicer-like1

DMI1 – Doesn't make infection

ESD4 – Early in short days 4

ESI – electrospray ionisation

FCM – flow cytometry

feSEM – field emission scanning electron microscopy

FG-repeat Nups – phenylalanine-glycine repeat domains nucleoporins

FPPs – Filament-like plant proteins

GCP – γ-tubulin complex protein

GDP – guanosine diphosphate

GIP – GCP3-interacting proteins

GTP – guanosine triphosphate

HYL1 – hyponastic leaves

INM – inner nuclear membrane

IPG – immobilized pH gradient

KASH – Klarsicht/Anc1/Syne1 homology

LC – liquid chromatography

LCA – lycopersicum calcium pumping ATPase

LINC – linker of nucleoskeleton and cytoskeleton

LINC1-4 – little nuclei 1-4

MAF1 – MFP1 associated factor

MALDI – matrix assisted laser desorption/ionisation

MAPs – microtubule associated proteins

MBDs – microtubule binding domains

MFP1 – Matrix attachment region (MAR)-binding filament-like protein 1

MS – mass spectrometry

MTs – microtubules

MTOC – microtubule organizing centre

NE – nuclear envelope

NES – nuclear export signal

NLS – nuclear localisation signal

NMCP1 – nuclear matrix constituent protein 1

NOR – nucleolus organising region

NPs – nuclear proteins

NPC – nuclear pore complex

NUA – nuclear pore anchor

NuMA – nuclear mitotic apparatus protein

Nups – nucleoporins

ONM – outer nuclear membrane

pI – isoelecric point

PMSF – Phenylmethylsulfonyl fluoride

PP1 – protein phosphatase1

RAE1 – mRNA export factor 1

RanGAP – RanGTPase activating protein

RanGDP – Ran guanosine diphosphate

RanGTP – Ran guanosine triphosphate

RCC1 – Ran guanine nucleotide exchange factor

RIPA – Radio-immunoprecipitation assay

SDS-PAGE – sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SINE – SUN-interacting nuclear envelope

SMN – survivor motor neuron protein

snRNPs – small nuclear ribonucleoproteins

SUMO – Small ubiquitin-like modifier

SUN – Sad1/UNc84

TCA – trichloroacetic acid

TM – transmembrane domain

TOF – time-of-flight

TPX2 – targeting protein for Xenopus kinesin-like protein 2

TREX2 – transcription-coupled export 2

WIP – WPP-domain interacting protein

WIT – WPP-domain interacting tail-anchored protein

WPP domain – tryptophan/proline/proline motif domain

γ-TuCs – γ-tubulin complex

γTuRC – γ-tubulin ring complexes

1-DE – one dimensional

2-DE – two dimensional

12. Supplements

Supplement I

Proteomic analysis of barley cell nuclei purified by flow sorting

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Proteomic Analysis of Barley Cell Nuclei Purified by Flow Sorting

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Key Words
Cell cycle · Chromatin · Flow cytometry · Gel electrophoresis · Mass spectrometry · Nuclear proteome · Protein analysis

Abstract
Many proteins are present in the nucleus; some are involved with its structural and functional organization, some with gene expression, and some with cell division. The plant nuclear proteome has not been well explored. Its characterization requires extraction methods which minimize both the artifactual alteration of the proteins and the extent of contamination with non-nuclear proteins. The conventional multi-step fractionation procedure is both laborious and prone to contamination. Here, we describe a single-step method based on flow sorting. The method allows the separation of G1, S and G2 phase nuclei and minimizes the risk of contamination by non-nuclear proteins. Preliminary results obtained using G1 phase cell nuclei from barley root tips indicate that flow sorting coupled with a protein/peptide separation and mass spectrometry will permit a comprehensive characterization of the plant nuclear proteome.

B.P. and H.J. contributed equally to this work.

The function of the nuclear genome cannot be completely understood without a secure knowledge of the composition, structure and behavior of nuclear proteins, the most abundant class of molecules present after the DNA [Sutherland et al., 2001]. The nuclear proteome is responsible for the spatial organization of the nucleus, both during its establishment and maintenance, and how this is related to cell function and gene expression. Nuclear proteins participate in a majority of the processes occurring in the nucleus, notably transcription and DNA replication, repair and recombination. Given the potentially high number of protein species involved in these processes, a high-throughput approach, such as that offered by current proteomic platforms, is needed to carry out a global analysis.

Recent advances in mass spectrometry (MS) have enabled the identification and sequencing of a majority, if not all, of the proteins represented in a given sample [Griffin and Schnitzer, 2011]. However, the data can only be meaningful if sample preparation does not compromise the integrity of the proteins, and if there is a minimum of (preferably no) contamination from neighboring tissue. In the context of nuclear proteins, the latter implies the avoidance of cytoplasmic remnants [Kodrzycki et al., 1989]. Protein extraction from plant cells has long been considered as being less tractable than from animal or

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prokaryote cells, primarily because of their high content of proteases and interfering secondary metabolites [Wang et al., 2008]. However, continuing technical improvements have largely overcome these difficulties in various explants, including the embryo [Masuda et al., 1991; Yamaguchi et al., 1992; Busk and Pages, 1997], endosperm [Ferreira et al., 2006; Li et al., 2008], seed [Riggs et al., 1989], seed coat [Renouard et al., 2012], leaf [e.g. Cushman, 1995; Zhang et al., 1995; Abdalla et al., 2009, 2010; Sikorskaite et al., 2013], and root meristem [Silva et al., 2010], and in in vitro cultured cells [Willmitzer and Wagner, 1981]. Currently used extraction protocols directed at nuclear proteins use mechanical homogenization, filtration to remove large detritus, pelleting, suspension in a non-ionic detergent, and finally separation by density gradient centrifugation [for review see Narula et al., 2013]. This sort of scheme has provided descriptions of the *Arabidopsis thaliana* [Bae et al., 2003; Jones et al., 2009], hot pepper [Lee et al., 2006], rice [Tan et al., 2007; Li et al., 2008; Aki and Yanagisawa, 2009; Choudhary et al., 2009], barrel clover [Repetto et al., 2008], maize [Casati et al., 2008], black-stick lily [Abdalla et al., 2010; Abdalla and Rafudeen, 2012], soybean [Cooper et al., 2011], flax [Renouard et al., 2012], and chickpea [Pandey et al., 2006; Varshney et al., 2013] nuclear proteomes. Typically, however, the number of proteins identified has been rather low, ranging from 56 (hot pepper) to 657 (rice) [Lee et al., 2006; Aki and Yanagisawa, 2009]. Clearly, improved extraction protocols are needed, addressing efficiency, sensitivity and speed, while at the same time taking care to maintain protein integrity and to avoid contamination.

A feasible alternative approach exploits the ability of a flow cytometer to purify nuclei prior to protein extraction. The advantages of the approach in principle are firstly that it avoids contamination by cytoplasmic remnants, and secondly that it offers the possibility of studying the nuclear proteome at various phases of the cell cycle. Flow cytometry has found numerous applications in biomedical research, but its extension to plant science has been hindered by problems associated with sample preparation [Doležel et al., 2007a]. The requirement to generate a suspension of single particles is difficult to manage in most plant materials because the cells are typically joined to one another by a robust extracellular matrix, and their rigid cell walls [Cosgrove, 2005] hamper the release of the cellular content. Currently, the most widely used application of flow cytometry in plants has been to analyze the nuclear DNA content as a means to identify the level of ploidy and to estimate the size of the nuclear genome [Doležel and Bartoš, 2005; Doležel et al., 2007a]. The increasing use of this technology has been stimulated by the development of a simple, rapid protocol to obtain suspensions of intact nuclei from fresh plant tissue [Galbraith et al., 1983].

Flow cytometry has also been used to sort nuclei prior to transcriptomic analysis [Macas et al., 1998; Zhang et al., 2008; Bourdon et al., 2012] and for cell cycle analysis [Binarová et al., 1993; Kotogány et al., 2010; Petrovská et al., 2012; Doskočilová et al., 2013]. A protocol aimed at yielding suspensions of intact chromosomes [Doležel et al., 1992] is also suitable for the isolation of nuclei; the mild fixation with formaldehyde does not compromise the quality of the DNA in the context of preparing DNA libraries [Šafář et al., 2004; Kasprzak et al., 2006]. The method offers the opportunity to purify nuclei suitable for proteomic analysis in a single step, thereby avoiding the time-consuming fractionation steps included in more conventional protocols; it also reduces the risk of contamination by non-nuclear proteins. In this paper, we describe such a protocol and demonstrate its compatibility with downstream proteomic analysis. The success achieved using this approach indicates that flow cytometry coupled with protein/peptide separation and MS represents a promising alternative strategy for analyzing plant nuclear proteomes at various stages of the cell cycle.

Materials and Methods

Instruments

The flow cytometer employed was a FACSAria SORP (BD Biosciences, San Jose, Calif., USA), equipped with a blue laser (488 nm) for forward and side scatter signal detection and a UV laser (355 nm) for DAPI excitation. The 2 mass spectrometers used in parallel were as follows: (1) an ultrafleXtreme MALDI-TOF/TOF device equipped with a smartbeam-II laser operating at a repetition rate up to 2 kHz (Bruker Daltonik, Bremen, Germany) and operated in offline coupling with a Dionex UltiMate3000 RSLC-nano liquid chromatograph (Thermo Fisher Scientific, Germering, Germany) connected to a Proteineer fc II fraction collector (Bruker Daltonik); eluted fractions were collected on MTP AnchorChip™ 800-384 MALDI targets; (2) a tandem UHR-Q-TOF maXis device equipped with a nanoelectrospray ion source (Bruker Daltonik), connected to a nanocapillary liquid chromatograph (nanoEASY; Proxeon, Bruker Daltonik).

Solutions

Flow sorting required Hoagland's nutrient solution, Tris buffer, formaldehyde fixative, sheath fluid and DAPI, as specified by Doležel et al. [2011]. The LB01-P lysis buffer was composed of 15 mM Tris, 2 mM Na_2EDTA, 0.5 mM EGTA, 80 mM KCl, 20 mM NaCl, 0.1% (v/v) Triton X-100, 0.2 mM spermine, and 0.5 mM spermidine. The solution was passed through a 0.22-μm filter, then supplemented with 14 mM 2-mercaptoethanol and 0.1 mM PMSF

just prior to use. The lysis buffer used for protein extraction contained 25 mM HEPES/NaOH, pH 7.5, 150 mM NaCl, 1% (v/v) IGEPAL CA-630, 0.1% (w/v) sodium deoxycholic acid, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 1 mM DTT, 1 mM PMSF, 1× cOmplete EDTA free (protease inhibitor cocktail tablet, Roche), 1 mM Na_3VO_4, 10 mM NaF, 5 mM 2-glycerophosphate, 1 mM Na_2MoO_4, 4 mM sodium tartrate, and 10 mM sodium butyrate.

For the nanoLC-MALDI-MS and MS/MS (tandem MS), the mobile phase A was 0.05% (v/v) trifluoroacetic acid (TFA), and mobile phase B was 80% (v/v) acetonitrile/0.05% (v/v) TFA; the loading solvent was 2% (v/v) acetonitrile/0.05% (v/v) TFA; the matrix solution I comprised 748 µl of 95% (v/v) acetonitrile/0.1% (v/v) TFA mixed with 36 µl of a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA) in 90% (v/v) acetonitrile/0.1% (v/v) TFA, 8 µl 10% (v/v) TFA, and 8 µl 100 mM $NH_4H_2PO_4$. For external MS calibration, the Peptide Calibration Standard II (Bruker Daltonik) was first dissolved in 125 µl of 0.1% (v/v) TFA. The matrix solution II was prepared by mixing 748 µl of 85% (v/v) acetonitrile/0.1% (v/v) TFA, 36 µl of the saturated CHCA solution, 8 µl 10% (v/v) TFA and 8 µl 100 mM $NH_4H_2PO_4$; to 300 µl of this solution, 2 µl of the Peptide Calibration Standard II solution was added. For the nanoLC-ESI-MS and MS/MS, the mobile phase solution A was 0.4% (v/v) formic acid and mobile phase B was 0.4% (v/v) formic acid in 70% (v/v) methanol/20% (v/v) isopropanol. All reagents were of analytical or biochemical/molecular biology purity grade.

Preparation of Nuclei and Flow Sorting

To obtain nuclei at each of G1, S and G2, ~300 grains of barley cv. Morex (obtained from the Agricultural Research Institute, Kroměříž, Czech Republic) were imbibed on moist filter paper at 25°C in the dark until the emerging seedling roots had reached a length of ~3 cm [Doležel et al., 1992]. The roots were removed to ddH_2O and fixed in formaldehyde at 5°C for 10 min, then washed twice in Tris buffer at 5°C for 5 min. Cell nuclei were isolated in smaller samples which were used individually for sorting. The number of samples prepared depended on the number of sorted nuclei needed for proteomic analyses. One sample was prepared from about 60 root tips (length 1–2 mm), which were dissected from roots and immersed in 1 ml LB01-P lysis buffer. The material was immediately homogenized using a Polytron PT1200 homogenizer (15,000 rpm, 13 s); the homogenate was filtered through a 20-µm nylon mesh and stored on ice. The suspension was stained with 2 µg/ml DAPI, and the nuclei were subjected to flow cytometry. Dot plots of forward scatter versus side scatter were used to discriminate between nuclei and cell detritus. Nuclei at various phases of the cell cycle (fig. 1) were sorted into tubes containing 1 ml LB01-P buffer supplemented with 100 mM PMSF, pelleted by centrifugation (300 *g*, 4°C, 30 min) and stored at –20°C.

Protein Extraction

Crude protein was extracted from the pelleted nuclei (5 million) using a modified approach involving DNase digestion in a lysis buffer [Wang and Zhu, 1990]. Briefly, 50 µl of the nuclei lysis buffer was added to the pelleted nuclei. After short vortexing to disperse clumps of nuclei, the suspension was sonicated for 10 min, 10 µl of 1 U/µl DNase I (Sigma-Aldrich, Steinheim, Germany) was added and the mixture was incubated at 37°C with shaking (900 rpm). Any remaining clumps of nuclei were dispersed by squeezing them against the tube wall with a syringe needle. After 2 h of incubation, a further 10-µl aliquot of DNase I was added, and the incubation with shaking resumed overnight. The following day, the sample was centrifuged (25,000 *g*, 15 min). Proteins present in the supernatant were recovered by adding 4 volumes of cold acetone (–20°C) and keeping at –20°C for at least 24 h. The precipitate was collected by centrifugation (25,000 *g*, 15 min) and dissolved in 50 µl of Laemmli's sample buffer containing mercaptoethanol, sonicated for 10 min and heated at 100°C for 10 min. This fraction is referred to hereafter as the 'precipitate fraction'. The same volume of Laemmli's sample buffer was used to make an extract from the pellet, which was subjected to sonication and heating as above; this fraction is referred to hereafter as the 'pellet fraction'. This fraction contains proteins, which could not be released directly by the DNase reaction and remained associated with the sediment. They were finally extracted by Laemmli's buffer and separated by SDS-PAGE without any further purification. The DNA and protein content of a plant nucleus is around 20% and 60% of the nucleus dry weight, respectively [Tautvydas, 1971]. Then, the total weight of 5 million G1 nuclei is estimated to 250 µg giving a total protein amount of 150 µg. In the precipitate fraction, the protein content can be measured directly, for example by the 2-D Quant Kit (GE Healthcare, Uppsala, Sweden). The rest, which remains in the pellet fraction, can then be calculated based on the above estimation as the accuracy of a direct spectrophotometric quantification in this material is doubtful because of the content of insoluble parts.

One-Dimensional Electrophoretic Separation and in-Gel Digestion of Nuclear Proteins

One-dimensional SDS-PAGE of nuclear proteins was performed using a standard procedure based on a 10% polyacrylamide separating gel and a 4% stacking gel [Laemmli, 1970]. Proteins were stained with Coomassie Brilliant Blue R-250 [Bennet and Scott, 1971]. The procedure used for in-gel digestion (including reduction and alkylation) and subsequent peptide extraction followed Shevchenko et al. [2000], employing a thermostable trypsin modified by raffinose [Šebela et al., 2006]. Prior to the nanoLC-ESI-MS and nanoLC-MALDI-MS procedures, the extracted peptides were purified and recovered in 30 µl 0.1% (v/v) TFA. Either stop-and-go tips [Rappsilber et al., 2007] or Zip-Tips (Millipore) were used for the purification step.

NanoLC-ESI-MS and MS/MS

Sample aliquots (7 µl) containing desalted peptides were injected onto a precolumn (75 µm × 30 mm, 5 µm C18 Reprosil GOLD 300 particles; Dr. Maisch, Ammerbuch-Entringen, Germany) with an integraFrit (New Objective, Inc., Woburn, Mass., USA), and washed by injection of 15 µl 0.1% (v/v) TFA. Bound peptides were eluted onto an analytical column (75 µm × 150 mm, 3 µm C18 Reprosil GOLD particles; Dr. Maisch) with a silica tip (New Objective) using a 125-min multi-step gradient at a constant flow rate of 200 nl/min. The gradient was: 0 min, 2% mobile phase B; 3 min, 8% B; 50 min, 18% B; 79 min, 28% B; 85 min, 32% B; 93 min, 45% B; 99 min, 65% B; 100 min, 95% B; 105 min, 95% B; 108 min, 2% B; 125 min, 2% B. The MS analysis was operated in data-dependent acquisition mode, using the top 4 precursors and the CID fragmentation technique for peptide identification. The whole system was controlled by QTofControl 3.2 software (Bruker Daltonik). The MS analyzer settings included source settings (capillary voltage: 1,900 V; dry gas: 6 l/min, dry temperature: 160°C)

and tune page settings (ion funnel RF: 400 Vpp; multipole RF: 400 Vpp; quadrupole ion energy: 5 eV; collision energy: 8 eV; collision RF: 1200 Vpp; ion cooler RF: 350 Vpp; transfer time: 85 µs; prepulse storage: 7 µs). The MS/MS settings were: auto MSMS: on; 4 precursor ions; threshold for switching from MS to MSMS mode: 5000 cts; active exclusion after 5 spectra for next 18 s; excluded mass range of precursors: 50–350 amu and 1500–2200 amu. The data were collected over the mass range of 50–2200 amu with an acquisition time of 500 ms for MS and 250–750 ms for MS/MS according to precursor intensity.

NanoLC-MALDI-MS and MS/MS

Peptide sample aliquots (5 µl) were injected from the autosampler onto a Nano Trap precolumn (100 µm × 20 mm) for pre-concentration and then separated on an analytical column (75 µm × 150 mm). Both columns were packed with Acclaim PepMap100 C18 particles (5 and 2 µm, respectively; Thermo Fisher Scientific). The flow rate of the loading solvent was 10 µl/min; both the preconcentration and separation on the analytical column were achieved in a run time of 70 min at a constant flow rate of 300 nl/min. The gradient was: 0 min, 4% mobile phase B; 7 min, 4% B; 45 min, 60% B; 48 min, 90% B; 57 min, 90% B; 59 min, 4% B; 70 min, 4% B. The eluate was collected in 15-second fractions starting from 20 min and spotted after mixing with the CHCA matrix solution I (the total drop volume was 420 nl) onto an AnchorChip™ 800-384 target plate. The chromatograph and spotter were controlled by HyStar 3.2 software (Bruker Daltonik).

The preparation of MALDI targets containing 120 sample matrix spots for each nanoLC-separated sample (3 nanoLC runs per a single target) was achieved by manual pipetting of the peptide standard solution (containing the CHCA matrix) for crystallization in the respective target positions. The target was then introduced into the MALDI-TOF instrument. Prior to spectra acquisition, a manual 3-position target geometry teaching was performed. The mass spectrometer was controlled by flexAnalysis 3.4 software for acquisition, flexControl 3.4 for spectra processing and WarpLC 1.3 for automatic measurement of nanoLC-MALDI fractions. MS spectra were acquired in automatic mode with the laser firing rate set at 2000 Hz and summed over 2,500 satisfactory shots. In MS/MS mode, the following acquisition method settings were applied: primary choice mass range (of precursors): 900–2500, number of precursor masses: 10; peak intensity: >800; peak quality factor: >30; signal/noise: >7; FAST minimal fragment mass: 250; LIFT: measure fragments only.

Proteomics Data Analysis

Raw MS data were processed by DataAnalysis 4.2 SP4 (Bruker Daltonik), uploaded to ProteinScape 3.1 (Bruker Daltonik) and searched against the NCBI non-redundant 'Viridiplantae' database with the Mascot 2.2.07 search engine (Matrix Science, London, UK). For the nanoLC-ESI-Q/TOF data, the search was split into 2 steps, the first applying a wide mass tolerance for both precursors and corresponding fragment ions (±50 ppm and ±0.1 Da, respectively). The second search was limited to the first round selections and used more stringent settings (mass tolerances of ±10 ppm and ±0.05 Da for precursors and fragments, respectively). The search parameters were as follows: trypsin was set as a protease with 1 missed cleavage allowed; carbamidomethylation of cysteine was set as a fixed modification and methionine oxidation as a variable modification; +1, +2 and +3 was set as a peptide charge; other settings: mass cut-off: 700 Da; minimum ion peptide score: 15 (with a minimum peptide length of 5 amino acids at a significance threshold of $p < 0.05$). For the nanoLC-MALDI-TOF/TOF data, the search employed mass tolerances for precursors and fragment ions of ±25 ppm and ±0.5 Da, respectively, and a peptide charge of +1. The other parameters were as above. Validated proteins were merged and grouped according to shared peptides using the ProteinExtractor function of ProteinScape.

Gene Ontology

Gene ontology (GO) terms were obtained by querying the obtained protein sequences against the *A. thaliana* subset of the Swiss-Prot database, utilizing the BLASTP search algorithm (E value cut-off of –10, minimal sequence identity of 70%). Subsequently, all significantly over-represented GO terms were extracted employing a modified Fisher's exact test and the DAVID gene functional classification tool [Huang et al., 2009].

Results and Discussion

The protocol presented here has several advantages compared to conventionally used ones. The volume of initial plant material is in the mg range, and so extraction is very rapid. While the current report describes the isolation of nuclei from root cells, the same method has also been used previously to isolate nuclei from leaf tissue [Šafář et al., 2004]. Here, a set of ~900 root tips (15 samples) generated 5×10^6 nuclei at each of the cell cycle stages G1, S and G2. The seedling root is a particularly convenient source material, since seeds are readily available for most plant species and are generally straight-forward to handle and germinate. Moreover, actively growing root tips contain nuclei in all stages of the cell division cycle, making them particularly suitable for the study of nuclear proteome dynamics during progression through the cell cycle.

Mechanical homogenization was used to release the nuclei from formaldehyde-fixed cells following the protocols of Doležel et al. [1992] and Gualberti et al. [1996]. The key modification to allow for proteomic analysis was shortening the time of fixation to 10 min, as this minimizes the extent of protein crosslinking. It was also important to add PMSF to the LB01 lysis buffer to inhibit any endogenous protease activity. DAPI was preferred to common DNA intercalating dyes, such as ethidium bromide or propidium iodide, to stain DNA of nuclei in preparation for flow cytometry as it does not bind to dsRNA, and hence RNase treatment is not needed. Moreover, DAPI staining produces a high resolution quantification of the DNA content [Doležel et al., 2007b]. The resulting DNA content histogram (fig. 1A) showed a clear separation between nuclei at

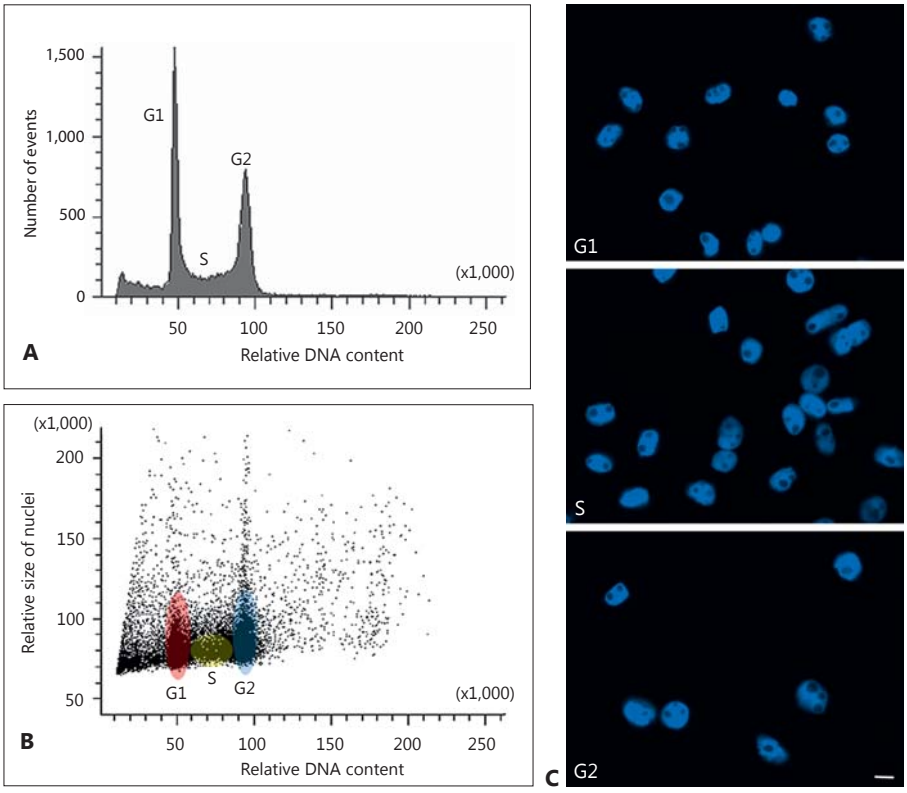

Fig. 1. A Distribution of the relative DNA content in interphase barley root tip cell nuclei obtained after flow cytometric analysis of DAPI-stained samples. The histogram shows 2 peaks, corresponding to G1 and G2 nuclei and a spread of S phase nuclei containing intermediate amounts of DNA. **B** Dot plot used for flow sorting, showing the positions of the sorted regions. **C** Micrographs of flow-sorted purified nuclei in different cell cycle stages stained with DAPI. Note that the nuclei are intact and the sorted fractions free of contaminating particles. The images were obtained using an IX81 motorized inverted research microscope CellR (Olympus) equipped with a DSU (Disk Scanning Unit) and a digital monochrome CCD camera CCD-ORCA/ER. Bar = 10 μm.

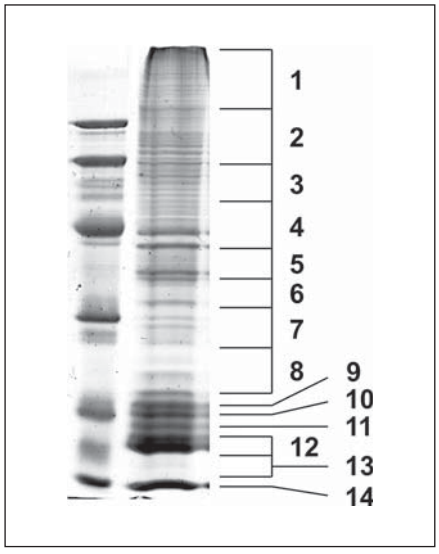

Fig. 2. One-dimensional SDS-PAGE separation of proteins extracted from flow-sorted G1 phase barley nuclei. The left-hand lane is a molecular mass marker (from the top: 97.4, 66.2, 45, 31, 21.5, and 14.4 kDa), the right-hand lane is the extract of the pellet fraction. The calculated protein amounts of the standard and sample were 6 and 15 μg, respectively. The numbers on the right show the 14 slices prepared for subsequent in-gel digestion.

G1 and at G2, while the S phase nuclei had an intermediate DNA content. Thus, flow sorting was able to generate 3 distinct populations of intact nuclei (fig. 1C), based on fluorescence strength and the length of the particle. Sorting speed was ~200 nuclei/s, so that the target number of 5×10^6 nuclei was obtained in ~3 h.

The nuclei lysis buffer used was adapted from the RIPA (radio-immunoprecipitation assay) buffer described by Ngoka [2008], which was found to efficiently solubilize protein without causing large-scale degradation. The buffer is compatible with protease and phosphatase inhibitors, minimizes non-specific protein binding and is suitable for protein quantification. A standard 1-dimensional SDS-PAGE separation was preferred to a 2-D electrophoretic separation to save time in a manual gel processing for in-gel digestion of proteins. Anyway, a reversed-phase chromatography of peptides from in-gel digests ensured a 2-dimensional separation of the complex proteome sample. A further reason to use 1-dimensional SDS-PAGE was that the reproducible separation of histones requires special protocols [Görg et al., 1997]. A typical SDS-PAGE separation of the pellet fraction obtained from G1 phase nuclei is shown in figure 2. The

complete separation lane on a 10% polyacrylamide gel corresponds to proteins with a mass distribution between 14 and ~300 kDa [Garfin, 2003]. Except for the histones H4, H2B, H2A, and H3 visualized in the region covering molecular masses of 14–20 kDa, relatively abundant non-histone proteins appeared in the region of 30–45 kDa consistent with the size range present in chicken nuclei extracts [Wang and Zhu, 1990].

Following SDS-PAGE separation, the gel was sliced into 14 parts, with the slices numbered in ascending order from the top of the gel (fig. 2). The content of each slice was then subjected to in-gel digestion with modified trypsin, and the digests were processed to obtain desalted and purified peptides. Prior to the MS analysis, the complex peptide mixtures were first separated by reversed-phase nanoflow liquid chromatography (fig. 3). The chromatographic separation was followed by MS and MS/MS analysis of eluate fractions performed simultaneously on different hybrid mass spectrometers to employ the 2 soft ionization techniques, ESI and MALDI, which are commonly used in proteomics studies. The ESI instrument was an online-coupled Q-TOF equipped with a collision cell, the MALDI mass spectrometer was a TOF/TOF comprising a LIFT cell, which was coupled offline to nanoLC via a fraction collector.

As confirmation that the flow cytometry sorting-based purification of nuclei was compatible with proteomic analysis, the proteome of G1 nuclei was characterized. Over 60,000 MS/MS spectra were acquired, of which 3,818 could be unambiguously assigned to peptides. The peptides matched 803 distinct proteins (fig. 4A), covering a wide range of protein abundance (fig. 5). Surprisingly, the overlap between the 2 parallel MS analyses involved only 147 proteins, leaving >80% of the identifications as instrument-unique (fig. 4A; online suppl. tables 1, 2; see www.karger.com/doi/10.1159/000365311 for all online suppl. material), presumably because of differences in peptide ionization and fragmentation (peptides with several basic amino acids tend to have a higher charge state and so are more likely to be detected by an ESI-based instrument). A GO term enrichment analysis of the identified proteins confirmed that the majority (about 99%) were nucleus-related (fig. 4B). Ribosome-associated proteins in the mid-abundant class (fig. 5) perhaps were found because of the presence of ribosome subunits in the nucleolus [Kressler et al., 2010]. Others related to endoplasmic reticulum-associated proteins, suggesting a level of unavoidable contamination. The ~1% non-nuclear proteins included as yet uncharacterized and/or only predicted proteins, so they may not all represent contaminants.

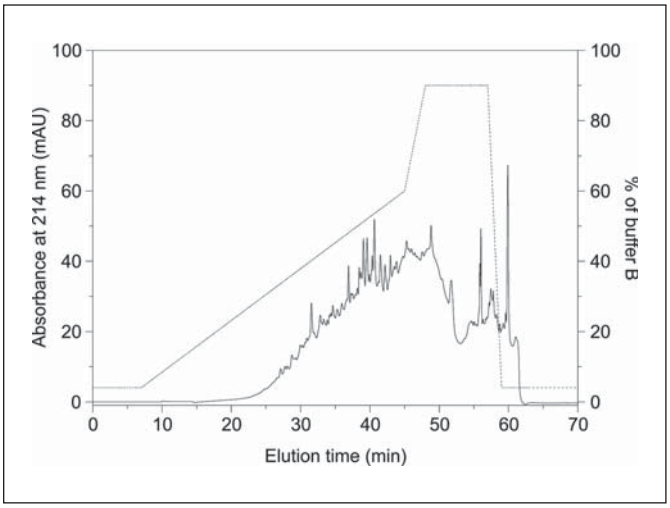

Fig. 3. Gradient reversed-phase nanoflow chromatographic separation of tryptic peptides produced by in-gel digestion with trypsin. The samples were separated by nanoLC. The chromatogram shown was generated from slice No. 6 in figure 2. The solid line represents absorption at 214 nm, the dotted line refers to the gradient of mobile phase B.

To date, the plant nuclear proteome has been characterized relatively poorly. The best characterized species is rice [Tan et al., 2007; Aki and Yanagisawa, 2009] with just over 900 proteins identified, followed by *A. thaliana* [Bae et al., 2003; Jones et al., 2009] and maize [Casati et al., 2008] with ~500 apiece. Among legume species, ~150 proteins have been identified in both barrel clover [Repetto et al., 2008] and chickpea [Panday et al., 2006]. The latter authors noted only a small overlap between the nuclear proteomes (as defined at that time) of chickpea, *A. thaliana* and rice. The current protocol has enabled the identification of over 800 proteins in the G1 nucleus of barley. Although we have not yet completed analogous analyses of the S and G2 phase nuclei, a similar volume of data can be expected from these.

Root tips are an ideal material to isolate nuclei from non-differentiated cells at different stages of the cell cycle. However, root tips may not be readily available in some plants, and there may be a need to analyze the proteome of nuclei from other cell and tissue types. Our protocol should also be applicable in these cases. For example, nuclei isolated from leaf tissues are the most common sample type in plant flow cytometry [Vrána et al., 2014]. Flow cytometric analysis has also been done with nuclei isolated from seeds [Matzk et al., 2000; Śliwinska et al., 2005], fruit tissues [Cheniclet et al., 2005], flower petals [Kudo

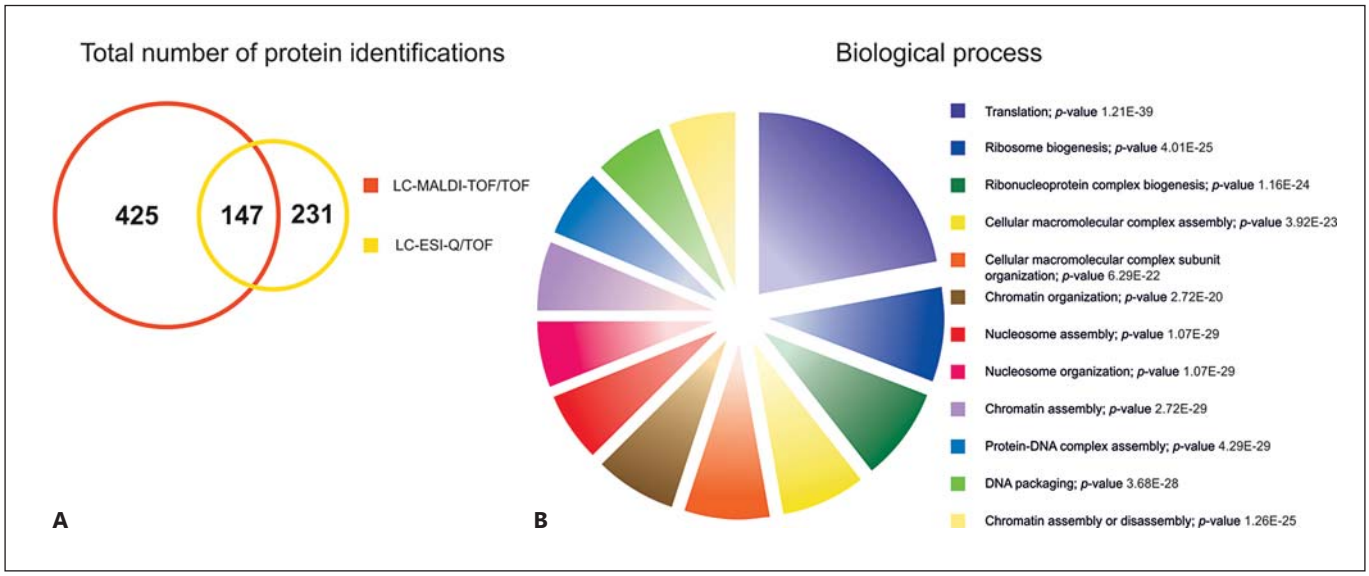

Fig. 4. The G1 nuclear proteome. **A** Number of proteins/peptides identified following the tryptic digest, using either ESI-MS or MALDI-MS. Both unique and overlapping identifications are displayed. **B** Most significantly enriched GO terms ($p < E^{-20}$) among the identified barley nuclear proteins. The distribution is based on the number of proteins connected with the particular GO term.

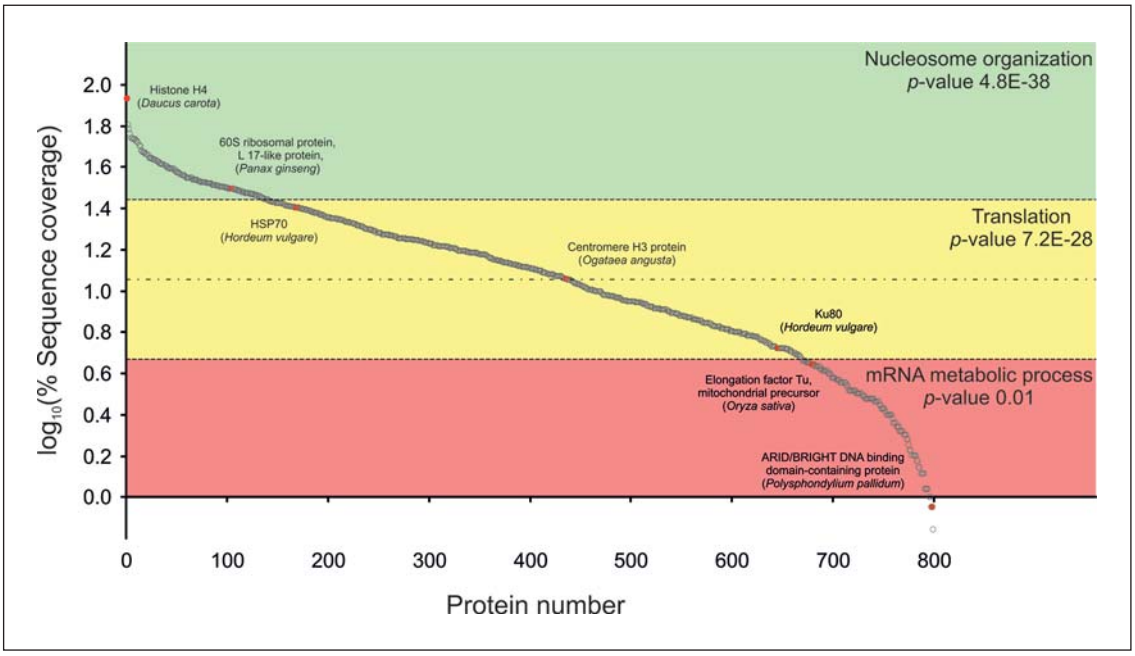

Fig. 5. Abundance of components of the barley G1 phase nuclei proteome. The relative amounts of nuclear proteins were characterized by their sequence coverage values (in %) from the database search revealing an S-shaped distribution. Based on their relative abundance, the proteins were classified into 3 groups (highlighted by shading).

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and Kimura, 2002], and pollen [Kron and Husband, 2012]. Flow cytometry is particularly suited to identify nuclei which underwent one or more endoreduplication cycles [Vrána et al., 2014]. Thus, our method provides an attractive opportunity to study proteins of nuclei in differentiated endopolyploid cells at different levels of endoreduplication. Our approach can be extended further to studying the chromosomal proteome, since mitotic chromosomes can be readily isolated using the same basic sorting procedure [Doležel et al., 2012]. As yet, such an analysis has only been attempted in human and animal chromosomes [Gassmann et al., 2005; Uchiyama et al., 2005; Ohta et al., 2010]. However, the elaboration of the flow sorting technique opens the possibility of widening the spectrum of species amenable to nuclear proteome charting.

Here, we have demonstrated that proteomic analysis is feasible from a flow-sorted population of plant cell nuclei treated with a mild formaldehyde-based fixation. Coupling flow cytometry, protein/peptide separation and MS provides an elegant and powerful means for obtaining the composition of the nuclear proteome at defined phases of the cell cycle, at the same time succeeding in avoiding large-scale contamination by cytoplasmic proteins.

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Supplement II

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

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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

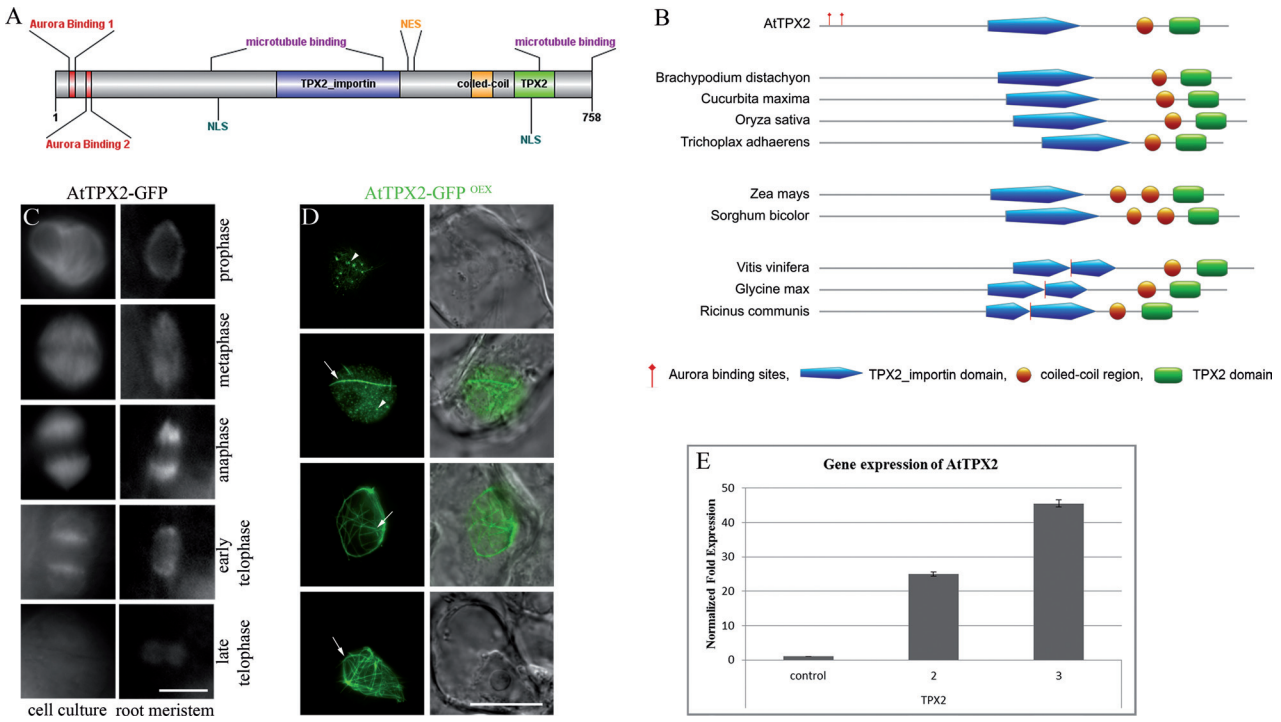

Fig. 1. Localization of AtTPX2 protein in *Arabidopsis* cells overexpressing AtTPX2. (A) Domains, putative interaction sites, and binding motifs in the amino acid sequence of *Arabidopsis* TPX2 protein. For putative interaction sites, binding motifs as well as domain graph, ELM (Dinkel *et al.*, 2012), 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.

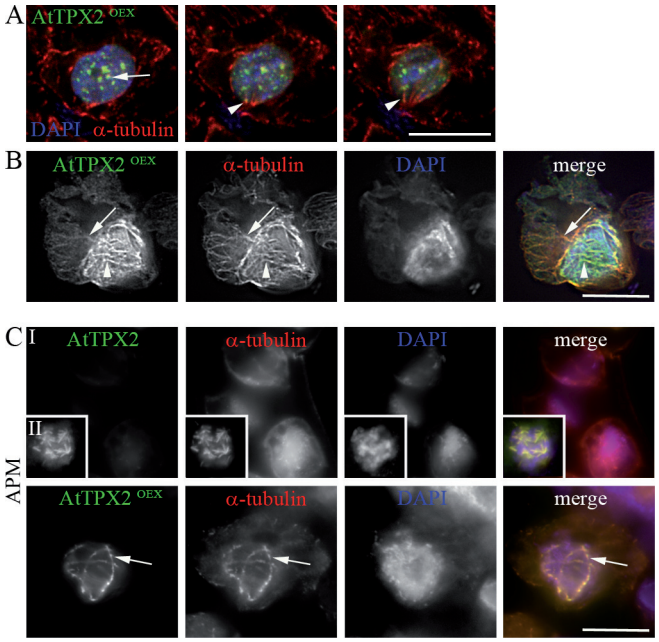

Fig. 2. AtTPX2 localization with ectopic perinuclear and nuclear microtubules in *Arabidopsis* cells overexpressing AtTPX2-GFP. (A) AtTPX2-GFP foci localized with α-tubulin in the nucleus (arrow) and AtTPX2-GFP partially loaded on α-tubulin fibres (arrowheads) in cells with foci of overexpressed AtTPX2-GFP. 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

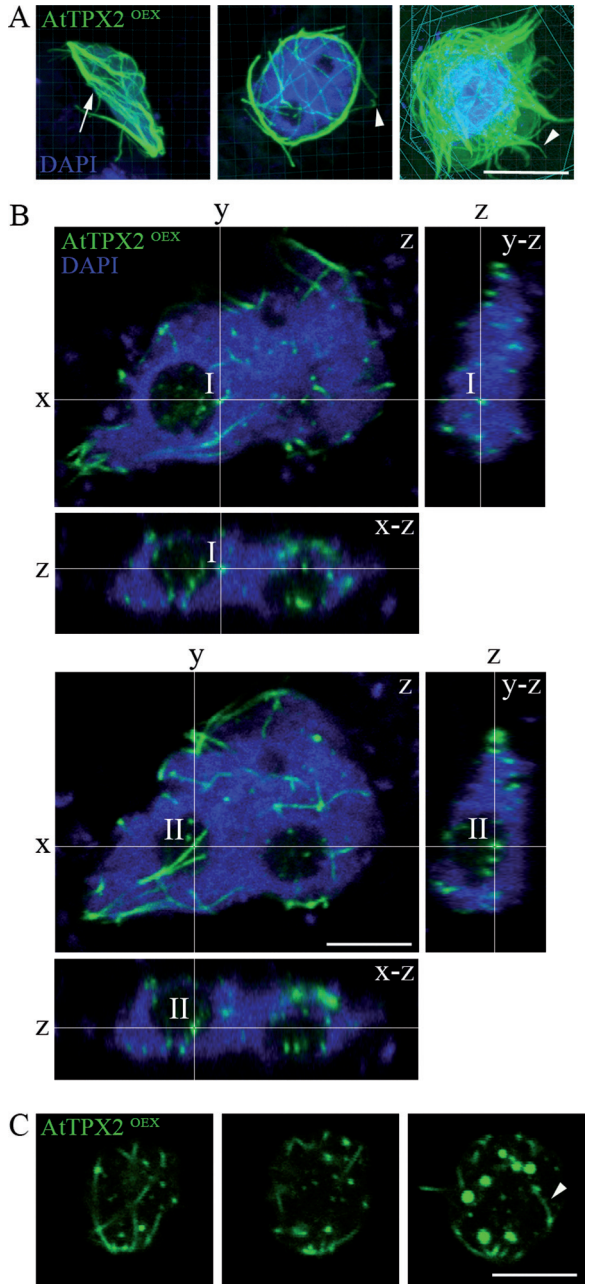

Fig. 3. Three-dimensional reconstruction and analysis of microtubular arrays in *Arabidopsis* cells overexpressing AtTPX2-GFP. (A) Representative images from 3D reconstruction of cells with AtTPX2-decorated fibres around and inside nuclei; Microtubular fibres in the perinuclear area are twisted (arrow), 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

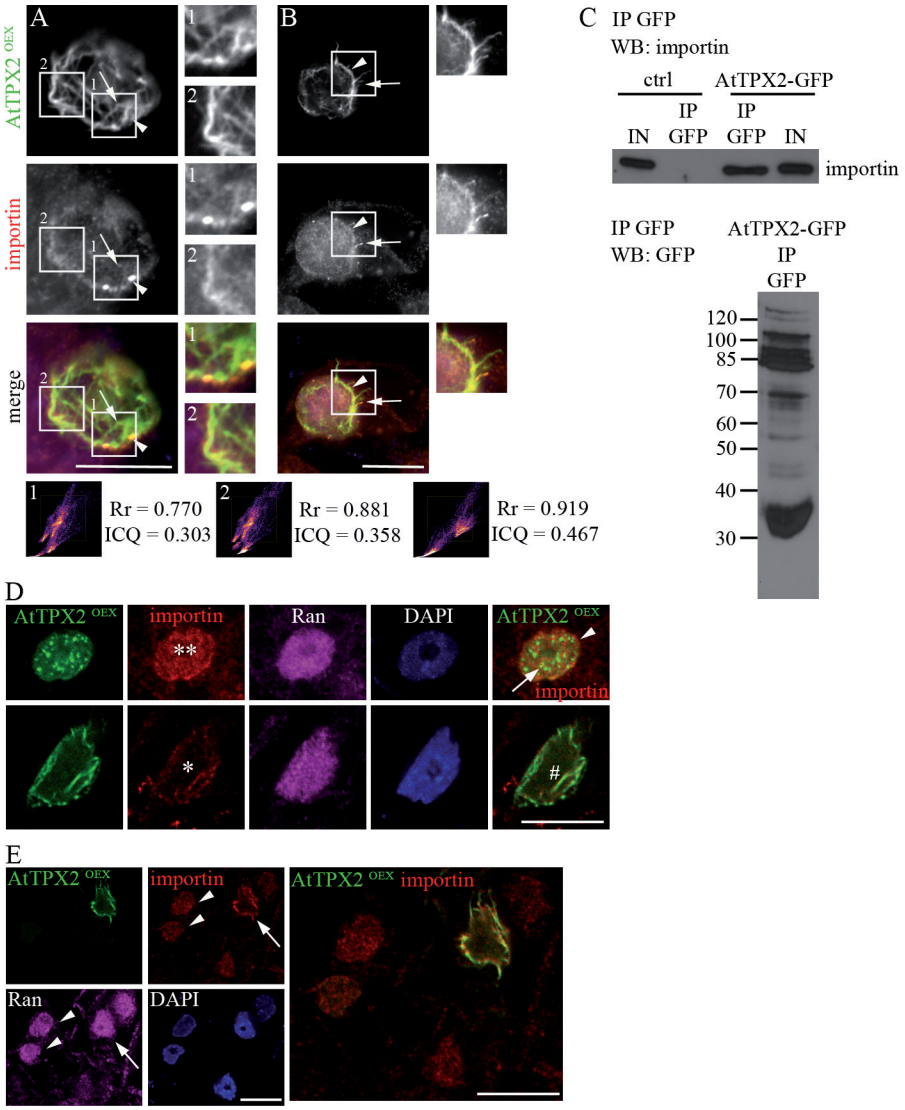

Fig. 4. Immunolocalization of importin and Ran in AtTPX2-GFP-overproducing cells. (A, B) AtTPX2 and importin colocalized in the vicinity of the nuclei (B, arrowhead), with the AtTPX2-decorated microtubular fibres (A, B, arrows), and in intranuclear foci (A, arrowhead). Colocalization was analysed by 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

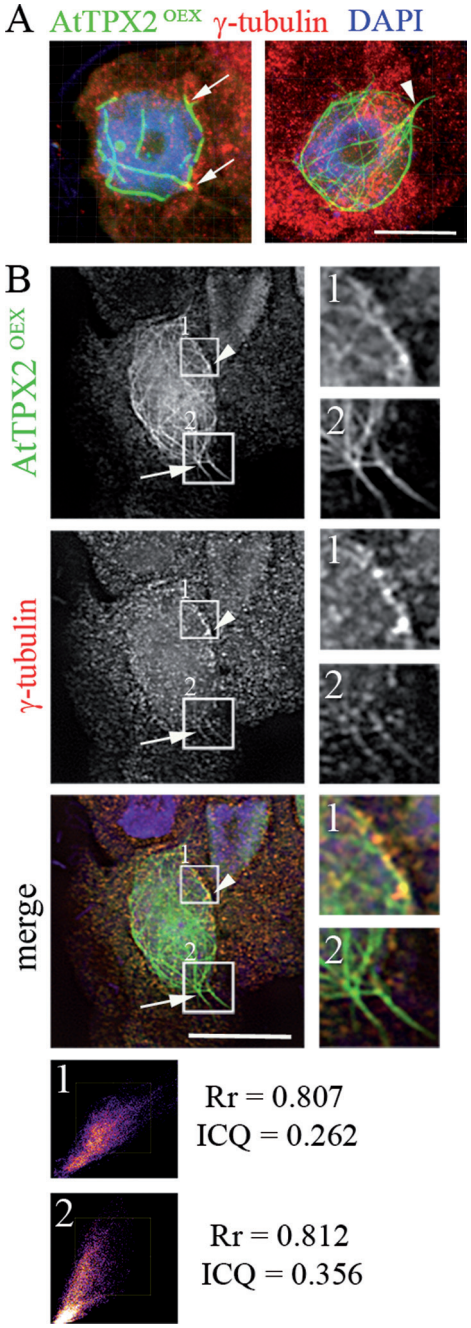

Fig. 5. Colocalization of AtTPX2 and γ-tubulin in *Arabidopsis* AtTPX2-GFP-overproducing cells. (A, B) γ-Tubulin localized together with AtTPX-GFP. (A) 3D analysis of cells overexpressing AtTPX2-GFP stained with anti-γ-tubulin antibody (red), DAPI (blue) obtained by laser scanning microscopy and 3D-reconstructed (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

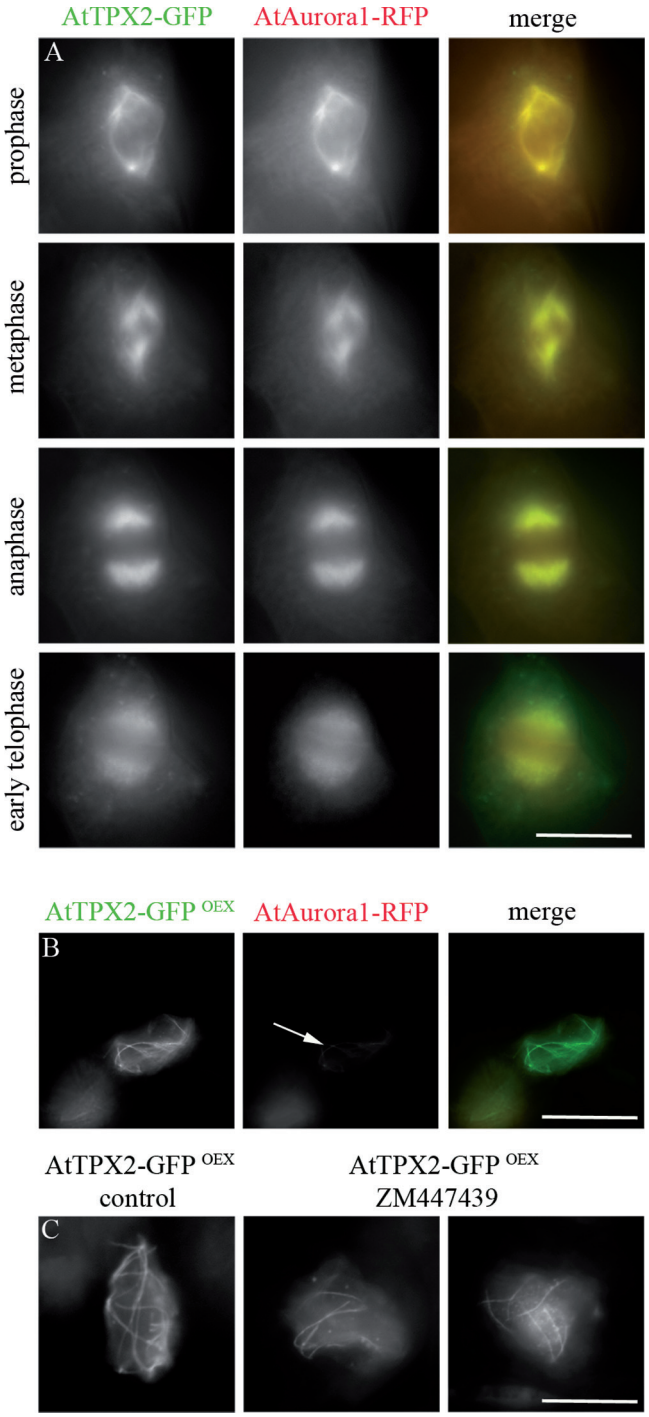

Fig. 6. Localization of AtTPX2 and AtAurora1 in control and ZM447439-treated cells overproducing AtTPX2. (A) Localization of AtTPX2-GFP and AtAurora1-RFP from the prophase until the early telophase in *Arabidopsis* cells. (B) Weak signal of AtAurora1 with AtTPX2 (arrow) in the nuclei of *Arabidopsis*. (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.

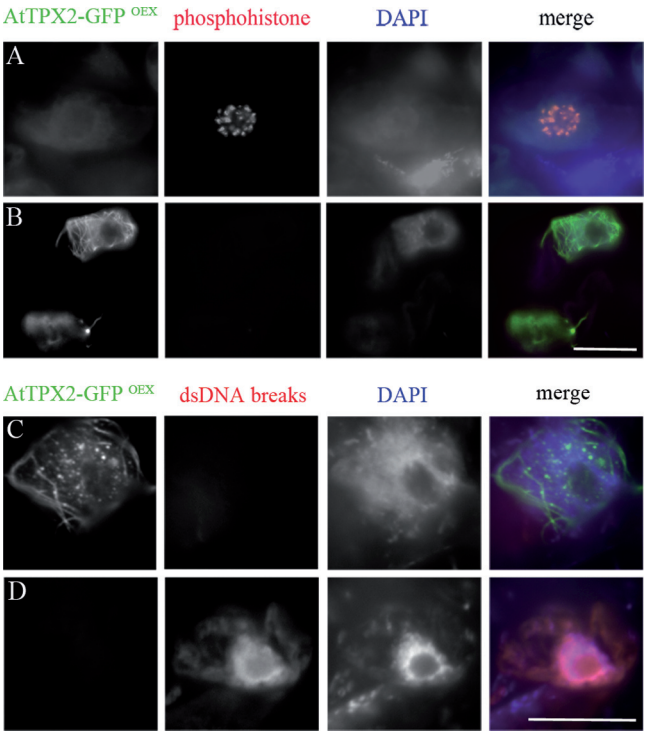

Fig. 7. Formation of AtTPX2 fibrillar structures was not dependent on mitotic status of the chromatin or connected with programmed cell death. (A, B) Immunolabelling of AtTPX2 (green) and phosphohistone (red) in prophase (A) and interphase (B) cells overexpressing AtTPX2. AtTPX2-decorated ectopic microtubules 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.

Acknowledgements

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

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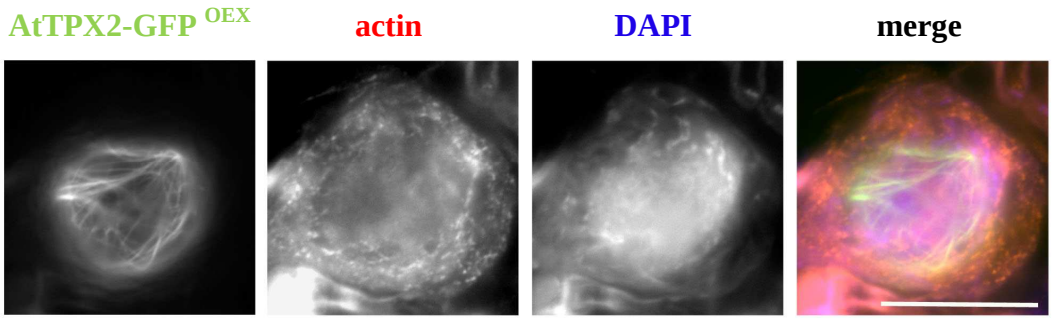
Supplementary Table S1. *In silico* analyses of AtTPX2 protein.

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

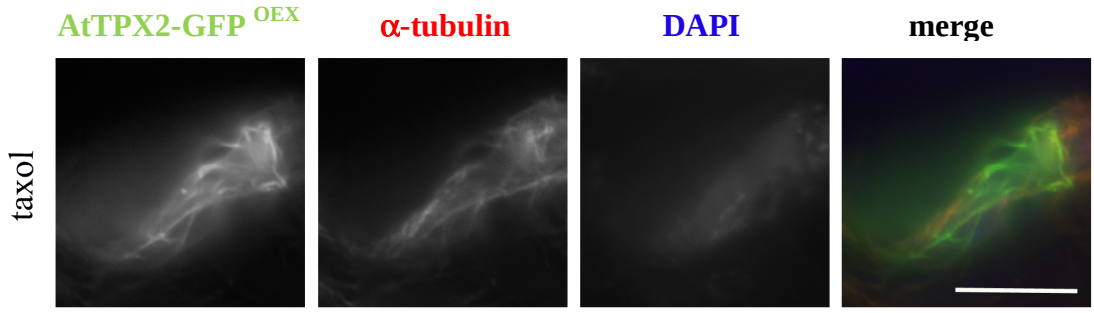
To predict putative interaction sites and binding motifs Pfam (Punta *et al*., 2012), ELM (Dinkel *et al*., 2012) and SUMOsp 2.0 (Ren et al., 2009) databases were used.

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



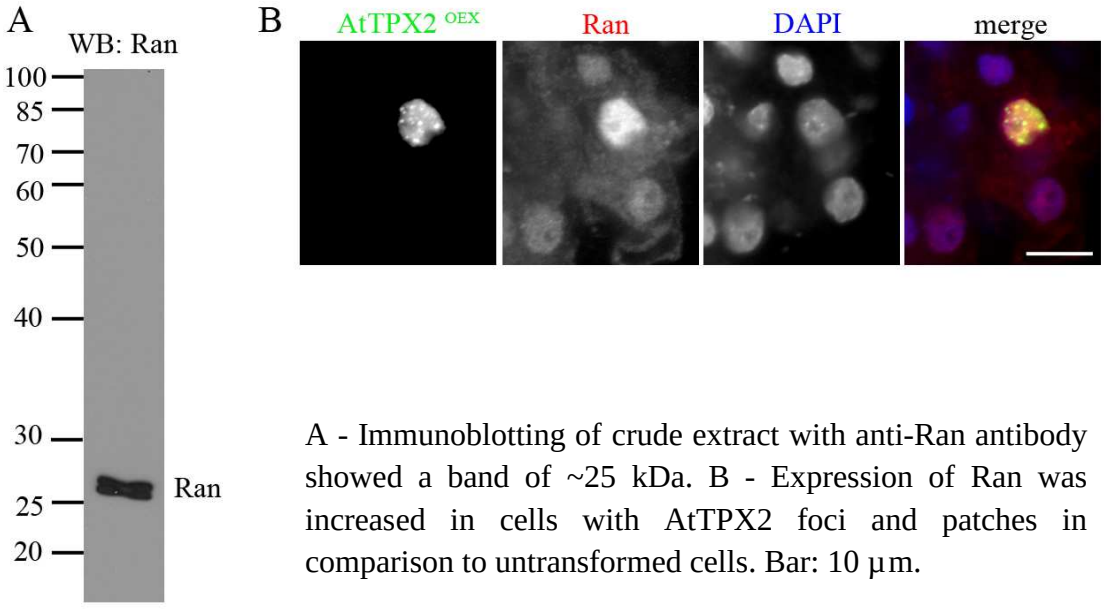
AtTPX2 fibres were not positive for actin immunolabelling. Bar: 10 µm.

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



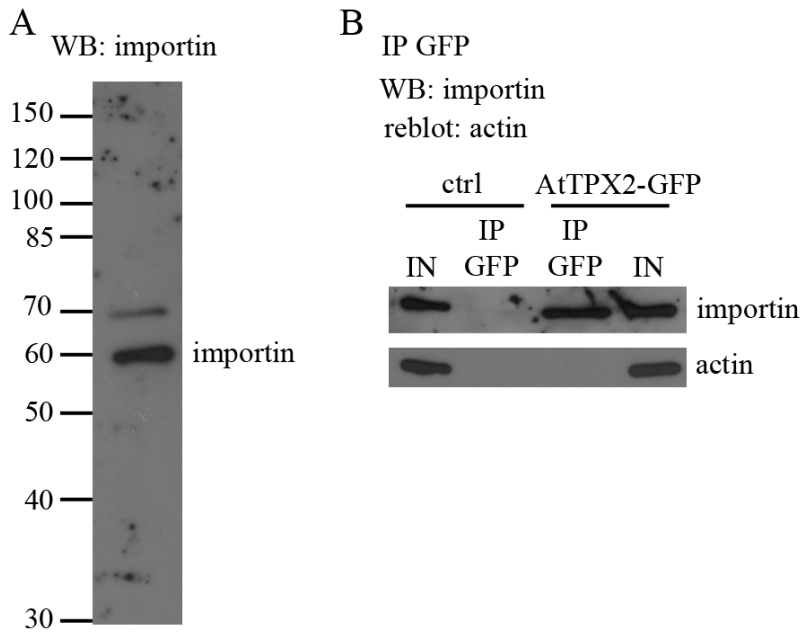
Further bundling of AtTPX2 decorated microtubules was not observed after treatment with 5 µM taxol for 3 hours. Bar: 10 µm.

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



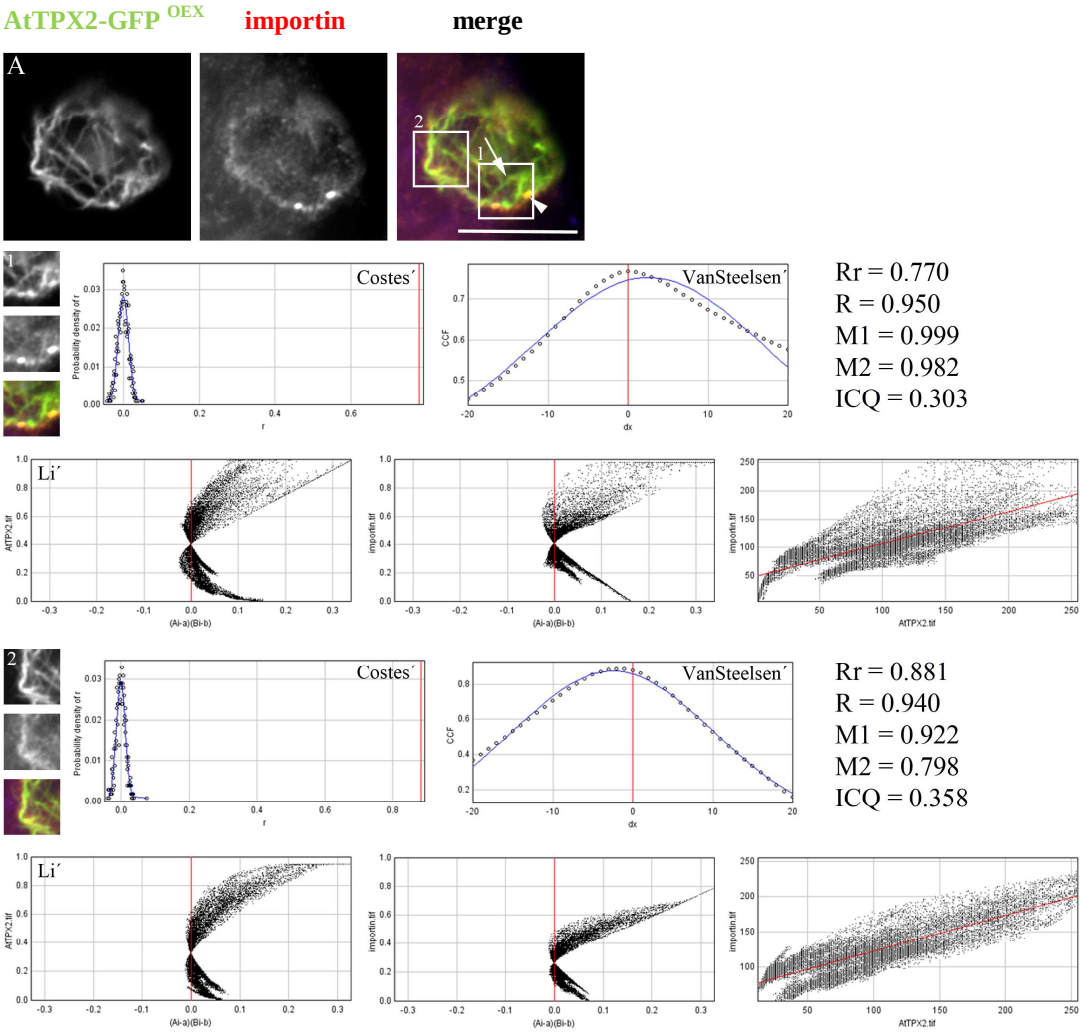
A - Immunoblotting of crude extract with anti-Ran antibody showed a band of ~25 kDa. B - Expression of Ran was increased in cells with AtTPX2 foci and patches in comparison to untransformed cells. Bar: 10 µm.

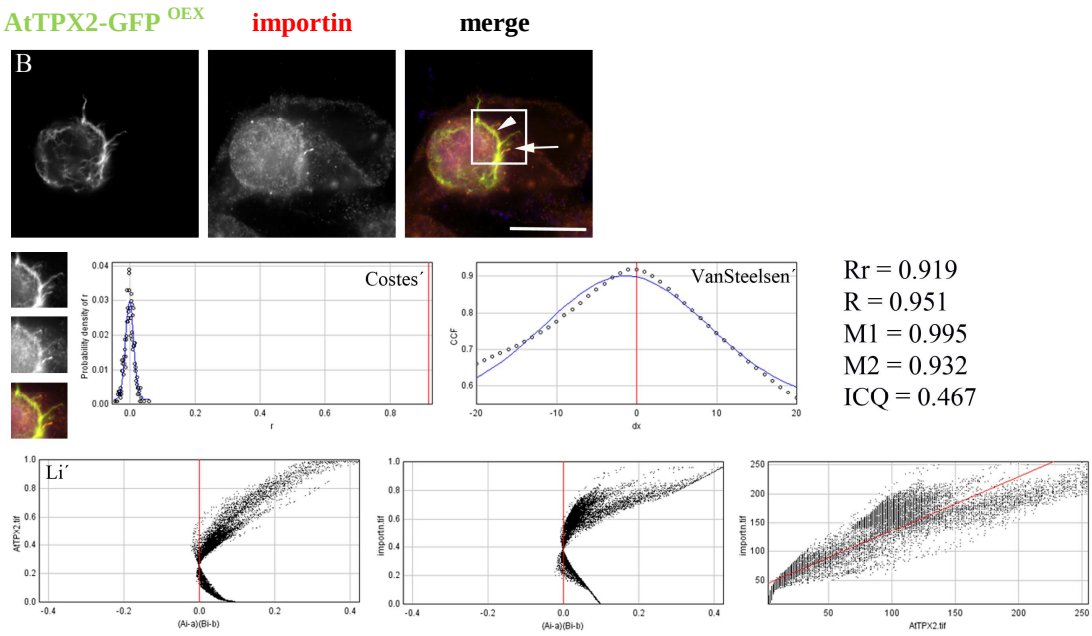
Supplementary Figure S4. Importin copurified with AtTPX2-GFP from *Arabidopsis* cultured cells.



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.

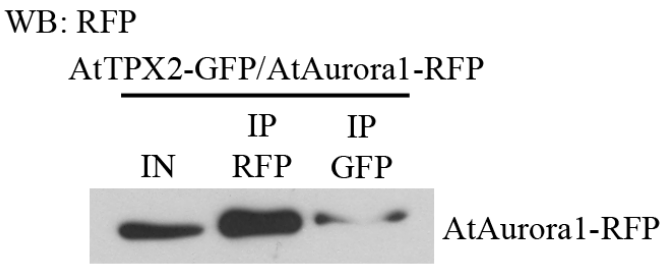
Supplementary Figure S5. Colocalization analyses of AtTPX2 and importin in *Arabidopsis* cultured cells.





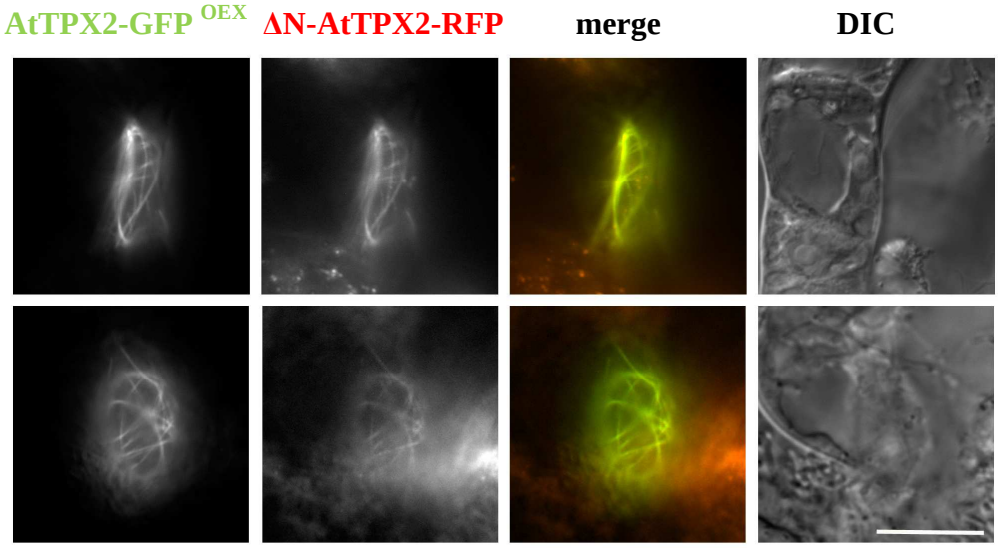
AtTPX2-GFP decorated fibres were present in close vicinity to the importin decorated nuclear envelope (A, B, arrowhead). Importin was present with AtTPX2 in dots on the nuclear envelope (A, B, arrowhead) and in the fibres (A, B, arrows). Pearson´s correlation coefficients (Rr) in Figure S8A (insets 1, 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.



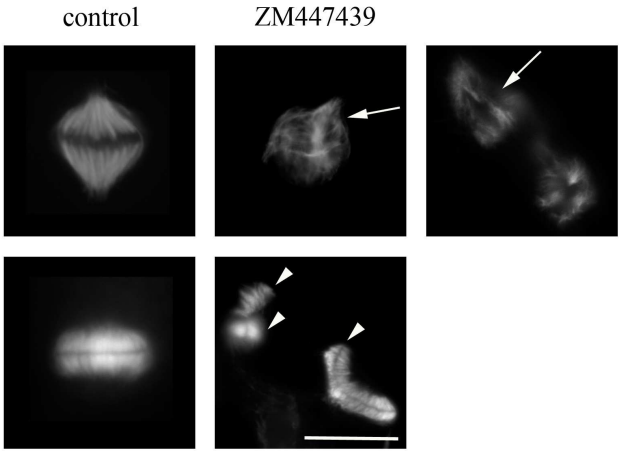
Immunoblotting with anti-RFP antibody showed that AtAurora1-RFP was copurified with AtTPX2-GFP using GFP trap (IP GFP) from extract prepared from cell culture co-expressing AtAurora1-RFP and AtTPX2-GFP. AtAurora1-RFP was purified by RFP trap (IP RFP).

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



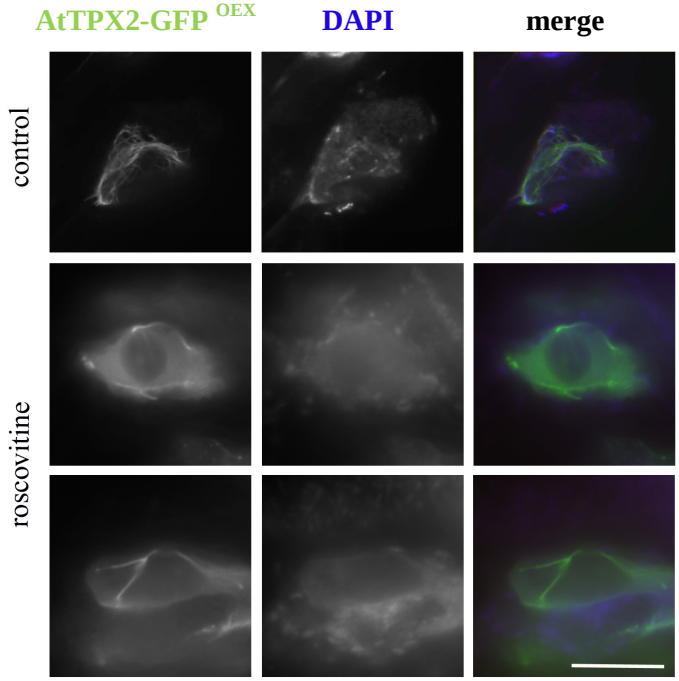
AtTPX2-GFP and its truncated version ΔN-AtTPX2-RFP localized together on microtubular array in the *Arabidopsis* nuclei. Bar: 10 µm.

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



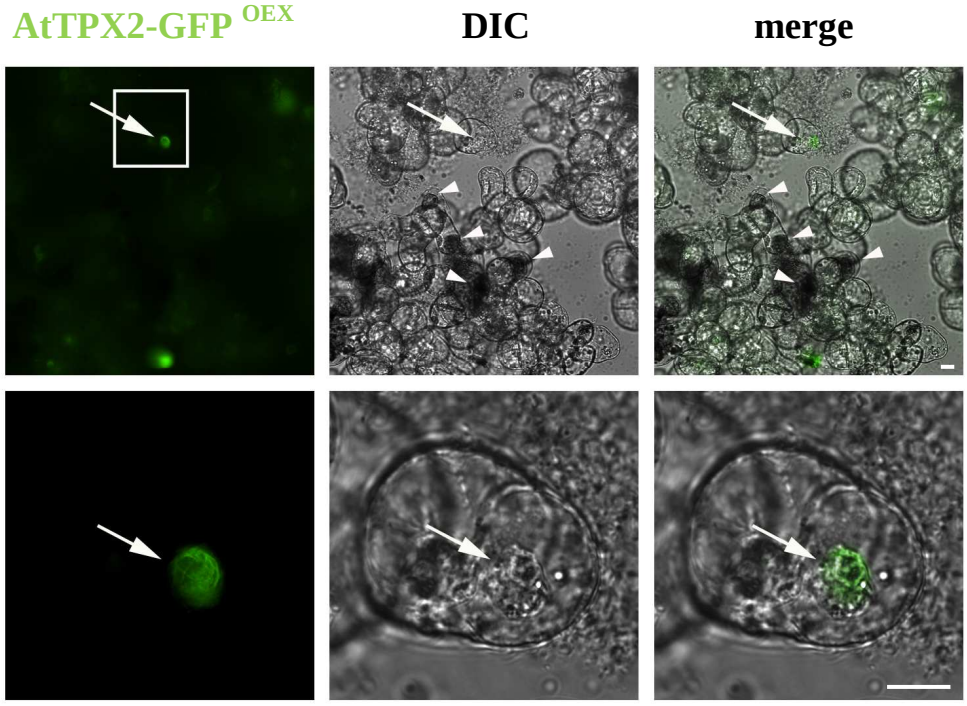
Microtubular arrays in the control (wild type) cells were affected after to the Aurora kinase inhibitor ZM447439 treatment. Multipolar mitotic spindle (arrows), fragmented phragmoplast (arrowheads) were often observed. Bar: 10 µm.

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.

Supplement III

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

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ORIGINAL PAPER

TPX2 Protein of *Arabidopsis* Activates Aurora Kinase 1, But Not Aurora Kinase 3 In Vitro

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Abstract 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. Such role in plants is not known. Here, we have assessed the ability of TPX2 of *Arabidopsis* to regulate Aurora family members in vitro. *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. 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.

Keywords Aurora kinase · Targeting protein for Xklp2 · In vitro kinase assay · Kinase activation

Introduction

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,

Eva Tomaštíková and Dmitri Demidov contributed equally to this work.

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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 and Vernos 2004). TPX2 is localized to cell nucleus during interphase, and it is released from importin by RanGTP after nuclear envelope breakdown. 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* (Wittmann 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). Binding of full-length TPX2 or N-terminal part of TPX2 (amino acids 1–49) 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 (Aurora1 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 serine 10 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. 2012). 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 phosphorylate *At*TPX2. Furthermore, we found that autophosphorylation activity of *At*Aurora1 is dramatically increased after addition of *At*TPX2 and Aurora binding domain of TPX2 is responsible for the activation. Increased autophosphorylation results in overall increase in the kinase activity towards histone H3, one of its most important mitotic targets. Our data suggest that similar mechanism of Aurora kinase activation occurs in plants and metazoans.

Material and 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 *Escherichia 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. 2012). 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. For *At*TPX2_100, entry vector of *At*TPX2 was used, and the first 300 bp comprising Aurora binding domain were amplified with primers REV1 3′-TTTCCTCCGGTTGCTTTCCTAG-5′ and FOR1 3′-GGCTCCAAGGAAGCAGCTGGCGAC-5′. Amplification was performed by High-Fidelity DNA Polymerase (Roche). PCR product was cloned into Gateway bacterial expression vector pET55DEST (Novagen, Madison, WI, USA).

*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). *At*histone H3 with replaced serine 10 (S10) to alanine was prepared by

site-directed mutagenesis from plasmid pET28::H3 (Demidov et al. 2009) using Phusion Site-Directed Mutagenesis Kit (Thermo Scientific).

Production of Recombinant Proteins

Recombinant *At*TPX2, *At*TPXΔN, *At*TPX2_100, *At*histone H3, and *At*histone H3S10/A were produced in *E. coli* BL-21 and purified under denaturing 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 h, 37 °C, and 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 h, 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, and 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 Tris-HCl, 50 mM KCl, 2 mM $MgCl \cdot 6H_2O$, 0.2 mM EGTA, pH 7.5) using 7 K MWCO Zeba Spin Columns (Thermo Scientific, Waltham, MA, USA). *At*Aurora1 or 3 was preincubated at 30 °C, 30 min with 0.5× 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*TPX2_100, *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). For detection Aurora1 kinase activity towards histone H3, *At*Aurora1 was incubated with 1× kinase buffer, 0.8 mM ATP, activators *At*TPX2 and *At*TPX2_100, and substrates H3 and mutated H3S10/A at 30 °C for 60 min. Kinase reactions were terminated by freezing and processed for Western blot analyses with histone H3 rabbit polyclonal antibody (dilution 1:1000, Abcam, ab1791) and H3S10ph mouse monoclonal (dilution 1:1000, Abcam, ab14955) primary antibodies and secondary antibodies IRDye 800 CW donkey-anti mouse IgG (H+L) (dilution 1:5000, LI-COR, 926-32212) or donkey-anti rabbit IgG (H+L) (dilution 1:5000, LI-COR 926-32213, LI-COR, NE, USA). Briefly, kinase reaction mixtures were diluted 1:10 mixed with 2× Laemmli protein loading buffer and 30 μl of mixtures were used as described in Demidov et al. (2009). Signals were detected by Odyssey CLx Imager (LI-COR, NE, USA).

Accession Numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers BAE00019 (AGI locus identifier *At*4g32830), BAE00020 (*At*2g25880), NP_182073 (*At*2g45490) for *At*Aurora1, 2, and 3 (respectively); NP_973754 (*At*1g03780) for *Arabidopsis* *At*TPX2; and NC_003070.9 (*At*1g09200) for *Arabidopsis* histone H3.

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 (Appendix S1, Appendix S2). 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 (Appendix S1). Some potential TPX2 interaction sites were observed in plant *At*Aurora3 homologues as well (Appendix S2). At the same time, some of the amino acids in such interaction sites differ from those of *At*Aurora1 and 2 (Appendix S1, Appendix S2). 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 (Fig. S3), suggesting that TPX2 acts as activator and/or regulator 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 (Fig. S4). Aurora A binding sites in *At*TPX2 have already been described by Evrard et al. (2009) and additionally, five of nine family members of TPX2-like proteins exhibit potential binding sites (Fig. 1). Different splicing variants of these proteins 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 (Fig. 1, Fig. S5). Since there is a similar domain composition among TPX2 and abovementioned proteins, we suggest to rename them as TPX2-like protein 1 (TPXL1, *At*3g01015), TPX2-like protein 2 (TPXL2, *At*4g11990), TPX2-like protein 3 (TPXL3, *At*4g22860), TPX2-like protein 4 (TPXL4, *At*5g07170), TPX2-like protein 5(TPXL5, *At*5g15510), TPX2-like protein 6 (TPXL6, *At*5g37478), TPX2-like protein 7 (TPXL7, *At*5g44270), and TPX2-like protein 8 (TPXL8, *At*5g62240) (Fig. 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 *A. 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 acids 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, Fig. S6B), we prepared a truncated version of the protein, lacking the Aurora binding sites and one of the microtubule binding domains (Petrovská et al. 2012). The truncated version of the protein, *At*TPX2ΔN covers amino acids 442–758 (around 45 kDa, Fig. S6B) 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. Here, it should be emphasized that TPX2 protein is highly unstable with a half time of degradation of around 10 min, making protein purification difficult (Stewart and Fang 2005). Nevertheless, we were able to purify enough *At*TPX2 for our kinase assays (Fig. S6B).

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

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	-	-	+

Fig. 1 Different variants of *Arabidopsis* TPX2-related proteins and their domains. AtTPX2 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 AtAurora1 localization at microtubules

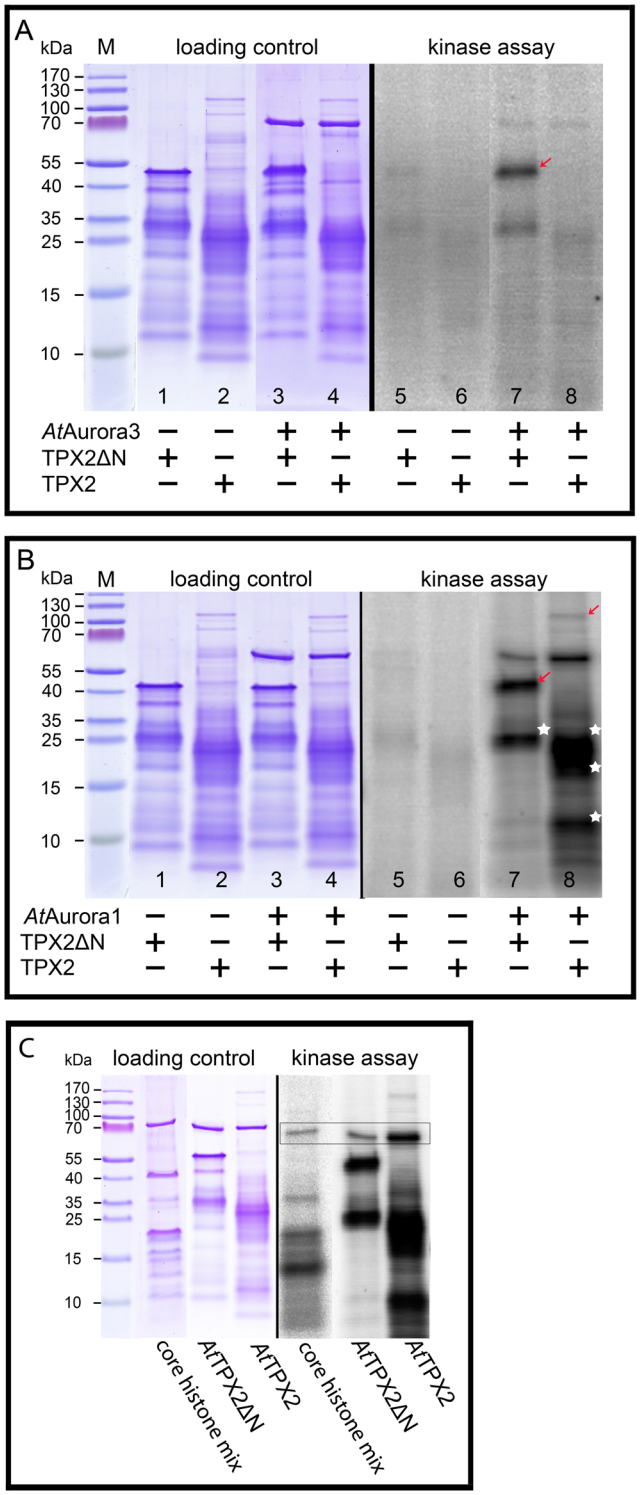

Fig. 2 Phosphorylation of *At*TPX2 and *At*TPX2ΔN by *At*Aurora 1 and *At*Aurora3 in vitro. Radioactive kinase assays were performed with *At*Aurora1 and *At*Aurora3 kinase using full-length and truncated *At*TPX2 proteins. **a**, **b** CBB staining (*1–4*) and autoradiographs (*5–8*) of kinase assays. 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*). Lanes *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. Lanes *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 (lane *3*) but not *At*TPX2ΔN (lane *2*), kinase activity of *At*Aurora1 is increased in comparison to control—core histone mix, well-known substrate for Aurora kinases (lane *1*)

(Fig. 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 (Fig. 2b).

Aurora Binding Domain of *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 (Fig. 2c).

To prove whether the Aurora binding domain of *At*TPX2 is involved in activation of the Aurora kinase 1, we cloned a construct containing first 100 amino acids of *At*TPX2 which comprise Aurora binding domain. Indeed, recombinant *At*TPX2_100 was able to activate Aurora kinase 1, therefore pointing to importance of Aurora binding domain of TPX2 in kinase activation (Fig. 3).

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. Recombinant histone H3 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 and *At*TPX2_100. Efficiency of Aurora kinase activation was detected by Western blot analyses with antibodies against histone H3 as loading control, H3S10, and H3S28 phosphorylation-specific antibodies. Consistent with our previous findings, full-length *At*TPX2 was able to increase activity of *At*Aurora1 resulting in increased phosphorylation of H3 specifically at serine 10. As negative control, H3 with serine 10 position mutated to alanine was used (Fig. 4a, b). Increase in H3S28 phosphorylation was

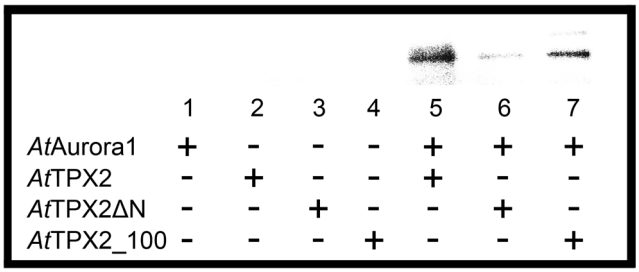

Fig. 3 Aurora binding domain of TPX2 increase *in vitro* autophosphorylation activity of *At*Aurora1. Radioactive kinase assay was performed with full-length TPX2, TPX2ΔN, and TPX2_100 proteins. *At*Aurora1 autophosphorylation activity was markedly increased after addition of full length TPX2 (lane *5*) as well as TPX2_100 (lane *6*) pointing to importance of TPX2 Aurora binding domain in *At*Aurora1 activation

not observed (data not shown). To show whether Aurora binding domain of TPX2 is involved in increased Aurora kinase activity, we performed kinase assay with additional activator, TPX2_100. Similar to full-length *At*TPX2, TPX2-100 was able to increase AtAurora1 activity (Fig. 4c, d). No increase in H3S28 phosphorylation was observed (data not shown). Hence, *At*TPX2, particularly its Aurora binding domain, 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

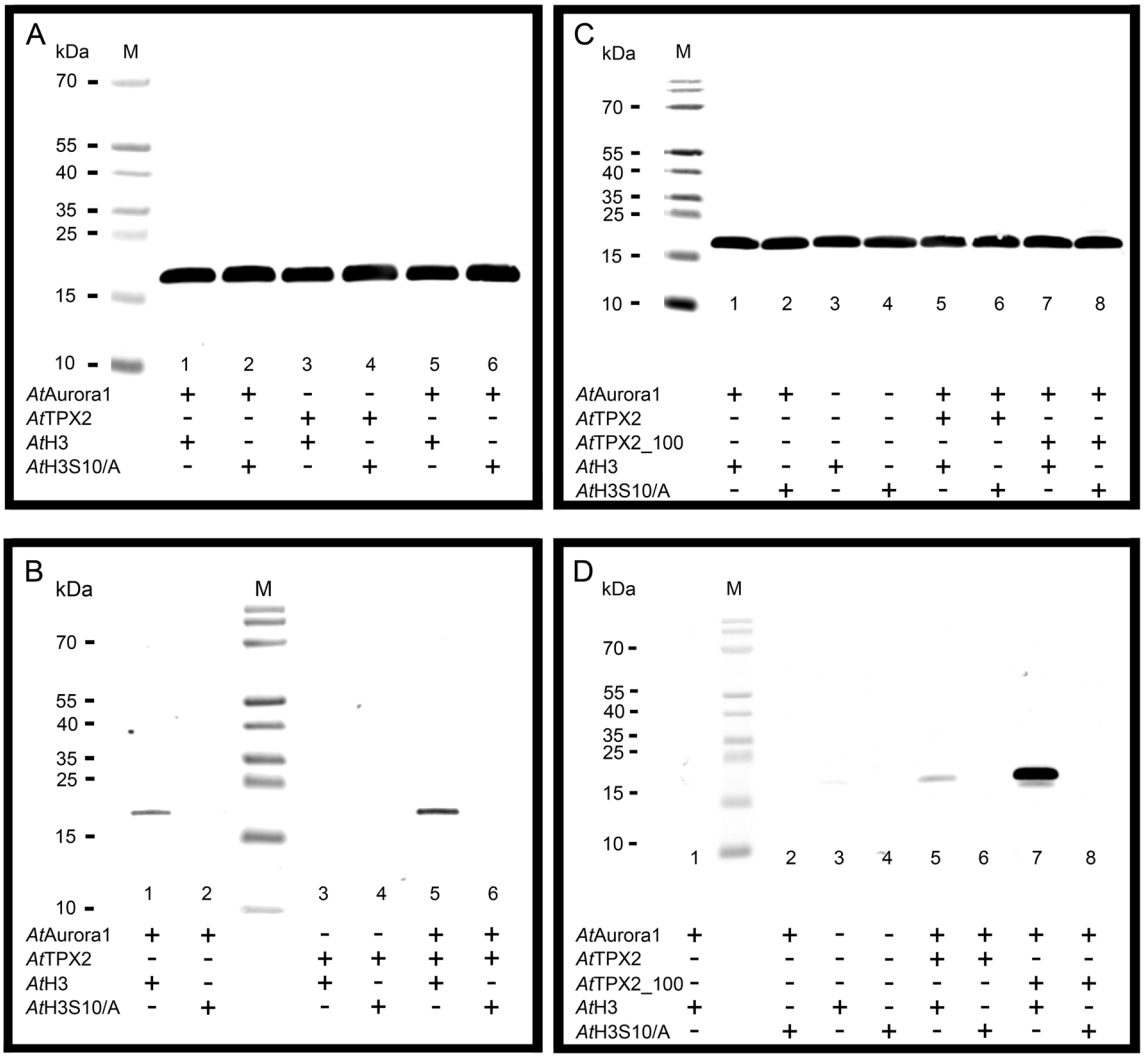

Fig. 4 In vitro activation of *At*Aurora1 by *At*TPX2 causes increased phosphorylation of downstream targets. Phosphorylation of histone H3 at position serine 10 was detected by phospho-specific antibodies. As negative control, H3S10/A was used. **a**, **c** Loading control—antibodies against total histone H3,;**b** H3S10ph antibodies. In vitro phosphorylation of histone H3 is markedly increased after activation of *At*Aurora1 by *At*TPX2. Comparison of *At*Aurora1 activity without (lane *1*) and with (lane *5*) addition of *At*TPX2 as kinase coactivator. **d** Aurora binding domain of histone H3 is involved in activation of *At*Aurora1. In comparison to full-length *At*TPX2 (lane *5*), TPX2_100 increase phosphorylation activity of *At*Aurora1 towards H3S10 (lane *7*)

negative regulators (Walter et al. 2000; Eyers et al. 2003). In the present study, we identified the microtubule-associated protein *At*TPX2 (*At*1g03780) 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 (Fig. 1). Some splicing variants are characterized by the absence of Aurora binding domain (Fig. 1, *At*4g22860 of 306 amino acids and *At*5g62240 of 327 amino acids) and some by the absence of a TPX2 motif (Fig. 1, *At*4g22860 of 501 amino acids or *At*5g15510, *At*3g01015, *At*5g37478, *At*5g44270). 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 not only interacts 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 (Figs. S1, S2). 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 (Fig. S3). No members of plant proteins of chromosomal passenger complex (CPC) still have been identified, especially INCENP as *At*Aurora3 activator. Because *At*Aurora3 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, more precisely its N-terminal part containing Aurora binding domain, stimulates autophosphorylation of *At*Aurora1 in vitro. *At*TPX2 has no effect on *At*Aurora3 activity, pointing towards 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. Interaction between *At*Aurora1 and *At*TPX2 has already been shown by co-immunopurification of *Arabidopsis* extracts (Petrovská et al. 2012, 2013). 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 phosphorylation of histone H3 at serine 10 and not serine 28. Nevertheless, lack of increased phosphorylation of serine 28 might be caused by generally lower activity of *At*Aurora1 towards H3S28 position. 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 kinetochor/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.

*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.

Acknowledgments We thank Oda Weiss for the excellent technical support. This research was supported by grants from the Czech Science Foundation (14-28443S) and the National Program of Sustainability I (LO1204); 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 and HJ. DD and AH are supported by the DFG (SFB 648). Grant from the Czech Science Foundation P501-12-2333 for PB. We thank Dr. H. P. Mock for helpful discussion about protein purification.

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

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Supplement IV

Interplay between Aurora kinases and TPX2 in plant cell nucleus

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

In: Plant Biotechnology: Green For Good III, p. 82, Olomouc,
Czech Republic, 2015

Interplay between Aurora kinases and TPX2 in plant cell nucleus

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

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Mitotic regulators play an important role in accurate progression of mitosis and division of genetic material to daughter cells. Aurora kinases, a group of conserved serine/threonine kinases, are involved in regulation of mitosis and cytokinesis in yeast, plant and animals. The targeting protein for Xklp2 (TPX2), a microtubule associated coiled-coil protein, functions in chromatin-induced microtubule nucleation and bipolar spindle assembly. The significance of Aurora A and TPX2 in spindle assembly and microtubule nucleation has been well documented in animal cells. TPX2 was found to activate animal Aurora A and, at the same time, to protect the kinase from dephosphorylation. Examining the Aurora kinases-TPX2 interaction in *Arabidopsis* cells we found colocalization of AtTPX2 and AtAurora1 at microtubules in cell cycle-dependent manner. Co-immunoprecipitation experiment confirmed AtTPX2 and AtAurora1 association. Data obtained from immunolocalisation and immunoprecipitation studies suggest that AtTPX2 may guide Aurora kinase to microtubules and, similarly with other systems, TPX2 may spatially and temporally modulate the function of AtAurora1. The evaluation of *in vitro* regulation of Aurora kinase family members by AtTPX2 showed AtTPX2 as a substrate and activator of Aurora1, but not Aurora3. These results may point to a specific regulation of plant Aurora1 and Aurora3 kinases, which may have its significance in regulation of cell cycle and signalling cascade transduction.

This research was supported by grants from the Czech Science Foundation (14-28443S), the National Program of Sustainability I (LO1204), the European Social Fund (Operational Program Education for Competitiveness CZ.1.07/2.3.00/20.0165) for BP, ET, HJ. DD and AH are supported by the DFG (SFB 648).

Interplay between Aurora kinases and TPX2 in plant cell nucleus

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

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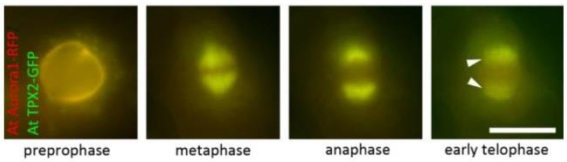
INTRODUCTION

Metazoan Aurora kinases, a family of conserved serine/threonine kinases, belong to the important regulators of mitotis and cytokinesis. The targeting protein for Xklp2 (TPX2), a microtubule associated coiled-coil protein, functions in chromatin-induced microtubule nucleation and bipolar spindle assembly. In animal cells the interplay of Aurora kinase A and TPX2 in the process of spindle assembly and microtubule nucleation is well documented. TPX2 was found to activate animal Aurora A and, at the same time, to protect the kinase from dephosphorylation. Here we present the results of examination of plant Aurora kinases and TPX2 relationship.

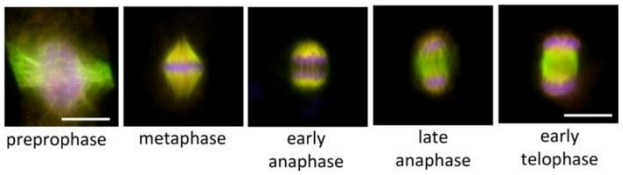
RESULTS

*At*TPX2 and *At*Aurora kinase1 localisation at microtubules

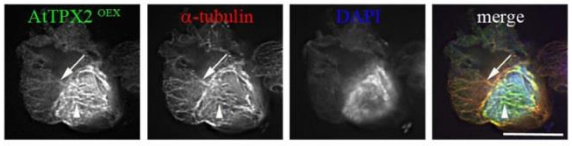
***At*TPX2-GFP and *At*Aurora1-RFP colocalised on mitotic microtubules (MT) and with early phragmoplast MT (arrowheads).**



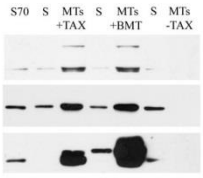
***At*Aurora1(red) and α-tubulin (green) were localised along MTs from metaphase to anaphase in dividing cells.**



***At*TPX2^{OEX} (green) localised with α-tubulin (red) in the interphase nucleus.**



Association of *At*Aurora1 and *At*TPX2 with microtubules confirmed biochemically.



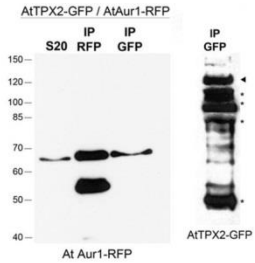
***At*Aurora1 and *At*TPX2 proteins pulled down with in vitro polymerized plant MTs prepared from extracts from the cell line expressing *At*Aurora1-RFP and *At*TPX2-GFP by taxol-driven polymerization and analysed by immunoblotting for α-tubulin, GFP, RFP**

AtTPX2-GFP* indicates the proteolysis degradation product

S70 -70 000 g supernatant; S - supernatant after the taxol polymerization; MTs+TAX, microtubular pellet; MTs+BMT-microtubular pellet from the assay supplemented with brain microtubules (BMTs); MTs-TAX - the negative control

Interaction of *At*TPX2 and *At*Aurora kinases

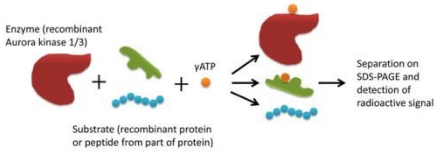
***At*Aurora1-RFP and *At*TPX2-GFP interaction confirmed by their co-purification.**



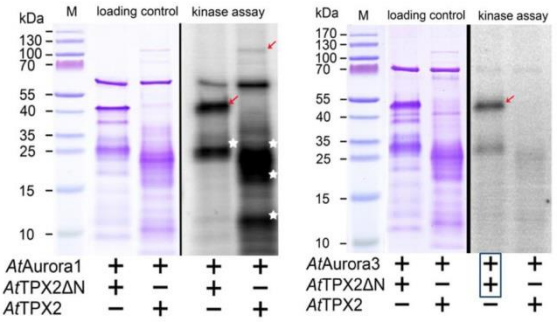
band of 120 kDa corresponded to full length *At*TPX2-GFP (arrowhead) was purified with GFP trap (IP GFP)

* indicates degradation products of AtTPX2

Scheme of *in vitro* kinase assay.



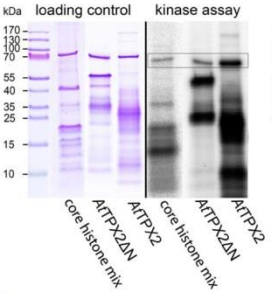
***At*TPX2 is phosphorylated by *At*Aurora1 but not *At*Aurora3.**



*At*Aurora kinase1 phosphorylates both *At*TPX2 and *At*TPX2ΔN* while *At*Aurora kinase3 phopsphorylates only *At*TPXΔ2N *in vitro* (red arrows).

* truncated version of *At*TPX2 without N-terminal Aurora binding domain

***At*TPX2 proved as coactivator of *At*Aurora1 autophosphorylation activity.**



Kinase activity of *At*Aurora1 was increased after addition of *At*TPX2 contrary to *At*TPX2ΔN in kinase assay compared to control core histone mix, a substrate for Aurora kinases

CONCLUSIONS

- **Association of *At*TPX2 and *At*Aurora kinase1 and their presence at microtubules in cell cycle-dependent manner was confirmed by co-immunoprecipitation or immunolocalisation experiments.**
- ***At*TPX2 was shown as a substrate and activator of *At*Aurora1, but not of *At*Aurora3 using *in vitro* kinase assay.**
- **Aurora binding domain of *At*TPX2 caused enhanced activity of *At*Aurora1**
- **Different mechanisms of activation of *At*Aurora1 and *At*Aurora3 may point to a specific regulation of both kinases, which may play important role in cell cycle regulation and signalling cascade transduction.**

This research was supported by grants from the Czech Science Foundation (14-28443S, P501/12/G090), the National Program of Sustainability I (LO1204), the European Social Fund (Operational Program Education for Competitiveness CZ.1.07/2.3.00/20.0165), and Internal Grant Agency of Palacky University, Olomouc (Prf/2013/00 and IGA_PrF_2014001).

Supplement V

The relationship between Aurora kinases and TPX2 protein in plants as revealed using multidisciplinary approaches

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

In: 6. Metodické dny (Bulletin České společnosti experimentální biologie rostlin a Fyziologické sekce Slovenské botanické společnosti), p. 86, Seč, Czech Republic, 2014

The relationship between Aurora kinases and TPX2 protein in plants as revealed using multidisciplinary approaches

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

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[2]*Leibniz Institute of Plant Genetics and Crop Plant Research, Corrensstrasse 3, D-06466 Gatersleben, Germany*

Correct progression of mitosis and transmission of genetic information to daughter cells depends on proper function of mitotic regulators. Aurora kinases, a family of conserved serine/threonine kinases, play important role in regulation of mitosis and cytokinesis in yeast, plants and animals. The targeting protein for Xklp2 (TPX2) was found to activate animal Aurora A and, at the same time, to protect the kinase from dephosphorylation. The significance of Aurora A and TPX2 in spindle assembly and microtubule nucleation in animal cells has been documented.

To examine the Aurora kinases–TPX2 interaction in *Arabidopsis* cell cultures, we used several state of art methodological approaches. Using immunofluorescence, we observed colocalization of AtTPX2 and AtAurora1 at microtubules in cell cycle-dependent manner. The AtAurora1-AtTPX2 association was confirmed by co-immunoprecipitation experiment. Data obtained from immunolocalisation and immuniprecipitation studies suggest that AtTPX2 may guide Aurora kinase to microtubules and, analogically with other systems, TPX2 may spatially and temporally modulate the function of AtAurora1.

In order to evaluate *in vitro* regulation of Aurora kinase family members by AtTPX2, we used *in vitro* kinase assays with -RFP/-GFP trap. The results showed AtTPX2 as a substrate and activator of Aurora1, but not of Aurora3. Different mechanisms of activation of AtAurora1 and AtAurora3 may point to a specific regulation of both kinases, which may play important role in cell cycle regulation and signalling cascade transduction.

This research was supported by grants from the Czech Science Foundation (14-28443S, P501/12/G090), the National Program of Sustainability I (LO1204), the European Social Fund (Operational Program Education for Competitiveness CZ.1.07/2.3.00/20.0165), and Internal Grant Agency of Palacky University, Olomouc (Prf/2013/00 and IGA_PrF_2014001).

THE RELATIONSHIP BETWEEN AURORA KINASES AND TPX2 PROTEIN IN PLANTS AS REVEALED USING MULTIDISCIPLINARY APPROACHES

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

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SUMMARY

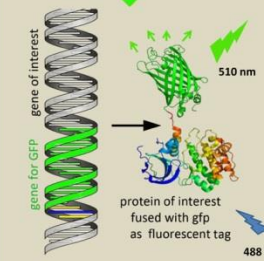
Metazoan Aurora kinases (A, B and C) belong to the important mitotic regulators. Aurora kinase A together with its activator TPX2 (targeting protein for Xklp2) function as significant components of spindle assembly process and microtubule nucleation. In addition, TPX2 was found to protect the kinase from dephosphorylation.

For functional characterisation of plant Aurora kinases (1,3) and TPX2 multidisciplinary approaches were performed – fluorescence visualisation, co-immunoprecipitation and *in vitro* kinase assay.

METHODS

Fluorescence methods for visualisation of proteins of interest

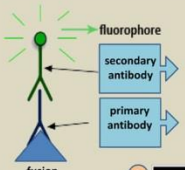
1 Live cell imaging



At Aurora kinase1, AtTPX2, α-tubulin were fused with -GFP/-RFP fluorescent tags.

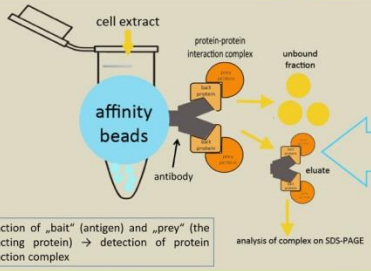
2 Immunofluorescence

We used specific primary and secondary antibodies to amplify fluorescent signal of AtAurora kinase1, AtTPX2 and α-tubulin in cell suspension.



3

Co-immunoprecipitation

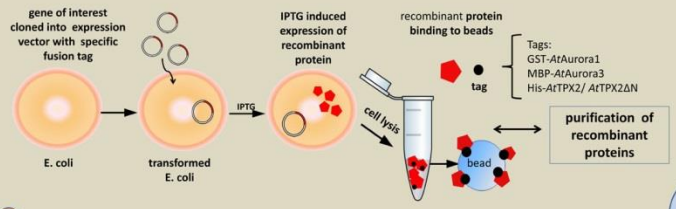


interaction of „bait" (antigen) and „prey" (the interacting protein) → detection of protein interaction complex

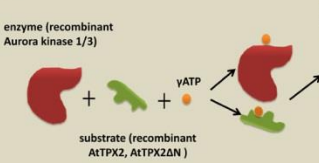
Extracts from *A. thaliana* cells co-expressing proteins AtTPX2-GFP and AtAurora1-RFP were incubated with GFP-Trap A or RFP-Trap A beads. The immunoprecipitated proteins were eluted, resolved by SDS-PAGE and immunobloted with relevant antibodies.

In vitro kinase assay for measuring phosphorylation activities of AtAurora kinases

1 Production of recombinant proteins



2 In-vitro kinase assay



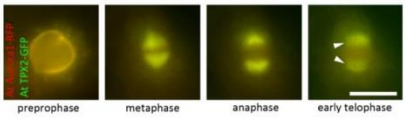
Radioactive γATP[32] is transferred by the kinase during *in vitro* kinase assay onto the substrate-*At*TPX2 or *At*TPX2ΔN (truncated version without Aurora binding domain)

RESULTS

Functional characterisation of AtAurora kinase1 and AtTPX2

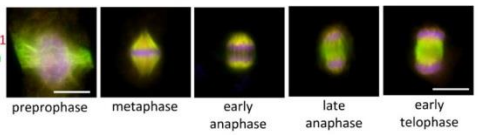
AtTPX2-GFP colocalised with AtAurora1-RFP on mitotic microtubules (MT) and with early phragmoplast MT (arrowheads).

Live cell imaging

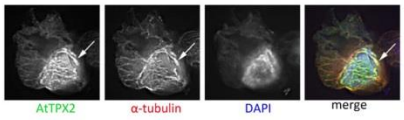


Immunolabelled At Aurora1(red) and α-tubulin (green) in dividing cells. At Aurora1 localised along MTs from metaphase to anaphase.

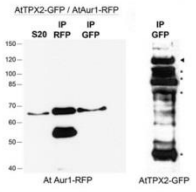
Immunofluorescence



AtTPX2^{OEX} (green) localised with α-tubulin (red) in the interphase nucleus.



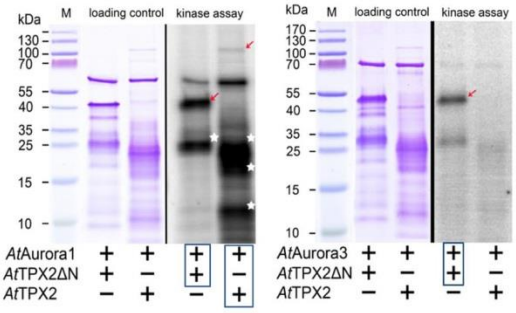
AtAurora1-RFP and AtTPX2-GFP interaction confirmed by their co-purification.



← band of 120 kDa corresponded to full length AtTPX2-GFP (arrowhead) was purified with GFP trap (IP GFP)

* indicates degradation products of AtTPX2

AtTPX2 is phosphorylated by AtAurora1 but not AtAurora3



AtAurora kinase1 phosphorylates both AtTPX2 and AtTPX2ΔN while AtAurora kinase3 only TPXΔ2N *in vitro* (red arrows).

CONCLUSION

- **Association of AtTPX2 and AtAurora kinase1 and their presence at microtubules in cell cycle-dependent manner was confirmed by co-immunoprecipitation or immunolocalisation experiments.**
- **AtTPX2 was shown as a substrate and activator of AtAurora1, but not of AtAurora3 using *in vitro* kinase assay.**
- **Different mechanisms of activation of AtAurora1 and AtAurora3 may point to a specific regulation of both kinases, which may play important role in cell cycle regulation and signalling cascade transduction.**

This research was supported by grants from the Czech Science Foundation (14-28443S, P501/12/G090), the National Program of Sustainability I (LO1204), the European Social Fund (Operational Program Education for Competitiveness CZ.1.07/2.3.00/20.0165), and Internal Grant Agency of Palacky University, Olomouc (Prf/2013/00 and IGA_PrF_2014001).

Supplement VI

Arabidopsis TPX2 activates Aurora kinase 1 *in vitro*

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

In: SEB Annual Meeting, p. 89, Manchester University, UK, 2014

Arabidopsis TPX2 activates Aurora kinase1 *in vitro*

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

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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 AtTPX2 as AtAurora1 regulator *in vitro*. AtTPX2 acts as a substrate as well as an activator of AtAurora1, but not AtAurora3. Although the truncated version of AtTPX2 lacking the Aurora binding domain can phosphorylate the kinase, it is unable to activate it. The activation of AtAurora1 results in dramatically increased phosphorylation of downstream targets, histone H3 in particular. The differences in the activation mechanisms of both AtAurora1 and AtAurora3 point to a specific regulation of both kinases, a fact that might play an important role in cell cycle regulation and signaling cascade transduction.

Our data indicate that similar mechanisms of Aurora kinase activation occur in plants and metazoans. Furthermore, activation of AtAurora1 by AtTPX2 could represent a mechanism for translation of RanGTP signaling to phosphorylation cascade performed by Aurora kinases at kinetochores. Increased AtAurora1 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]

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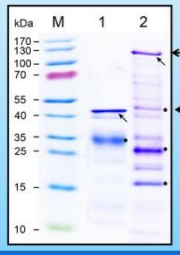
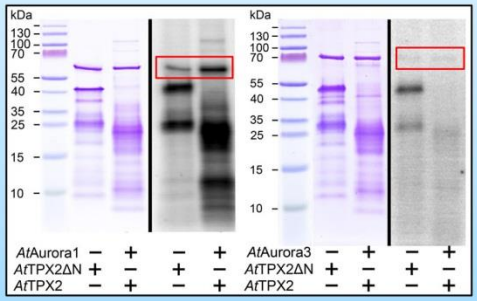
Introduction: Aurora kinases belong to the family of serine/threonine kinases. They play a role in execution a variety of mitotic events, such as chromosome segregation and bipolar mitotic spindle assembly. The function and regulation of Aurora kinases in animal systems have already been described. It 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.

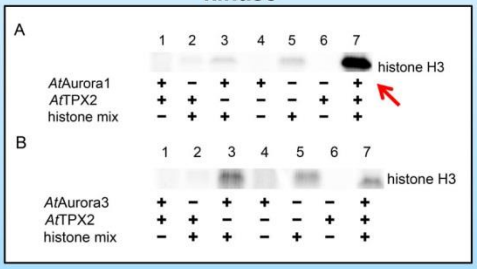
In order to elucidate whether activity of plant Aurora kinases is influenced by *At*TPX2 *in vitro*, kinase assay was performed with recombinant proteins.

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

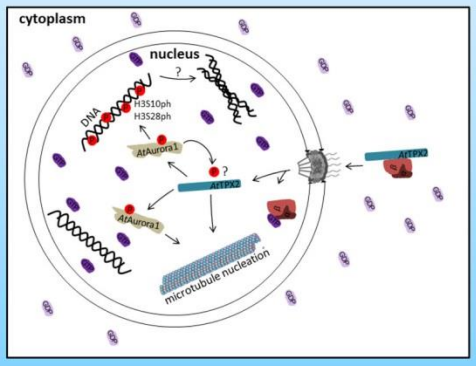


The activation of *At*Aurora1 results in dramatically increased phosphorylation of downstream targets, histone H3 in particular. Hyperphosphorylation of histone H3 by *At*Aurora1 was found in the presence of *At*TPX2 (marked with red arrow) (A). In contrast, no elevated level of H3 phosphorylation was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

Supplement VII

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

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

In: SEB Annual Meeting, p. 89, Manchester University, UK, 2014

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

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]

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The targeting protein for Xklp2 (TPX2) is a microtubule associated coiled-coil protein with functions in chromatin-induced microtubule nucleation regulated by RanGTPase pathway and bipolar spindle assembly. In contrary to the animal systems the information about plant TPX2 functions is still not complete. Plant TPX2 has been found as a nuclear protein with important functions during mitosis. Our previous study showed AtTPX2 localised with AtAurora1 kinase which acts as its interactor in the process of mitotic microtubules nucleation. What is the reason for accumulation of AtTPX2 in interphase nuclei remains to be resolved. Recently we showed that significant overproduction of AtTPX2-GFP is associated with ectopically nucleated stable microtubular arrays. Signals of AtTPX2-GFP were observed as dots and fibrilar structures decorating the microtubules on the surface of nuclear envelope and in the nuclei. AtTPX2-GFP fibres often extended to the cell periphery. AtTPX2 fibres were positive for α-tubulin and γ-tubulin immunolabelling. Using TUNEL test it was proved that AtTPX2 stabilized microtubules are not a manifest of apoptosis. 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 comprising γ-tubulin and TPX2 protein. The data suggested the AtTPX2 overproduction amplifies the ability of nuclear envelope to promote the microtubule nucleation which is typical for acentrosomal plant cells. Moreover, co-localisation and copurification of AtTPX2 with importin and Ran, respectively, determined that RanGTPase pathway which is involved in the process of TPX2 induced microtubules formation in animal cells is present also in plant cells.

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

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

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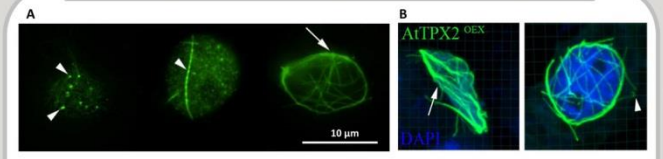
INTRODUCTION

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.

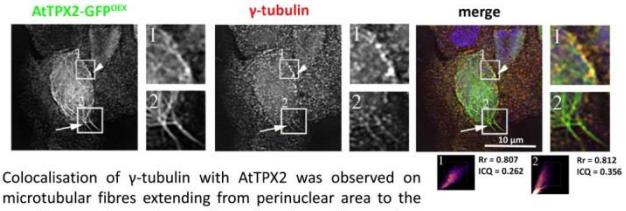
The role of plant TPX2 in MT assembly during mitosis has been suggested, nevertheless, its function during interphase is still unclear. In this study we analysed *Arabidopsis* interphase cells overexpressing AtTPX2-GFP to obtain further insights into TPX2 mediated MT formation in plants. To determine 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



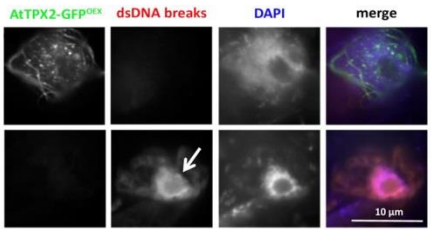
A) Dots (arrowheads) and fiber-like structures (arrows) decorated with AtTPX2-GFP were formed 72 hours after transformation around and inside interphase nuclei of AtTPX2^{OEX} cells.
B) 3D reconstruction of AtTPX2 decorated microtubular arrays twisted in perinuclear area (arrow) and extended to cell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

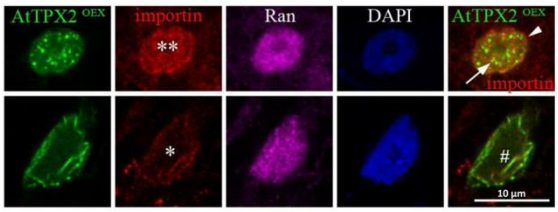


No ectopic AtTPX2–decorated arrays were observed in cells that were positive for TUNEL labelling (arrow), which was used for detection of apoptosis.

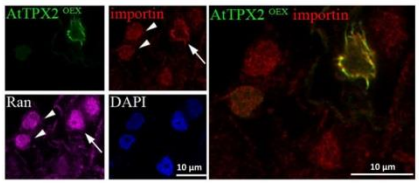
CONCLUSIONS

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.

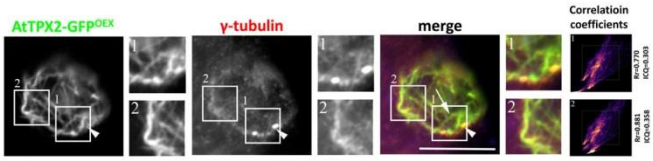
AtTPX2–GFP colocalised with importin in a Ran-GTPase dependent manner



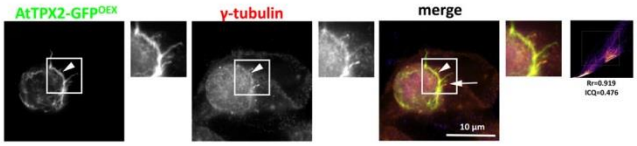
The accumulation of importin and Ran in the nuclei of AtTPX2^{OEX} cells indicates a nuclear import of AtTPX2^{OEX}. Importin signal was present in nuclei (**), around nuclei (arrowhead) and colocalized with some of the AtTPX2 foci (arrow). Nuclear signal for importin was reduced (asterisk) in cells 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).

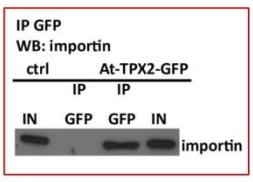


AtTPX2^{OEX} and importin colocalized at microtubular fibres (arrow) and visibly in intranuclear foci (arrowhead). Bar =10 µm.



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.

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.].

Supplement VIII

Proteomic analysis of plant cell nuclei purified by flow sorting

Petrovská B., Jeřábková H., Chamrád I., Vrána J., Lenobel R., Uřinovská J., Šebela M., Doležel J.

In: EMBO/FEBS Lecture Course Nuclear Proteomics, p. 111, Kos Island, Greece, 2014

Proteomic analysis of plant cell nuclei purified by flow sorting

Beáta Petrovská[1], Hana Jeřábková[1], Ivo Chamrád[2], Jan Vrána[1], René Lenobel[2], Jana Uřinovská[2], Marek Šebela[2], Jaroslav Doležel[1]

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Hundreds of eukaryotic proteins are involved in cell nucleus, some with its structural and functional organization, some with gene expression, and some with cell division. However, except for histones and a few other nuclear proteins, only a fraction of these proteins is known in plants. The plant nuclear proteome has not been well explored yet. Biochemical composition of plant sub-cellular components may be altered during their isolation and during subsequent protein purification. Typically, a series of fractionation steps is needed to obtain purified nuclei. However, this is time consuming, laborious and the isolated fractions can be contaminated by cytoplasmic proteins. We have developed an alternative and efficient method for the isolation of plant nuclei, which does not affect their protein content and which comprises only a single step. The new protocol involves flow cytometric sorting –the method which found many important applications in biomedical research. Flow cytometry allows discrimination between G1, S and G2 phase nuclei and their purification, and minimizes the risk of contamination by non-nuclear proteins. Our preliminary results indicate that flow sorting coupled with protein/peptide separation and mass spectrometry will permit a comprehensive characterization of the proteome of plant cell nuclei in various phases of cell division cycle.

This research was supported by grants from the Czech Science Foundation (14-28443S, P501/12/G090), the National Program of Sustainability I (LO1204), the European Social Fund (Operational Program Education for Competitiveness CZ.1.07/2.3.00/20.0165), and Internal Grant Agency of Palacky University, Olomouc (Prf/2013/003).

Proteomic Analysis of Plant Cell Nuclei Purified by Flow Sorting

Beáta Petrovská[1], Hana Jeřábková[1], Ivo Chamrád[2], Jan Vrána[1], René Lenobel[2], Jana Uřinovská[2], Marek Šebela[2], and Jaroslav Doležel[1]

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SUMMARY

Many proteins are involved in maintaining nuclear organization, gene expression and nuclear and cell division. However, except for histones and a few other nuclear proteins, only a fraction of these proteins is known in plants. The **plant nuclear proteome** has not been well explored yet. Biochemical composition of plant sub-cellular components may be altered during their isolation and during subsequent protein purification. The conventional multi-step fractionation procedure is both laborious and liable to contamination. We have developed a single step method based on **flow sorting.** The method allows purification of G1, S and G2 phase nuclei, and minimizes the risk of contamination by non-nuclear proteins. Preliminary results obtained using G1 phase barley root tip cell nuclei indicate that **flow sorting coupled with a protein/peptide separation and mass spectrometry** will permit a comprehensive characterization of the plant nuclear proteome.

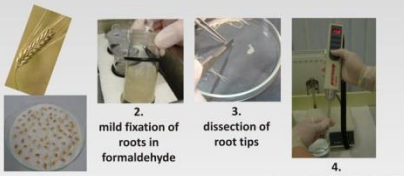
AIM OF THE STUDY

I. Developing a new protocol for purification of plant nuclei:

- More efficient (identification of as yet unknown plant nuclear proteins),
- More sensitive (low abundance proteins),
- Less time consuming (avoiding mechanical homogenization of tissues, filtration, nuclei solubilization, separation on a density gradient),
- Not altering nuclear proteins,
- Avoiding contamination by non-nuclear proteins.

II. Identification of the plant nuclear proteins

1. Sample preparation

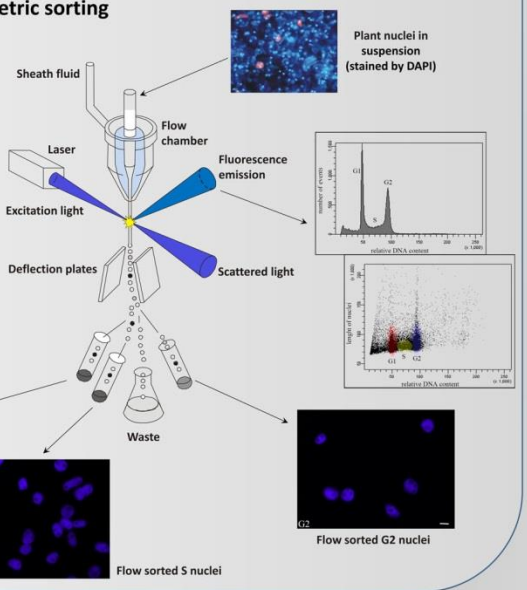


1. barley seedlings
2. mild fixation of roots in formaldehyde
3. dissection of root tips
4. release of nuclei to LB01 buffer by mechanical homogenization

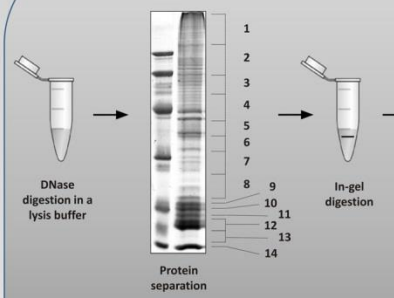
2. Flow cytometric sorting

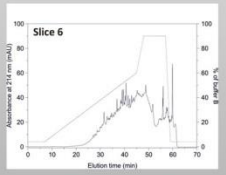
FACSAria SORP

- Provides the opportunity to study nuclear proteome in different phases of cell cycle
- 5 million of sorted nuclei for protein/peptide separation and MS
- Sorting: 200 nuclei/sec >>> 5 million nuclei sorted from 12 samples in ~180 min



3. Proteomic analyses



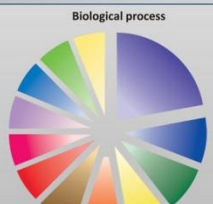


Separation by reversed-phase nanoLC

NanoLC-ESI-MS and MS/MS

NanoLC-MALDI-MS and MS/MS

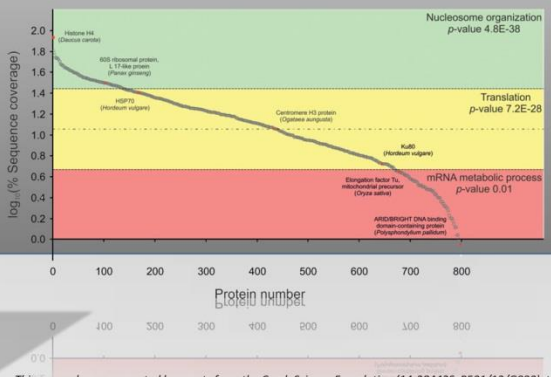
GO



- Translation; *p*-value 1.21E-39
- Ribosome biogenesis; *p*-value 4.01E-25
- Ribonucleoprotein complex biogenesis; *p*-value 1.16E-24
- Cellular macromolecular complex assembly; *p*-value 3.92E-23
- Cellular macromolecular complex subunit organization; *p*-value 6.29E-22
- Chromatin organization; *p*-value 2.72E-20
- Nucleosome assembly; *p*-value 1.07E-29
- Nucleosome organization; *p*-value 1.07E-29
- Chromatin assembly; *p*-value 2.72E-29
- Protein-DNA complex assembly; *p*-value 4.29E-29
- DNA packaging; *p*-value 3.68E-28
- Chromatin assembly or disassembly; *p*-value 1.26E-25

803 distinct proteins identified in G1 phase barley nuclei

GO term enrichment analysis = 99 % nucleus-related !!!



CONCLUSION

- Proteomic analysis is feasible using flow sorted population of plant cell nuclei.
- Coupling FCM, protein/peptide separation and MS provides an elegant and powerful means to determine the composition of nuclear proteome at defined phases of the cell cycle.
- Our approach can be extended further to studying the chromosomal proteome.

This research was supported by grants from the Czech Science Foundation (14-28443S, P501/12/G090), the National Program of Sustainability I (LO1204), the European Social Fund (Operational Program Education for Competitiveness CZ.1.07/2.3.00/20.0165), and Internal Grant Agency of Palacky University, Olomouc (Prf/2013/003).

Supplement IX

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: Olomouc Biotech 2013. Plant Biotechnology: Green for Good II, p. 77, Olomouc, Czech Republic, 2013

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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

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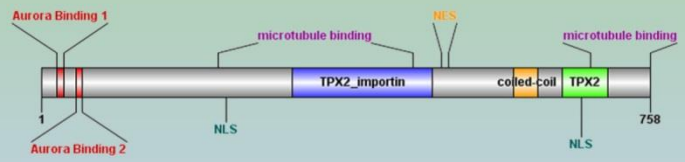
INTRODUCTION: The targeting protein for Xklp2 (TPX2) is a microtubule associated coiled-coil protein with multiple functions in microtubule nucleation and spindle organization. In plant systems, proteins with coiled-coil signature are less understood. Previously we showed that AtAurora1 kinase and AtTPX2 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.

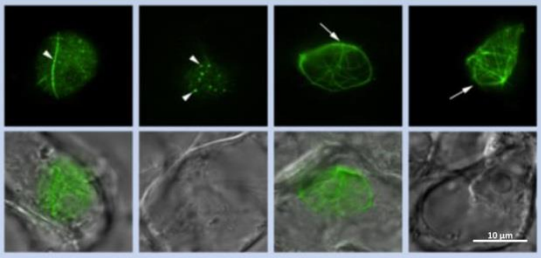
RESULTS:

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



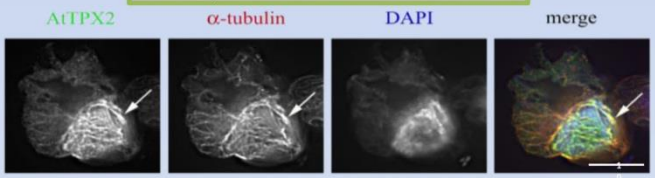
The main features of AtTPX2 protein domains are TPX2_importin and TPX2 domain, two nuclear localisation signals (NLS), nuclear export signal (NES), two Aurora kinase binding sites, coiled-coil domain, microtubule binding domains.

Localization of overproduced AtTPX2-GFP in nuclei



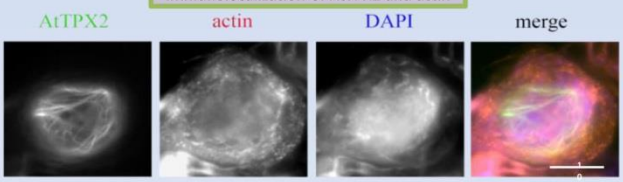
Dots (arrowheads) and fiber-like structures (arrows) decorated with AtTPX2-GFP were formed 72 hours after transformation around and inside the interphase nuclei of cells overexpressing AtTPX2.

α-tubulin positive fibres colocalized with AtTPX2-GFP



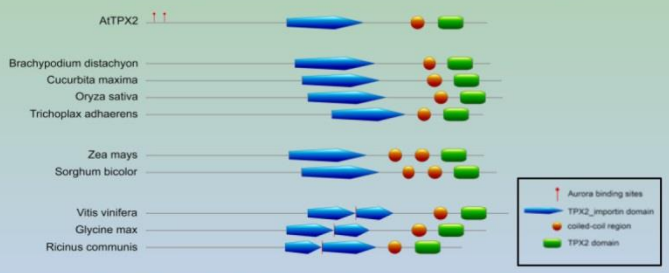
α-tubulin immunolabeling corelated with AtTPX2-GFP signal; thinner AtTPX2 positive fibres were decorated with α-tubulin along the entire length, thicker fibres showed patchy or weak pattern of decoration with α-tubulin (arrows).

Immunolocalization of AtTPX2 and actin



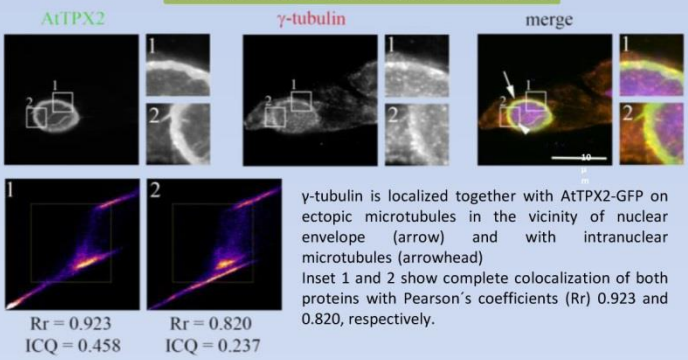
To exclude the localisation with actin fibres, an anti-actin antibody was used. AtTPX2 fibers were not positive for actin immunolabeling.

AtTPX2 protein domain organization and its plant homologs



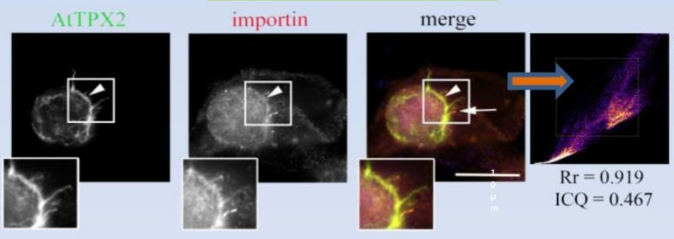
Similarly to the AtTPX2 protein the coiled-coil region together with TPX2_importin and TPX2 motif was found in various plant protein homologs, mostly yet uncharacterised or only hypothetical.

γ-tubulin colocalized with overexpressed AtTPX2-GFP



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.

AtTPX2-GFP colocalized with importin



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.

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.].

Supplement X

Plant Aurora kinases are required for maintaining the level of endoreduplication

Jeřábková H., Petrovská B., Cenklová V., Suchánková P., Binarová P.

In: Bulletin České společnosti experimentální biologie rostlin a Fyziologické sekce Slovenské botanické společnosti (of the Czech Society of Experimental Plant Biology and the Physiological Section of the Slovak Botanical Society), 12, p. 97, ISSN 1213-6670, 2012

Plant Aurora kinases are required for maintaining the level of endoreduplication

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Aurora kinases, a group of conserved serine/threonine kinases, play important role in mitosis and subsequent cytokinesis. In metazoans Aurora kinases A, B, and C are important oncogens overexpressed in many types of cancer cells. Three types of Aurora kinases – AtAurora 1, 2 and 3 have been identified in *Arabidopsis* genome. In our work we down-regulated *Arabidopsis* Aurora kinases using RNA interference (RNAi) method and carried out detailed phenotypic analysis of Aurora RNAi plants. RNAi silenced plants showed strong developmental defects. Arrested primary meristems, ectopic meristem formation, trichomes with supernumerary branches, cells with large nuclei or cells with several nuclei of irregular shape in roots as well as in aboveground part were often observed. DNA content measurement of seedlings with down-regulated Aurora kinases using flow cytometry analysis (FCM) showed a high proportion of cells with 8C and 16C DNA, while 2C and 4C cells remained similar to the controls. Subsequent chromocentres counting confirmed that the increased amount of DNA content was caused by extra replication cycles (endoreduplication). Our results revealed an essential role of plant Aurora kinases in the control of cell division and their requirement for maintaining of meristematic activity and for the switch from meristematic cell proliferation to the differentiation and endoreduplication.

Acknowledgement: This work was supported by grants 204 / 07 / 1169, 204 / 09 / P155 and GD 204 / 09 /H084 from the Grant Agency of the Czech Republic, grants LC06034 and LC545 from the Ministry of Education, Youth and Sports of the Czech Republic, grant IAA500200719 from the Grant Agency of the Czech Academy of the Sciences, IGA UP PrF/2012/001 for H.J., grant No. CZ.1.05 / 2.1.00 / 01.0007 to H.J., B.P., and P.S. from the Centre of the Region Haná for Biotechnological and Agricultural Research.

Plant Aurora kinases are required for maintaining the level of endoreduplication

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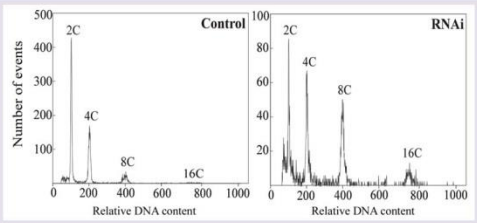
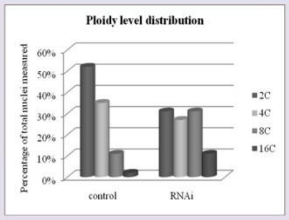
INTRODUCTION: Metazoan Aurora kinases (A, B and C) belong to the major regulators of cell cycle and cytokinesis and are expressed in many types of cancer cells. In *Arabidopsis thaliana* (L.) Heynh. three Aurora kinases (1, 2 and 3) were identified. AtAurora 1 and 2 with high level of homology were localized to the interphase nuclei, during mitosis to the spindle and to the cell plate during cytokinesis. AtAurora 3 engages the role of chromosomal passenger-like protein with its localisation to chromosomes. The initiation of endoreduplication process is crucial during development in *Arabidopsis thaliana* seedlings. RNA interference (RNAi) approach revealed increased levels of endoreduplication and requirement of AtAurora kinases for maintaining meristematic activity and controlling the cell differentiation.

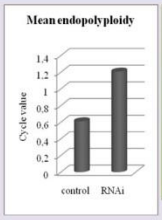
RESULTS:

I. Levels of endoreduplication were higher in RNAi seedlings compared to wild-type.



The DNA content measurement of 14 days-old seedlings using flow cytometry analysis showed a high proportion of cells with 8C and 16C DNA, while 2C and 4C remained similar to the controls.





Ploidy level distribution (in percentage) and mean endopolyploidy (analyses performed according to Barow & Jovtchew) in control and AtAurora RNAi seedlings. Mean edopolyploidy was increased twice.

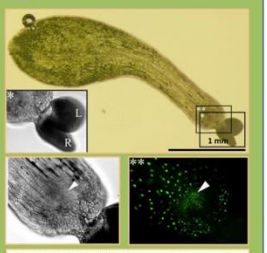
CONSEQUENCE

II. Plant AtAurora kinases are implicated in maintaning meristematic cells.

SHOOT SYSTEM

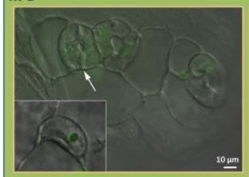
II. A

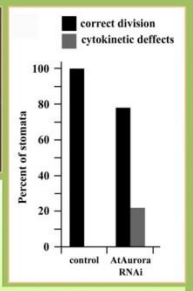
6-d-old RNAi seedlings showed defective development of primary root, detailed in (*), and shoot meristems.



A 6-d-old RNAi seedling with arrested primary root (R) and cotyledon leaf (L) development (*), detailed (**): an ectopic meristem formed from the base of a cotyledon leaf (arrowheads).

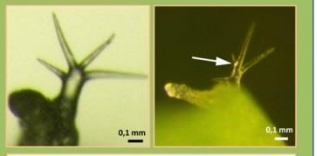
II. B





Effects of silenced AtAurora kinases on stomata development: clustered stomata, single guard cells (inset) and incomplete guard cell division (arrow). Frequencies of stomata with cytokinetic defects in RNAi plants were higher compared to the control (graph).

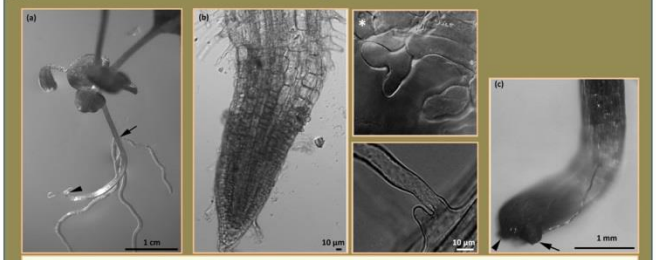
Curled, fragile lea - ves and needle-like leaves (arrow) were often observed in RNAi plants.



Irregular shape of trichome with increased branch points number (arrow) indicates increased level of endoreduplication.

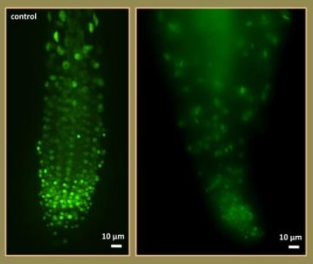
ROOT SYSTEM

II. C

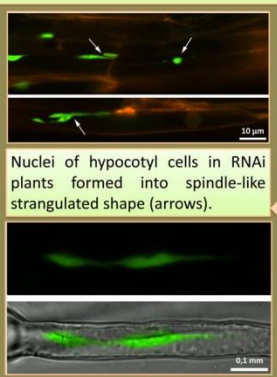


Developmental abnormalies were observed also in roots: arrested primary root meristem (arrowhead) and adventitious roots formed from the central part of the hypocotyl (arrow) (a); swollen root hairs with loss of polarity and root hairs with two growth axes (*) b); the lateral root meristem (arrow) initiated close to the arrested primary meristem (arrowhead) (c).

II. D



Development in meristematic zone was impaired: irregular division of cell files and nuclei in roots of At Auroras down-redulated plants of *Arabidopsis thaliana*.



Nuclei of hypocotyl cells in RNAi plants formed into spindle-like strangulated shape (arrows).

endoreduplicated nuclei

RNAi root hairs with filamentous nuclei.

CONCLUSION: Phenotypic analysis of RNAi plants revealed developmental defects in both root and aerial parts. Defective cell division, arrested primary meristems, ectopic meristems, trichoms with supernumery branches, cells with large nuclei or cells with several nuclei of irregular shape were often observed. Down-regulating Aurora kinases in *Arabidopsis thaliana* plants also resulted in higher levels of endoreduplication. Thus it can be assumed that defects in cell division and primary meristematic proliferation are accompanied by entry into endoreduplication programme.

Results of this study reveal the importance of plant AtAurora kinases in the control of cell division, their requirement for maintaining of meristematic activity and for the switch from meristematic cell proliferation to the differentiation and endoreduplication.

REFERENCES: Petrovská et al. (2012). Plant Aurora kinases play a role in maintenance of primary meristems and control of reduplication. New Phytologist 193: 590–604; Barow, M., and Jovtchew, G. (2007). Endopolyploidy in plants and its analysis by flow cytometry. 349-372. In: Doležel, J., Greilhuber, J., Suda, J. (eds.): Flow Cytometry with Plant Cells., Pp. 454. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, 2007.

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Palacký University Olomouc

Faculty of Science

Department of Botany

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Centre of the Region Haná for Biotechnological

and Agricultural Research

Institute of Experimental Botany ASCR

Analysis of nuclear proteins in plants

Mgr. Hana Jeřábková

P1527 Biology - Botany

Summary of Ph.D. Thesis

Olomouc 2015

This Ph.D. Thesis was carried out within the framework of internal Ph.D. studies at the Department of Botany, Faculty of Science, Palacký University Olomouc and Centre of the Region Haná for Biotechnological and Agricultural Research, Institute of Experimental Botany ASCR during the years 2011-2015.

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The review of the Ph.D. Thesis has been written by Department of Botany, Faculty of Science, Palacký University Olomouc.

The summary of Ph.D. Thesis was sent out on 29th September 2015

The oral defense will take place in front of the Commission for Ph.D. Theses for Study subject Botany in the conference room at the Institute of Experimental Botany, Šlechtitelů 31, Olomouc - Holice, on 21st December 2015

The Ph.D. Thesis is available in the Library of Biological Department of Faculty of Science at the Palacký University Olomouc, Šlechtitelů 27, Olomouc – Holice, and at the Institute of Experimental Botany, Šlechtitelů 31, Olomouc – Holice.

Prof. Ing. Aleš Lebeda, DrSc.
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Contents

1. Introduction

Cell nucleus is one of the distinctive features of eukaryotic cell. Bi-layer nuclear envelope constitutes the interface between cytoplasm and nucleoplasm comprising chromatin, nucleolus, nuclear bodies and other substructures. The complex of nuclear pores imbedded in the envelope mediates the transport of macromolecules and confers the important regulation function.

The nucleus has been the subject of a research for a long time due to the presence of genetic material and apparatus for DNA replication and transcription. The fast progress in sequencing technologies has enabled assembling of complete genome sequences in many species and disclosing of function of genes and non-coding sequences. However, to completely understand the genome function, the knowledge about nuclear proteome is essential. Proteins represent the most abundant molecules in the cell nucleus and provide structural and functional framework for the majority of nuclear processes, whether it is the regulation of gene expression, regulation of the cell cycle and molecular transport, or the structural support for these processes. Although the nuclear proteins perform a large scale of functions, there is a limited knowledge about them. Within plant kingdom, nuclear proteome has been studied in a few model plants and crop plants with known genome sequences. They are represented by model plant *Arabidopsis*, *Xerophyta*, and crop plants such as rice, soybean, hot pepper, chickpea and barley (Bae *et al*., 2003; Khan and Komatsu, 2004; Lee *et al*., 2006; Pandey *et al*., 2006; Casati *et al*., 2008; Li *et al*., 2008; Repetto *et al*., 2008; Choudhary *et al*., 2009; Abdalla *et al*., 2010; Cooper *et al*., 2011; Petrovská *et al*., 2014). Moreover, mapping of plant nuclear proteome focused so far mostly on stress-condition related protein composition (reviewed in Petrovská *et al*., 2015).

Various tools for proteomic analysis have been developed in the past

few decades. Most of them were developed for studying animal proteome and later were adopted for plant proteomics (Anguraj Vadivel, 2015). Contemporary proteomics utilizes high-throughput techniques. High-resolution separation methods (two-dimensional gel electrophoresis) and liquid chromatography provide a material for protein identification with mass spectrometry. Modern mass spectrometers utilize different sources of ionisation and mass analyzers and represent a technique with a high sensitivity, accuracy and resolution (Yates, 2004). Mass spectrometry can be the then employed not only in protein identification, but also protein quantification (Bantscheff *et al.*, 2012).

The previous studies of plant nuclear proteome have used predominantly fractionation methods for nuclei isolation. These are time-consuming, but more importantly there is a high probability of contamination with non-nuclear proteins (Petrovská *et al*., 2014). Other method which can be utilized for nuclei purification is flow cytometry (FCM). FCM enables a high speed analysis of microscopic particles such as intact cell nuclei and mitotic chromosomes and achieving a maximum yield and purity of specimen with a minimal contamination with non-nuclear particles and proteins. This method can provide a material suitable for proteomic analysis. With the implementation of FCM approach before proteomic analysis we are able to obtain a comprehensive data on nuclear proteome in individual phases of the cell cycle. Consequently we can quantify a number of proteins specific for individual phases and perform functional analysis of selected proteins.

Regarding the functional analysis, the aim of this Thesis was to contribute to the characterisation of nuclear protein TPX2 (Targeting Protein for Xklp2), which belongs to the group of microtubule associated proteins. Metazoan TPX2 is a key factor that promotes microtubule nucleation process regulated by RanGTPase (Gruss *et al*., 2001; Kufer *et al*., 2002). After

releasing from the complex with importins α and β in RanGTP-dependent manner, TPX2 activates Aurora kinase A which is followed by microtubule nucleation and spindle assembly. Aurora kinase A has been established as a one of the key regulators of mitosis and cell cycle progression, and being detected in many types of cancer cells, its research earns a big importance (Kufer *et al.*, 2002; Bayliss *et al*., 2003). TPX2 has several functions in relation to Aurora A. Binding to TPX2 increases *in vitro* autophosphorylation activity of Aurora A and, at the same time, prevents Aurora A from dephosphorylation by protein phosphatase1 (PP1) (Eyers and Maller, 2004). TPX2 was also identified as a new scaffolding protein and co-activator of Aurora B in chromosomal passenger complex (Iyer and Tsai, 2012).

In *Arabidopsis thaliana*, there were three Aurora kinase orthologs identified, AtAurora1, 2 and 3. These phosphorylate serine 10 in histone H3 *in vitro*. AtAurora kinases share similar localisation as their animal orthologs. AtAurora1 and 2 localize to the nuclei during interphase and to the mitotic spindles during cell division (Kawabe *et al.*, 2005). AtAurora3 localizes with chromosomes, which seems to indicate its chromosomal-passenger-like role similar to animal Aurora B (Kawabe *et al*., 2005; Demidov *et al*., 2005). Plant Aurora kinases are designated as one of the main coordinators of mitosis and also regulation factors for acentrosomal microtubule nucleation at dispersed sites within nuclei and on membrane.

Plant TPX2 shares localisation patterns similar to its animal counterparts. It is accumulated in the cell nuclei during interphase and actively transported out of the nuclei before the nuclear envelope breakdown. During mitosis, it localises with the microtubular arrays from prophase to early telophase and is degraded at the end of mitosis (Vos *et al.*, 2008). The role of *Arabidopsis* TPX2 in spindle assembly and mitosis promotion in plant cells was first proposed by Vos *et al*. (2008). AtTPX2 association with importin-α in

Xenopus egg extracts (Vos *et al*., 2008) and its ability to promote spindle assembly *in vitro* indicates that also plant TPX2 is involved in RanGTP pathway-regulated microtubule nucleation in plant cells. Moreover, the observed moderately elevated concentration of AtTPX2 at the nuclear envelope during interphase may point to the activity of AtTPX2 in MT nucleation at this area.

The work of Petrovská *et al*. (2012) contributed to the elucidation of the AtAurora kinase-AtTPX2 association. Based on colocalisation data, it was suggested that AtTPX2 may guide AtAurora kinase1 to microtubules and contribute to spatio-temporal modulation of kinase function. Also the presence of Aurora1, its regulator AtTPX2 and γ-tubulin on microtubules suggested the linkage towards γ-tubulin mediated MT formation in acentrosomal plant cells (Petrovská *et al*., 2012).

The nuclear TPX2 protein has an indispensable role in both animal and plant cells. Its mitotic function has been rather well studied, albeit predominantly in animal cells. Nevertheless, what is the function of TPX2 in plant interphase nucleus is still not fully elucidated. Manifestations of overexpressed TPX2 in *Arabidopsis* interphase cells and its interplay with plant Aurora kinases are examined in this Thesis. It can bring the further insight into the mechanism of microtubule assembly and regulation of mitotic processes in plant cells.

2. Aims

This Thesis aimed at contributing to the characterisation of so far poorly explored plant nuclear proteomes. It provides a general overview of the organisation of plant cell nuclei and their protein composition, plant nuclear proteome research and fundamental methods of proteomics. The experimental part of the Thesis covered the two complementary research topics. The first of them focused on development and application of a new approach to characterisation of plant nuclear proteome, which is based on nuclei purification by flow sorting and protein identification using soft ionisation MS techniques. The second part of the Thesis focused on functional analysis of plant nuclear proteins, namely on AtTPX2 protein and its relationship with plant Aurora kinase 1 and 3.

Particular aims of the Thesis were:

1) Development of an alternative strategy for purification of plant cell nuclei for proteomic analysis addressing the efficiency, sensitivity and speed, while paying particular attention to maintaining protein integrity and avoiding contamination by other cellular components.
2) Sub-proteomic analysis of barley cell nuclei using the state of the art proteomic approaches.
3) Functional characterisation of plant nuclear protein TPX2.

3. Results

3.1. Proteomic analysis of barley cell nuclei

For the characterisation of barley (*Hordeum vulgare* L. cv. Morex) nuclear proteome, a novel approach for nuclei purification was developed. Method based on flow cytometric sorting enabled isolation of nuclei in G1, S and G2 phases of the cell cycle with the negligible contamination by non-nuclear elements and proteins. Subsequent proteomic analysis of intact nuclei in G1, S and G2 phases was performed simultaneously on mass spectrometers using different ionization techniques, ESI and MALDI. Altogether 803 distinct proteins were identified in G1 phase. Analogously, G2 phase nuclei analysis resulted in 2003 identified proteins (unpublished data). An overlap between ESI and MALDI made 147 proteins in G1 and 113 proteins in G2, which points to the uniqueness of each MS technique. The highest proportion of G1 proteins belonged to the class of translation and ribosome and ribonucleoprotein complex biogenesis. In contrast, functions of G2 nuclear proteins were predominantly linked to chromatin assembly, nucleosome assembly and organisation, and DNA packaging (data not published). These results are in accordance with the processes that are known from the respective cell cycle phase.

3.2. Functional characterisation of nuclear protein TPX2 in *Arabidopsis thaliana*

Within the functional characterisation of plant TPX2 protein, the effect of AtTPX2 overexpression in the interphase suspension cells of *Arabidopsis thaliana* was examined. AtTPX2 overexpression resulted first in formation of nuclear dots and patches that were gradually assembled into fibrillar structures in and around cell nuclei. AtTPX2 decorated structures represented

microtubule fibres and were resistant to drug-induced depolymerisation. AtTPX2 induced microtubule bundling was neither a manifestation of apoptosis nor related to the onset of mitosis and AtAurora1 association. Immunofluorescence analysis confirmed co-localization of AtTPX2 with Ran and importin, respectively, and showed a decrease of Ran and importin signal after assembly of microtubule bundles. The co-immunoprecipitation experiment also proved the interaction of AtTPX2 with importin and Ran respectively. Observed AtTPX2 colocalisation with γ-tubulin may point to the role of AtTPX2 in the mechanism of microtubule assembly from MTOCs comprising γ-tubulin, nevertheless any interaction of AtTPX2 and γ-tubulin couldn't be demonstrated.

3.3. AtTPX2 *in vitro* regulation of Aurora kinase family members

The sequence similarity between human and plants' homologs of TPX2 enabled testing whether AtTPX2 and plant Aurora kinases share the similar regulatory patterns as their human counterparts. *In vitro* phosphorylation of AtTPX2 by AtAurora1 and 3 kinases showed that only AtAurora1 and not AtAurora3 (analog of mammalian Aurora B) reproducibly phosphorylates AtTPX2. Examination of the autophosphorylation activity of AtAurora1 after AtTPX2 binding revealed that only AtTPX2 including Aurora binding domain increased autophosphorylation activity of AtAurora1. Co-activation of AtAurora1 by AtTPX2 then enhanced the activity of the kinase towards one of its most important downstream target, histone H3.

4. Conclusions

This Thesis represents a contribution to the exploration of the plant nuclear proteome. It outlines the current methods for analysis of the plant nuclear proteome and provides an overview of constitutional proteins of particular plant nuclear structures. In the experimental part, the protocol for proteomic analysis of barley cell nuclei was developed. The innovative approach was based on flow cytometric sorting of nuclei in different cell cycle phases. Using flow cytometry for purification of nuclei, a maximum yield and purity of nuclei with a negligible contamination with non-nuclear proteins was achieved. Proteome of G1 and G2 phases nuclei was analysed utilizing two diverse mass spectrometry techniques, MALDI-TOF/TOF and ESI-Q/TOF, in order to maximize the number of identified proteins. Relatively low proportion of identical proteins identified in each type of spectrometer points to the uniqueness of each spectrometry technique. Thus we can conclude that utilization of both methods brings us the coverage of wide spectrum of identified proteins and becomes therefore a preferred strategy.

Next, this Thesis contributed to the description of nuclear microtubule-associated protein TPX2 and proposing its nuclear functions. *Arabidopsis* TPX2 localizes to the nuclei during interphase and to the microtubules during mitosis. It plays a role in mitotic spindle assembly process with the involvement of RanGTPase pathway. The patterns of microtubule assembly were examined in the interphase cells with overexpressed AtTPX2. Signal of overexpressed AtTPX2 in the interphase cells was initially observed in the form of dots and patches from which microtubules could grow and organise. Later, the arrays of intranuclear and perinuclear AtTPX2-decorated thick fibrils were formed, extending out to the cell periphery. After formation of microtubular arrays, the nuclear signal of importin was strongly reduced, which

may suggest the involvement of RanGTPase pathway in microtubule formation triggered by AtTPX2. It is also likely that overexpressed AtTPX2 may amplify the ability of nuclear envelope and chromatin to promote microtubule nucleation, which is typical for acentrosomal cells. The evidence of interaction of AtTPX2 and importin and Ran, respectively, indicates the involvement of Ran pathway in the process of microtubule assembly in the cell nuclei with overexpressed AtTPX2. AtTPX2 was further characterized as the regulator of AtAurora kinase1. Next to the targeting of the kinase to the mitotic spindle, AtTPX2 was shown to increase the autophosphorylation activity of AtAurora1 towards histone H3. We can thus consider AtTPX2 a possible element in translation of RanGTP signalling to phosphorylation cascade performed by Aurora kinases. AtTPX2 can be involved not only in microtubule nucleation but also in regulation of mitosis and cell cycle progression.

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8. Souhrn (Summary, in Czech)

Buněčné jádro je jedním z charakteristických organel eukaryotické buňky. Dvouvrstvá jaderná membrána představuje rozhraní mezi cytoplazmou a nukleoplasmou zahrnující chromatin, jadérko, jaderná tělíska a další struktury. Je protkána komplexem jaderných pórů a je tedy hlavním regulátorem importu a exportu molekul. Jádro bylo dlouhou dobu předmětem výzkumu především díky přítomnosti genetického materiálu a jeho replikačního a transkripčního aparátu. Rychlý vývoj sekvenačních technologií umožnil sestavovat úplné sekvence genomů u mnoha eukaryot a odhalovat funkci genů a nekódujících sekvencí. Pro úplné porozumění funkce genomu je však nutné získat znalosti o jaderném proteomu. Proteiny představují nejpočetnější molekuly v buněčném jádře a poskytují strukturní a funkční rámec pro většinu procesů odehrávajících se v jádře, ať už jde o regulaci genové exprese, buněčného cyklu a transportu molekul, nebo o strukturní podporu pro tyto děje. Ačkoli jaderné proteiny plní celou řadu důležitých funkcí, jejich znalost zůstává velmi omezená. U rostlin byl proteom buněčného jádra studován jen u několika modelových rostlin se známými genomovými sekvencemi a u několika hospodářsky významných plodin, u kterých byl sledován vliv stresových faktorů na změny jaderného proteomu.

Cílem předkládané práce je studium jaderných proteinů rostlin a přispění k odhalení vztahů mezi strukturou a funkcí jádra rostlinné buňky. Záměrem je získat ucelený přehled o složení jaderného proteomu v jednotlivých fázích buněčného cyklu a charakterizovat funkci vybraných proteinů. Proteomická analýza byla provedena na jádrech kořenových špiček ječmene (*Hordeum vulgare* L. cv. Morex). Přesná identifikace proteinů pomocí hmotnostní spektrometrie vyžaduje precizní přípravu materiálu. Dřívější práce využívaly pro izolaci jader převážně frakcionační metody. Ty však s sebou

nesou vysoké riziko kontaminace proteiny cytoplasmy a jsou časově náročné. Pro izolaci jaderných proteinů ječmene byl zde využit nový postup založený na třídění pomocí průtokové cytometrie. Průtoková cytometrie umožňuje poměrně rychlou analýzu mikroskopických částic, jako jsou buněčná jádra a mitotické chromozomy. Získané výsledky potvrdily vhodnost nového postupu, který umožňuje přípravu vzorků s minimální kontaminací nejadernými proteiny. S cílem identifikovat maximální počet proteinů byly použity dvě techniky hmotnostní spektrometrie, MALDI-TOF/TOF a ESI-Q/TOF a ukázala se jedinečnost každého typu spektrometru pro identifikaci určitých proteinů. Využití obou metod tak umožňuje pokrytí širokého spektra identifikovaných proteinů.

Funkční analýza byla zaměřena na charakterizaci jaderného proteinu TPX2 (Targeting Protein for Xklp2), který náleží do skupiny proteinů asociovaných s mikrotubuly. V živočišných buňkách působí TPX2 jako jeden z organizačních elementů mikrotubulů v oblasti centrozómu i chromozómů. Protein TPX2 po uvolnění z komplexu s importiny α a β aktivuje Aurora kinázu A, což je významný regulátor mitózy. Následně dochází k nukleaci mikrotubulů a tvorbě dělícího vřeténka. Bylo zjištěno, že TPX2 je asociovaný s kinázou Aurora1 také v rostlinných buňkách a že může působit jako regulátor kinázy Aurora1, která se podílí na organizaci mitotického vřeténka. K dalšímu objasnění funkce TPX2 byly analyzovány buňky *Arabidopsis thaliana* s nadprodukovaným TPX2. Nadprodukce vedla ke vzniku ektopických mikrotubulárních vláken v blízkosti jaderného obalu a uvnitř jádra. Toto uspořádání nebylo specifické pro přechod do mitózy a vyskytovalo se nezávisle na přítomnosti Aurora1 kinázy. Nebyla také prokázána spojitost mezi vytvářením mikrotubulárních svazků vlivem TPX2 a apoptózou, což odlišuje rostlinné buňky od buněk živočišných. Důležitým poznatkem je potvrzení asociace/interakce rostlinného homologu TPX2 s importinem a účasti Ran-

GTPasové dráhy při formování mikrotubulů u acentrosomálních rostlinných buněk.

Experimenty zameřené na regulační vztahy AtTPX2 a AtAurora kinázy 1 a 3 potvrdily AtTPX2 jako substrát, ale rovněž i aktivátor pouze u kinázy Aurora1. Aktivace kinázy Aurora1 pomocí AtTPX2 vedla ke zvýšené fosforylaci cílových proteinů, zvláště histonu H3. Odlišná regulace obou Aurora kináz může hrát významnou roli v regulaci procesů mitózy a buněčného cyklu.

Ve svém souhrnu znamená předkládaná disertační práce přispění k identifikaci jaderných proteinů rostlin a odhalování jejich funkce a vytváří předpoklady pro další intenzivní studium v této oblasti.