Functional divergence of TPX2 family members in *Arabidopsis thaliana*

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Research article

Keywords: *Arabidopsis thaliana*, Targeting protein for Xklp2, Aurora kinase 1, phylogeny, kinase assay, in vivo co-localization

Posted Date: July 29th, 2019

DOI: <https://doi.org/10.21203/rs.2.12059/v1>

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Abstract

Background TPX2 (Targeting protein for Xklp2) is an evolutionary conserved microtubule-associated protein important for microtubule nucleation and proper mitotic spindle assembly. The protein was also described as an activator of the mitotic kinase Aurora A in humans and the Arabidopsis AURORA 1 (AUR1) kinase. In contrast to animal genomes that encode only one TPX2 gene in their genome, higher plants genomes encode a family with several TPX2-LIKE gene members (TPXL). Results Arabidopsis genome comprise of 21 TPXL genes. To better understand the functional divergence within the TPXL gene family in plants, we characterized eight genes most closely related to the canonical TPX2 Arabidopsis. TPXL genes of Arabidopsis can be divided into two groups. Group A proteins (TPXL2, 3, 4 and 8) contain Aurora binding and TPX2_importin domains, while group B proteins (TPXL1, 5, 6 and 7) harbor an Xklp2 domain. Canonical Arabidopsis TPX2 contains all above mentioned domains. We confirmed using in vitro kinase assays that the group A proteins contain a functional Aurora kinase binding domain and are able to at least double the activity of recombinant AUR1 kinase. Transient expression of GFP-tagged Arabidopsis TPX2-like proteins in *Nicotiana benthamiana* revealed preferential localization to microtubules and nuclei, except TPXL7, which localized mainly to the nuclear envelope. Co-expression of AUR1 together with TPX2-like proteins changed the localization of AUR1, indicating that these proteins serve as targeting factors for Aurora kinases. Conclusion Taken together, we visualize the various localizations of the TPX2-LIKE family in Arabidopsis as proxy to their functional divergence and provide evidence of their role in the targeted regulation of AUR1 kinase activity.

Background

Cell cycle progression and timing of events are crucial for cell division and viability. To ensure equal chromosome segregation during mitosis, the interphase microtubule network represented by the cortical microtubules, undergoes a dramatic reorganization and reassembles into a functional mitotic spindle. The spatial and temporal coordination of events is therefore critical. In most cases, such regulation is carried out by post-translational control, such as phosphorylation and dephosphorylation of regulatory proteins. Aurora kinase family of serine/threonine protein kinases are known as very important mitotic kinases evolutionary conserved among eukaryotes (1,2).

The Aurora kinase family of *Arabidopsis thaliana* contains three members that are subdivided into two groups: α (*AURORA1* and *AURORA2*) and β (*AURORA3*). α -Aurora kinases are localized at spindle microtubules during mitosis and cell division plate in cytokinesis, while β -Aurora is localized at centromeres during metaphase (3–5). Interestingly, in human cells, the substitution of just one amino acid, close to the catalytic domain of Aurora A kinase, can functionally convert Aurora A into a B-like Aurora kinase (6,7).

Downregulation of Aurora kinases in Arabidopsis results in mitotic cell division defects and interferes with the meristem development (8). *aurora1/aurora2* double mutant plants display severe defects in formative cell divisions during lateral root formation (5). Furthermore, *aurora1/aurora3* double mutant

plants suffer from aberrant meiosis with the formation of micronuclei, unequal separation of chromosomes and defects in tetrad formation. These defects indicate the importance of Aurora kinases in meiotic cytoskeleton dynamics (9). Plant α -Auroras have also been shown to affect the microtubule binding properties of MICROTUBULE-ASSOCIATED PROTEIN 65–1 (MAP65–1). Phosphorylation control residues in the C-terminal part of MAP65–1 was shown to be important for the efficient cell cycle progression (10,11).

The TARGETING PROTEIN FOR XKLP2 (TPX2), is required to prevent inactivation of human Aurora A (6). TPX2 was first described as a microtubule-associated protein involved in chromosome-dependent spindle assembly of human cells (12). During mitosis, TPX2 is released from importin α/β heterodimers due to the high concentration of Ran-GTP at the vicinity of chromosomes. Free TPX2 subsequently activates Aurora kinase A, thus stimulating mitotic spindle assembly in human cells (13). Homologues of *TPX2* were found in different species, including plants. Similar to the human TPX2, Arabidopsis canonical TPX2 contains three conserved domains—the Aurora binding domain responsible for binding and activation of Aurora kinase, the TPX2_importin domain involved in transfer of protein to the cell nuclei and the TPX2_Xklp2 motif for binding to microtubules. Further, an additional microtubule-binding motif is present at the N-terminal part of plant TPX2 protein (14). Arabidopsis TPX2 was described as an activator of Aurora1 *in vitro* (15). *In planta*, Aurora1 colocalizes with TPX2 at spindle microtubules throughout mitosis and co-precipitates with TPX2 on microtubules in a cell cycle specific manner (16).

According to Evrard *et al.*, the N-terminal Aurora binding domain, TPX2_importin and C-terminal microtubule binding domain and TPX2_Xklp2 are the conserved domains that characterize true plant *TPX2* orthologues. The authors proposed that other proteins containing only some of the functional blocks may be considered as TPX2-related proteins (17). In contrast to analyzed genomes of animals, where only one *TPX2* gene was found, Arabidopsis encodes 20 genes with sequence homology to some of the domains of canonical TPX2. The first members of the *TPX2-LIKE PROTEIN (TPXL)* gene family in Arabidopsis, *TPXL2* and *TPXL3*, were functionally characterized only recently (18). In plants, outside Arabidopsis, a *TPXL* gene family containing 12 members was described in *Eucalyptus grandis* (19). However, the authors focused only on the presence of the TPX2_Xklp2 domain and did not take into consideration proteins with a TPX2_importin domain which is equally important for canonical TPX2 functions.

Here, we revise the phylogeny of the *TPXL* gene family. We identified a group of TPXL proteins with predicted Aurora kinase binding domain. *In vitro* kinase assays demonstrated that the Aurora binding domains of Arabidopsis TPXL homologs are able to activate recombinant AUR1. The TPXL members are characterized by different expression patterns and localization, suggesting diversification of *TPXL* genes for specific functions during plant development.

Results

The A. thaliana genome possesses 20 genes with similarity to TPX2

The EggNOG 4.5 database (20) was searched to discover proteins that contain typical TPX2- domains in Arabidopsis. In total, 20 proteins were identified, which can be classified into two groups based on their domain composition (Figure 1A, Additional File 1, 3). Group A comprises members with the TPX2_importin domain, while group B members contain the TPX2_Xklp2 domain.

We performed a maximum likelihood phylogenetic analysis with the identified proteins. In agreement with the domain composition, the phylogeny (Figure 1A; Additional File 3) revealed two major groups, A and B. Group A could be further divided into two clusters based on the absence (cluster I) or presence (cluster II) of a plant-specific KLEEK-motif. The KLEEK-motif is present in an already characterized group of WAVE-DAMPENED (WVD) and WAVE-DAMPENED-LIKE (WDL) proteins (21,22) and is typical for microtubule binding proteins (23) (Additional File 3).

The deepest clades (Additional File 2) comprised Arabidopsis orthologues of TPXL1, TPXL5, TPXL6 and TPXL7 (clades 1, 2 and 3 in (19)). It should be noted that TPXL7 formed a separate clade with only protein sequence from *A. thaliana*. Subsequently derived clade was composed of canonical TPX2 and TPXL2, TPXL3 and TPXL8. The canonical TPX2 clade (clade 3 after (19)) was subdivided into 5 groups representing metazoa, angiosperms, mosses, algae and fungi. Analyzed representatives of metazoa, algae and fungi contain a single copy of the canonical TPX2 (Additional File 3).

The group of plant-specific proteins with a KLEEK domain (WDLs) contains three major lineages. The first lineage consists of WDLs 5, 7, 9 and TPXL proteins. The second lineage contains WDLs 1, 2 and 3. Finally, the third lineage embraced WDLs 4, 5 and 6. Special attention should be paid to the moss *Physcomitrella patens*, which evolved a unique WDL paralog group (Additional File 3). Interestingly, the Aurora binding domain containing TPXL4 was grouped together with the WDL lineage (Additional File 3).

Each clade of plant TPX2 and TPXL proteins was divided into two subclades containing monocots and eudicots (Additional File 3), thus, there seems to be not a specific paralog for any of these groups.

Functional prediction of Arabidopsis Aurora binding domain

Activation of Aurora A kinases is a significant function of TPX2 proteins. Binding of TPX2 to human Aurora A activates the phosphorylation activity of the kinase and protects it from dephosphorylation by protein phosphatase 2A (6). Similarly, Aurora kinase binding domain of canonical Arabidopsis TPX2 is able to activate Aurora1 *in vitro* (15). Recently we showed that Aurora kinase binding domains of TPXL2 and TPXL3 also are able to activate Aurora1 *in vitro* (18). To characterize the Aurora binding domain of plant group A TPXL proteins, a multiple sequence alignment was performed with orthologues of Arabidopsis TPX2. Additionally to TPX2 the Arabidopsis genome contains four genes, *TPXL2*, *TPXL3*, *TPXL4* and *TPXL8* with both TPX2_importin and putative Aurora kinase binding domains (Figure 1).

The overall sequence conservation is very poor between human TPX2 and plant TPX2-like proteins, but the key residues important for binding of Aurora kinases are evolutionary conserved (Figure 2A, 2B). The Aurora binding domain of human TPX2 contains upstream and downstream helical stretches (6). Despite

some amino acid substitutions in the upstream helical stretch of TPXL4/TPXL8 or TPXL2/TPXL3 compared to human TPX2, the hydrophobic side chain involved in stacking interactions is preserved (Figure 2A, aminoacid 8). Similarly, the amino acid residues important for Aurora kinase activation (Figure 2A, aminoacids 34, 35) (6) are conserved, further indicating the functional conservation of the TPX2/Aurora kinase complex. Importantly, MEME (Multiple Em for Motif Elicitation; (24)) analyses confirmed the presence of a similar Aurora binding domain with key conserved residues in 45 proteins from different plants species (Figure 2B, Additional File 4).

All Arabidopsis TPX2 family members possessing an Aurora kinase binding domain activate AUR1 in vitro

The Aurora kinase binding domains of canonical Arabidopsis TPX2, TPXL2 and TPXL3 were shown to activate Aurora1 *in vitro* and increase its phosphorylation activity towards histone H3 as a physiological substrate (15,18). To address the functionality of the Aurora kinase binding domains of Arabidopsis TPXL proteins, we performed *in vitro* kinase assays. The Aurora binding domains of TPXL2, TPXL3, TPXL4 and TPXL8 were expressed in *E. coli*, purified (Additional File 5A) and combined with recombinant AUR1 as enzyme and histone H3 as a substrate. An increase in histone H3 phosphorylation detected by incorporation of radioactive isotope P³² into histone H3 was used as a means to measure the activity of AUR1 (26). The *in vitro* kinase assay showed that Aurora binding domains of all TPXL proteins are able to activate AUR1 kinase (Figure 2C, Additional File 5B). TPXL3 has the highest activation potential with up to 5-fold increase compared to Aurora1 kinase alone, which was significantly higher than AUR1 activation by canonical TPX2 (Figure 2C).

The eight closest homologues of canonical TPX2 are differentially expressed during Arabidopsis development

In order to investigate the expression of *TPXL* genes, we analyzed publicly available RNA sequencing data from different developmental stages of Arabidopsis (25). To profile gene expression patterns of selected *TPXL* genes, we analyzed the expression across all developmental stages (Figure 3, Additional File 5). These heat maps illustrate distinct gene expression of *TPXLs* during development. *TPXL2*, *TPXL3*, *TPXL5* and *TPX2* seem to be the most widely expressed *TPXLs*. In general, expression of *TPX2* was among the highest in all tissues. Strikingly, *TPXL4* was considered as pseudogene (17); however, transcriptome analyses showed specific expression of *TPXL4* in mature anthers. *TPXL6* expression was restricted to siliques and *TPXL7* was only detected in dry seeds. Taken together, our data confirmed the validity of our hypothesis that *TPXL* genes might have evolved different functions during plant development. Similar expression patterns for *TPX2*, *TPXL2*, *TPXL3* and *TPXL5* point to possible functional redundancy (18). Moreover, these four proteins are expressed in a similar pattern to *Aurora1* and *Aurora2* in agreement with the fact that they are physiological activators of the kinases.

TPXL proteins mostly localize on microtubules

The canonical TPX2 was shown to localize on microtubular arrays and the TPX2_Xklp2 domain is involved in microtubule binding (14,17). TPXL proteins of group A (Figure 1) contain a TPX2_importin domain important to interact with alpha importin (26) and an Aurora binding domain. Group B proteins contain a TPX2_Xklp2 domain with a kinesin-targeting signature. To uncover the localization patterns of the selected TPXL proteins, constructs to express fluorescently-labelled translational fusions were infiltrated into *N. benthamiana* leaves and visualized by confocal microscopy. Consistent with the presence of a TPX2_importin and an Aurora kinase binding domain, group A TPXL proteins showed strong microtubular labelling at the nuclei (Figure 4A). Moreover, the proteins also faintly labelled cortical microtubules (Figure 4A). It should be noted that two members group A TPXL2 and TPXL3 labelled microtubular fibers decorating the nuclear envelope like canonical TPX2 (Figure 4A). Group B TPXL proteins mainly localized with cortical microtubules, TPXL1, TPXL5 and TPXL6 showed very strong labelling resembling cytoskeletal filaments (Figure 4B). TPXL5 also labelled microtubules close to the nucleus (Figure 4B). Interestingly, TPXL7 showed a different localization pattern compared to all other TPXL proteins. TPXL7 lacks microtubular localization and mainly localized in the vicinity of the nuclear membrane (Figure 4B). The canonical TPX2 decorates cytoskeletal cables and bundles of microtubules around nuclei (Figure 4C). These results indicate that TPXL gene family has probably evolved differential targeting and different functions in the regulation of microtubule cytoskeletal dynamics.

TPXL proteins re-localize Aurora1 kinase by loading it on microtubular arrays

During interphase, AUR1 kinase is localized in in very low amounts in the nucleus, while the microtubular localization of AUR1 is a hallmark of cell division (5,8). To check whether TPXL proteins co-localize with AUR1, DNA constructs of fluorescently-labelled fusion variants of AUR1 and TPXL were co-infiltrated into *N. benthamiana* leaves. Normally, AUR1 shows diffuse nuclear and weak cytoplasmic labelling in infiltrated *N. benthamiana* epidermal cells (Figure 4D). Interestingly, after co-infiltration with TPX2 construct, AUR1-GFP is mostly localized on cortical microtubules (Figure 4G). Co-expression of TPXL proteins also re-localized AUR1. TPX2, TPXL1, TPXL2, TPXL3, TPXL4, TPXL6 and TPXL8 re-localize AUR to the nucleus as well as to cortical microtubules, while TPXL4 and TPXL5 relocalize it to microtubules (Figure 4E, 4F, 4G). Interestingly, co-expression of TPXL proteins with AUR1 not only changed the localization of the kinase, but also re-localized some TPXL proteins. The most striking redistribution was observed for TPXL7, which shared a strong nuclear localization with AUR1. These results indicate that co-localization of TPXL proteins with AUR1 is not only dependent on the presence of a functional Aurora binding domain, but other mechanisms must exist to regulate these proteins.

Discussion

Diversity of TPX in plants

TPX2 is a widely conserved microtubule-associated protein required for mitotic spindle assembly and function (12,14) although recent findings also show its involvement in DNA damage response (27,28). Functions of TPX2 are well characterized in animals including human; however, the knowledge from plant

systems is sparse. Moreover, unlike animals which contain a single TPX2 gene, plants contain a family of twenty TPX2-related proteins. In this work, we performed phylogenetic analyses of the whole group of Arabidopsis TPXL proteins. The canonical TPX2 contains an Aurora binding domain, a TPX2_importin and TPX2_Xklp2 domains. Only the presence of these three domains defines the *bona fide* homologs of TPX2 (17). In plants, TPXL proteins have been also identified in *Eucalyptus grandii* (19); however, the authors did not take into account the importance of the TPX2_importin domain and therefore missed the entire group A. Consequently, we show the presence of 16 proteins with a TPX2_Xklp2 domain and 4 proteins with TPX2_importin and Aurora binding domains in the *A. thaliana* genome. Strikingly, the all proteins containing the TPX2_importin domain also contain the Aurora binding domain.

In agreement with the predicted domain composition, group A TPXL proteins combine an Aurora binding domain and also a TPX2_importin domain. The functional relevance of the lack of the TPX2_Xklp2 domain compared to TPX2_importin remains to be determined. Group B proteins with TPX2_Xklp2 were all clustered together in a separate clade. Despite the presence of an Aurora binding domain, TPXL4 clustered together with the plant-specific lineage of WVD proteins containing the KLEEK domain and seems to be an ancestor of the WDL clade. Further sequence analyses did not reveal the presence of a KLEEK motif in TPXL4. This may cause a slight difference of TPXL4 function and therefore explain different localization of TPXL4 compared to other members of group A TPXLs. Possible explanations for the diversification of TPXL gene family in plants could be differences in organization of microtubule-organizing center in animals and plants. Some of the diversity also come from whole genome duplications as suggested by the similar exon-intron structure of some of the TPXL genes (Additional File 8). Also it should be noted that compared to animals, plants usually encode two genes of α -Aurora kinases (5). Although we do not have any data about specificity of complex formation between different α -Auroras and TPXL proteins during development we have already shown interaction of AUR1 and AUR2 with TPXL2 and TPXL3 (18). On the other hand, unlike animals, plants show a high level of endopolyploidization, which is highly specific for different tissues and different stages of development. Since activation of α -Aurora kinases and regulation of spindle assembly play a key role in endopolyploidization this can explain high number of TPXL genes in plant genome. Furthermore, the complex TPX2 gene family could represent a land plant adaptation strategy of the spindle assembly and positioning (29).

In addition to the canonical TPX2, we have described four additional Aurora binding domain-containing proteins. Until now, the canonical TPX2 of Arabidopsis was considered to consist of two adjacent Aurora kinase binding domains (14). However, crystallographic analyses of N-terminal part of human TPX2 with Aurora A catalytic domain showed the presence of two helical stretches separated with a short linker that are responsible for the interaction (6). Indeed, we were able to identify those two stretches in the Aurora binding domain of plant TPXLs. Although the overall plant sequences of the Aurora binding domain are highly divergent from the consensus sequence, residues important for binding and activation of the Aurora kinase in plant TPXL are highly conserved. Interestingly, the Aurora kinase binding domains of plants TPX2/TPXL, like in the case of animal TPX2 also localize at the N-terminus. Whether this is because of the sterical reasons or functional diversification remains to be determined.

Based on domain analysis of TPXL proteins, we observe a possible specialization of TPXL. Group A, without the kinesin domain (18) might be implicated in regulation of AUR1 and group B in regulation of kinesins in Arabidopsis. It seems that during the evolution plants divided TPXL members depending on the tissue specificity and developmental stage. Importantly, Aurora binding domain is always present together with TPX2_importin binding domain. This allows importin to bind TPXs of group A and prevent activation of α -Aurora kinases (14,26). This raises an additional level of regulation of α -Aurora kinases. The presence of a larger number of kinesin genes in plant genomes, in comparison to animals or human (30,31) can explain the diversity of TPXL isoforms in B group. At the same time, the reason for such evolutionary separation of the TPXL family based on functionality remains unclear.

Activation of Aurora kinase by TPX2 family proteins seems to be evolutionary conserved

Aurora kinases are known to phosphorylate various targets. However, in plants only histone H3 (32), MAP65-1 (10), microtubule-associated proteins TPX2 (15), TPXL2 and TPXL3 (18) were confirmed as targets of Aurora1 kinase. Additionally, a number of transcription factors were shown to be phosphorylated by both AUR1 and AUR3 kinases (33). Several of these transcription factors are closely related to the regulation of developmental processes. It is tempting to speculate that phenotypical similarities of both AUR1 and TPX2 mutants (5,8,16) are partially dependent on the activation of the AUR1 kinase by TPX2 family proteins. The *in vitro* kinase assay proved the activation potential of the Aurora binding domain of TPXL proteins on recombinant AUR1. TPXL3 was the strongest activator of Aurora1 with even higher activity than the canonical TPX2. Importantly, activation of Aurora kinase by TPX2 seems to be evolutionary conserved, since the Aurora binding domain of TPX2 from a distantly related *Brassicaceae* species *Eutrema salsugineum* is also capable of Aurora1 activation (Figure 2C, Additional File 5).

TPXL members with Aurora binding domain are strongly expressed

Although all the tested proteins were able to activate AUR1, not much was known about their expression during Arabidopsis development. Group A TPXL proteins showed generally stronger expression compared to group B. It is therefore possible, that plants need more TPXL proteins with Aurora binding domain. Despite this, there is always one of the proteins that seems to have generally higher expression and could probably fulfil housekeeping function. Consistently with the previous findings, TPX2 is expressed in highly dividing tissues such as flowers, flower buds and seedlings. The high expression of TPX2 is in agreement with the importance of AUR1 to phosphorylate various substrates, such as histone H3 during cell division (3) in Arabidopsis and during gametophyte development to phosphorylate CENH3 (Demidov et al., accepted to Frontiers in Plant Science). Moreover, the expression pattern of AUR1 and AUR2 is highly similar to that of TPX2, TPXL2 and TPXL3, suggesting a common regulation of these proteins. TPXL2 and TPXL3 were shown as interactors of AUR1 and AUR2 and TPXL3 is a primary activator of Aurora1(18) further supporting the importance of their common expression patterns.

The localization of TPXL is associated with their importance for spindle microtubules

We also speculate that activation of Aurora kinase by TPX2-related proteins TPXL2, TPXL3, TPXL4 and TPXL8 is related to its nuclear localization which is in agreement with the proposed function of TPX2 in chromatin-induced mitotic spindle assembly (16). On the other hand, we were not sure that TPXL without TPX2_Xklp2 domain can be localized on microtubules. Most likely similarly to canonical TPX2, other TPXL proteins contain a microtubule-binding domain in front of the TPX2_importin domain (13). Infiltration of *N. benthamiana* confirmed the functionality of the tested TPXL proteins. Most of the proteins localized both in the nucleus and on microtubules. It has previously been shown that the overexpression of TPX2 in Arabidopsis results in nuclear envelope and in nuclear localization (16). Relatively similar localization was also observed for other members of TPXL gene family.

Co-localization analyses showed especially strong overlap with AUR1 kinase for those TPXL with an Aurora binding domain. In humans, the interaction between TPX2 and Aurora A is not only important for its activation but also for targeting of the kinase to the spindle microtubules (34) and assembly of spindle of the correct length (35). Importantly, most of the TPXL proteins changed the localization of AUR1. This was a big surprise for us, because it is unclear how TPXL isoforms without Aurora binding domain can interact with Aurora kinases. This phenomenon could possibly be explained by the presence of the coiled-coil motif, a domain involved in protein dimerization and protein-protein interactions. The coiled coil motif is present in all TPXL proteins. Importantly, human TPX2 is known to provide a scaffold for the chromosome passenger complex (36). Similarly, TPXL might have a critical role for the recruitment of the microtubule nucleation complex by Aurora kinase.

Conclusion

The Arabidopsis *TPXL* gene family consists of 21 *TPXL* genes. Phylogeny of the TPXL gene family from selected animal and plant genomes revealed two major groups of TPXLs, A- with Aurora binding and TPX2_importin and B- with TPX2_Xklp2 domains. The Aurora1 binding domains of TPXL2, TPXL3, TPXL4 and TPXL8 were able to activate AUR1 *in vitro*. Analyses of publicly available gene expression data confirmed different expression profiles of *TPXL* family members. While TPX2, TPXL1, TPXL2, TPXL3 and TPXL5 showed expression throughout the plant development, TPXL4, TPXL6, TPXL7 and TPXL8 were expressed at specific developmental stages. Ectopic expression in *N. benthamiana* showed localization of all TPXLs except TPXL7 on cortical microtubules. Interestingly, co-expression with AUR1 changed its localization towards microtubules. Our findings suggest that for targeting of Aurora1, one or more TPXL proteins may respond as activation factors. We speculate that the expansion of this protein family arose to assist in the proper spatiotemporal regulation of α -Aurora kinases in plants.

Methods

Identification of TPXL proteins

The BLAST (Basic Local Alignment Search Tool, (37)) was used to identify protein homologues of canonical TPX2 protein (At1g03780) in Arabidopsis. To characterize the domain composition of TPXL

proteins, *in silico* analyses of protein sequences using PFAM30 (38) and SMART (Simple Modular Architecture Research Tool, (39)) domain prediction programs was performed.

Phylogenetic analysis

The EggNOG4.5 database (<http://eggnogdb.embl.de/>; (20)) was used to identify orthologs of TPX2_Xklp2 and TPX2_importin domain-containing proteins. The database EggNOG4.5 contains an orthologous group of genes, which were retrieved from eukaryotic, prokaryotic and viral sequencing projects. Each identified homologue of TPX2 (At1g03780) in Arabidopsis was submitted to search separately. Some identical sequences occurred in several orthologous groups and those were removed. The final dataset contained 458 protein sequences. Subsequently, multiple sequence alignment was performed in MUSCLE 3.8.31 (40). The maximum likelihood phylogenetic tree was inferred in RAxML HPC 8.2.9 (41) using PROTGAMMALG model via Cipres Science Gateway (42). The tree topology was tested using rapid bootstrapping by 500 replicates. The tree was rooted to TPX2 protein of *Naegleria gruberi*.

The maximum likelihood phylogenetic analysis of TPX2-like proteins in *A. thaliana* was performed in MEGA 7 (GAMMA+LG model; (43)). The tree topology was tested using bootstrapping by 1000 replicates.

Gene differential expression analyses

Med normalized raw counts of *A. thaliana* gene expression data were downloaded from (25). Obtained raw counts were further within-sample normalized using transcript per million (TPM) (44). Data were log-normalized and used for hierarchical clustering using function heatmap.2 in R (45) (Additional File

Plant material

A. thaliana ecotype Columbia plants were used in this study, originally obtained from European Arabidopsis Stock Centre (NASC ID: N3176). Plants were grown in growth chambers under short day conditions and after 2 weeks cultivated under long day conditions at 20°C. *N. benthamiana* plants (Accession number: NIC 660, IPK Gatersleben, Germany) were grown under a 12 h photoperiod at a constant temperature of 26°C.

Cloning of TPXL genes

TPXL sequences were obtained by PCR amplification from Arabidopsis cDNA or gDNA using Platinum Pfx DNA Polymerase (Thermo Fisher Scientific) using primers listed in Additional File 7. The amplified fragments were cloned into a Gateway donor vector pDONR207 as described previously (46). cDNA or gDNA of TPXL genes were subsequently cloned as fusion with GREEN or RED FLUORESCENT PROTEINS (GFP and RFP) into a Gateway destination vectors pH7FWG2.0 and pH7WGF2.0 for N- and C-terminal GFP fusion and pH7RWG2.0 and pH7WGR2.0 for RFP fusions. For expression of the recombinant Aurora binding domain, the first 300 bp of respective TPXL genes, comprising the Aurora binding domain, were cloned as 6xHis fusion into a pET55DEST expression vector using primers listed in Additional File 8.

Production of recombinant proteins

GST-Aurora1 was expressed in *E.coli* C-43 strain (Lucigen, www.lucigen.com) and purified under native conditions as described in (15). Aurora binding domains of TPXL genes were expressed in *E.coli* BL-21 (GE Healthcare Life Sciences, <https://www.gelifesciences.com>) and purified under denaturing conditions as described (32).

In vitro kinase assay

Purified recombinant proteins were desalted in kinase buffer using 7K MWCO Zeba Spin Columns (Thermo Scientific) and processed as described in (15). Briefly, samples with Aurora1 were incubated at 30°C, 30 min with 0.1 mM ATP for activation of the kinases. Subsequently, [³²P]ATP and substrates (Arabidopsis histone H3 ~10 µg, common substrate of Aurora1; (32)) were added and incubated for an additional 60 min at 30°C.

Infiltration of N. benthamiana and confocal microscopy

Transient infiltration of *N. benthamiana* leaf cells was performed as described in (47). For the infiltration of multiple constructs, bacterial cultures with an OD between 1 - 1.3 were mixed in a 1:1 ratio. Distribution of fluorescence signals within the nucleus was recorded as Z-stacks. For a co-localization analysis, probes were excited with dual 488 nm and 561 nm laser lines in combination with a 488/561 nm beam splitter. eGFP emission was detected over a 490–540 nm range, RFP emission was detected over a 570–620 nm range. Presence of both fluorophores was confirmed by photospectrometric analysis.

Accession numbers

Sequence data from this article can be found in the EMBL/GenBank data library under following accession numbers [Q5XVC4](#) (AGI locus identifier At3g01015, TPXL1), [Q4V3B0](#) (At4g11990, TPXL2), [Q4V3C5](#) (At4g22860, TPXL3), [F4K6K7](#) (At5g07170, TPXL4), [F4K9U0](#) (At5g15510, TPXL5), [F4K773](#) (At5g37478, TPXL6), [Q9FKW1](#) (At5g44270, TPXL7), [Q5XUX8](#) (At5g62240, TPXL8), and [F4I2H7](#) (At1g03780, TPX2) (Additional File 1).

Abbreviations

ATP: Adenosine TriPhosphate; *AUR1*: Aurora1; *AUR2*: Aurora2; *BLAST*: Basic Local Alignment Search Tool; *CENH3*: Centromeric Histone 3; *eGFP*: Enhanced Green Fluorescent Protein; *GFP*: Green Fluorescent Protein; *GST*: Glutathione S-Transferase; *H3*: Histone 3; *KLEEK*: short motif of aminoacids lysine, leucine, glutamic acid, glutamic acid, lysine; *MAP65*: Microtubule Associated Protein 65; *OD*: Optical Density; *PCR*: Polymerase Chain Reaction; *RFP*: Red Fluorescent Protein; *TPX2*: Targeting Protein for Xklp2; *TPXL (1–8)*: Targeting Protein for Xklp2-Like (1–8); *WVD*: Wave-Dampened; *WDL*: Wave-Dampened Like

Declarations

Acknowledgements

The authors thank Oda Weiss for excellent technical support. We thank Dr. Ales Pecinka (Institute of Experimental Botany, Czech Republic) for critical review and suggestions.

Funding

The work was supported from ERDF project “Plants as a tool for sustainable global development” (CZ.02.1.01/0.0/0.0/16_019/0000827), INTER-COST (LTC18026) from Ministry of Education, Youth and Sports of the Czech Republic, and German Research Foundation (SFB) 648 “Molecular mechanisms of information processing in plants”. Part of this work was supported by a grant from the Research Foundation of Flanders (G029013N).

Authors' contributions

EDT and DD conceived the study, EDT, DD, AT and KP performed experiments, TR performed confocal microscopy analyses, PD performed the phylogenetical analyses, AH, JD, BP and DVD provided funding, materials and contributed to the final manuscript. All authors read the manuscript.

Ethics approval and consent to participate

Arabidopsis thaliana seed stocks are available from the European Arabidopsis Stock Center (NASC, www.arabidopsis.info). *Nicotiana benthamiana* seed stocks are available from IPK Gatersleben genebank. Research here did not involve field studies.

Consent for publication

Not applicable.

Availability of data and materials

Multiple sequence alignments used for the phylogenetic tree was stored at https://figshare.com/articles/Untitled_Item/8798357 (accession numbers are included in the alignment). Plasmids are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Phylogenetic reconstruction of the TPXL gene family in Arabidopsis. EggNOG4.5 was used to identify orthologs of Arabidopsis canonical TPX2 (At1g03780). Multiple sequence was performed in MUSCLE 3.8.31 and used for phylogenetic reconstruction of TPX like gene family and the maximum likelihood phylogenetic tree was inferred in RAxML HPC 8.2.9 using the PROTGAMMALG model. The tree topology was tested using rapid bootstrapping by 500 replicates. *Negleria gruberi* TPX2 was selected as outgroup. Monophyletic clades were collapsed. Consistent with its domain composition, TPXL genes form two separate clusters, groups A and B – proteins containing Aurora binding domain (red square) and importin domain (blue bar) and TPX2_Xklp2 domain (green bar).

Figure 2

Group A TPXL proteins contain a functional Aurora kinase binding domain. A - Multiple sequence alignment of putative Aurora binding domains of TPX2, TPXL2, 3, 4 and 8 proteins. TPXL proteins with Aurora binding domain retained all key amino acids residues important for Aurora kinase binding and activation in human (Hs) TPX2. B - MEME (Multiple Em for Motif Elicitation; Bailey et al., 2009) analyses confirmed presence of similar Aurora binding domain with key conserved residues in 45 proteins from

different plants species C – In vitro kinase assay with recombinant Aurora1 and TPX2 proteins confirmed that all members of TPX2 family with Aurora binding domain are able to activate Aurora1. TPX2 of distantly related *Eutrema salsugineum* (EsTPX2) also activates *Arabidopsis* Aurora1. ***p-value < 0.001 in hypergeometric test, **p-value < 0.01 in hypergeometric test.

Figure 3

Expression analyses of TPXL and α -Aurora genes at different developmental stages of Arabidopsis. Heat map display differential expression profiles across various developmental stages. The color bar represents log10 expression values inferred from raw counts of (25); thereby white colour representing the lowest expression values and brown signifies the highest expression level. Black bars indicate a set of multiple samples from the same tissue. The dendrogram was computed and reordered based on gene expression values. Detailed information about tested developmental stages is available in Additional File

6.

Figure 4

Subcellular localization of Arabidopsis TPXL proteins in tobacco leaf epidermal cells. Images were acquired 2 days after infiltration using a laser scanning confocal microscope. A – Localization of Group A TPXL proteins on microtubules and in the nucleus. GFP – Green Fluorescent Protein, RFP – Red Fluorescent Protein. B - Localization of Group B TPXL proteins on microtubules and in the nucleus. C – Localization of canonical TPX2-GFP. D – Localization of Aurora1-GFP alone. E – Co-localization of Group A TPXL and Aurora1. F – Co-localization of Group B TPXL and Aurora1. Bar = 20 μ m.

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