Acentrosomal spindle morphogenesis is dependent on the unconventional TPX2 family protein TPXL3 and a Aurora kinase in Arabidopsis thaliana

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Article

Keywords: Arabidopsis thaliana, α Aurora kinase, mitotic kinase regulation

DOI: https://doi.org/10.21203/rs.3.rs-519922/v1

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Abstract

The α Aurora kinase serves as an essential mitotic regulator of spindle assembly in Arabidopsis thaliana. This indispensable function was thought to be dependent on an evolutionarily conserved targeting factor known as TPX2. However, our previous work indicated that the canonical TPX2 protein was dispensable but the plant specific TPX2 family protein TPXL3 became essential in A. thaliana. Here, we found that the TPXL3 polypeptide contains a microtubule-binding domain following the N-terminal Aurora-binding and activation motif, and its TPX2 signature domain with an importin-binding motif is followed by a novel short C-terminus. To test the hypothesis that TPXL3 dictated the essential mitotic function of α Aurora, we generated artificial microRNA lines in which TPXL3 expression was compromised. The amiR-TPXL3 mutants exhibited phenotypes of TPXL3 expression-correlated growth retardation. Due to the compromised TPXL3 expression, α Aurora delocalized from spindle microtubules and diffused in the cytoplasm. Such a phenotype was concomitant with defects in spindle morphogenesis as represented by disorganized spindle poles. A functional TPXL3-GFP fusion protein colocalized with α Aurora by exhibiting a highly dynamic cell cycle-dependent localization pattern highlighted by prominent appearance on the prophase nuclear envelope, spindle microtubules with biases towards poles, and reforming nuclear envelope during telophase and cytokinesis. However, TPXL3 was largely absent from phragmoplast microtubules that exhibited prominent γ-tubulin association. Finally, we found that the C-terminal domain was dispensable while other domains are required for its mitotic function. Hence, our discovery of the TPXL3 function shifted the TPX2-centered paradigm of mitotic kinase regulation in plants.

Introduction

The spindle apparatus drives the partitioning of genetic materials during eukaryotic cell division. Spindle microtubules are assembled into a bipolar array and converged towards two opposite poles. Developing converged spindles poles guide two sets of segregated chromatids to be incorporated into two and only two daughter nuclei. The Aurora family kinases serve as master regulators of spindle assembly and a wide spectrum of mitotic and meiotic events on the spindle apparatus by phosphorylating proteins associated with the cytoskeleton and chromosomes 1. They were discovered as centrosome-associated kinases of Eg2 in frog cells and Aurora in fly cells and their functions are indispensable for mitosis in their respective systems 2, 3. In fact, these Aurora kinases, often known as Aurora A or AURA in animal cells, not only are concentrated at the centrosomes but also decorated spindle microtubules with biases towards spindle poles, and they represent one of the two (or three) classes of Aurora family kinases 4. The Aurora A counterpart in plants is α Aurora that is detected on spindle microtubules during mitosis and plays an essential role in cell division as its loss causes gametophytic lethality in Arabidopsis thaliana 5.

Aurora A is targeted to spindle microtubules and centrosomes and activated by the microtubule-associated protein TPX2 (Targeting Protein of XKLP2) 6. The vertebrate TPX2 is one of the most
important proteins for mitosis because it regulates functions of many spindle assembly factors, e.g. acting in a Ran-GTP-dependent pathway for microtubule nucleation and polymerization, besides targeting and activating Aurora A\(^7,8,9\). Through direct interaction, TPX2 also plays a critical role in targeting the Kinesin-5, an essential mitotic motor, to spindle microtubules for the organization the two poles\(^10\). TPX2 is conserved in plants as referenced in \textit{A. thaliana}, and it is joined by a number of homologous proteins that exhibit different degrees of homology in regions like the N-terminal Aurora-binding site and the importin-binding TPX2 signature domain\(^11,12,13\). Over expression of TPX2 induces perinuclear and intranuclear microtubule formation possibly in a Ran-dependent manner in \textit{A. thaliana}\(^14\), that seemed to suggest a critical function of this highly conserved protein in acentrosomal microtubule nucleation. However, we recently demonstrated that TPX2 is dispensable for normal growth and reproduction as multiple null \textit{tpx2} mutants resembled the wild-type control in all examined aspects of growth and development\(^15\). This finding challenged the commonly perceived role of the canonical TPX2 in mitosis, especially regarding its essential function in Aurora activation and spindle assembly.

Our work also revealed two closely related TPX2-like proteins TPXL2 and TPXL3 which both contain segments of TPX2-related peptides but distinctly lack the ~ 150-amino acid C-terminal segment found in TPX2 for Kinesin-5 interaction\(^15\). TPXL3, but not TPX2L2, was found to be an essential protein. Because TPXL3, like many other TPXL proteins, contains the N-terminal Aurora-binding motif, it can activate the kinase activity of \(\alpha\) Aurora \textit{in vitro}. It has been intriguing whether TPXL3 serves as the primary regulatory protein of the kinase \textit{in vivo} and how its essential function is expressed in \textit{A. thaliana}.

To overcome the null mutation-caused lethality obstacle and discover the mitotic function of TPXL3, we generated artificial microRNA lines in which TPXL3 expression was repressed. These miR-\textit{TPXL3} lines revealed that TPXL3 plays an essential role in regulating \(\alpha\) Aurora activities on spindle microtubules and consequently its function is translated into spindle morphogenesis. Our results showed that morphological changes in mitotic spindles have a great impact on the robustness of plant growth as demonstrated by manipulations of the functions of TPXL3 in transgenic plants. Hence, flowering plants employ a TPX2-independent and TPXL3-dependent mechanism of mitotic regulation.

**Results**

**Repression of TPXL3 expression leads to retarded growth**

To repress the expression of \textit{TPXL3} in \textit{A. thaliana}, an artificial microRNA gene was designed to specifically target at this gene but not other related genes (Fig. 1A). These amiR-\textit{TPXL3} transformants produced dwarf plant that exhibited different degrees of growth inhibition when compared to the control plant (Fig. 1B). To test whether the growth phenotype was correlated to the expression level of the target gene \textit{TPXL3}, real-time RT-PCR experiments were carried out and showed that greater reduction of the mRNA level was associated with more severe growth defects (Fig. 1C). To further prove the linkage of the amiR-\textit{TPXL3} expression with the phenotype, we constructed an amiR-\textit{TPXL3}-resistant version of the
TPXL3 gene (TXPL3R) by introduction of silent mutations and delivered it into one of the amiR-TPXL3 mutant. The expression of the TPXL3R gene under the control of the TPXL3 native promoter and in fusion with GFP (green fluorescent protein) fully suppressed the growth phenotype in the mutant to render plants resembling the wild-type control (Supplemental Fig. 1). Therefore, we concluded that the growth phenotypes exhibited by the amiR-TPXL3 mutants were caused by the repression of TXPL3 expression and these mutants could serve as the genetic material for subcellular phenotypic analysis of the consequence of compromised TPXL3 expression.

TPXL3 exhibits a distinct dynamic localization pattern during mitosis

A previous interaction assay in yeast revealed that TPXL3 barely binds to α Aurora but that the homologous TPXL2 interacts strongly. However, it was hypothesized that the TPXL3 protein serves as the primary activator of α Aurora (AUR1 and AUR2) in A. thaliana in part because of the lethality caused by the loss of this gene. To learn their in vivo activities, we compared the localization patterns of TPXL3 and TPXL2 when fused with GFP and expressed in the corresponding homozygous mutant background. When surveyed by fluorescent microscopy, the two proteins exhibited different localization dynamics in dividing root cells (Fig. 2A, B). While TPXL3 showed pronounced association with mitotic microtubule arrays, TPXL2 did not show distinguishable localization patterns other than residing in some interphase nuclei. Therefore, this result was in line with the lack of growth phenotype upon the loss of the TPXL2 gene highlighted the essential contribution of TPXL3.

To gain insights into the dynamic localization of TPXL3 during mitosis, we observed the functional TPXL3-GFP fusion protein expressed in the homozygous ptxl3-1 mutant background, by time-lapse live-cell imaging. The TPXL3-GFP signal became concentrated on the nuclear envelope at prophase and gradually polarized towards two poles (3:00 & 9:00, Fig. 2C). Around the time of nuclear envelope breakdown (10:30), the GFP signal was largely concentrated at the poles. Concomitant with the development of the mitotic spindle, the TPXL3 signal spread along spindle microtubules (12:00–15:30). Following the anaphase onset (17:30), the signal retrieved from the middle zone together with the shortening of kinetochore fiber microtubules and eventually it became highly concentrated at the poles again (19:30). When the phragmoplast formed, TPXL3 became enriched in a region flanking the phragmoplast microtubules (22:00–27:00 and Supplemental Fig. 4). Such a pattern was later replaced by the returning of the protein to the reforming nuclear envelope (35:00–41:00). Therefore, TPXL3 exhibited microtubule-associated (along spindle microtubules) and microtubule-independent (phragmoplast flanking) patterns of distribution during mitosis and cytokinesis.

To discern whether TPXL3 dynamics mirrored that of α Aurora, we then performed co-localization experiments. To do so, the Aurora 1 (AUR1) gene was expressed in fusion with the FLAG peptide in the TPXL3-GFP transgenic lines. Like the GFP-AUR1 fusion, this FLAG-AUR1 fusion protein was fully functional as the transgene restored growth of the aur1 aur2 double mutant to the wild-type level (Supplemental Fig. 2). Throughout the mitotic cell cycle, the FLAG-AUR1 and TPXL3-GFP exhibited identical localization patterns on the prophase spindle and kinetochore fiber microtubules, and on the
reforming nuclear envelope, but not associated with microtubules in developing phragmoplasts (Fig. 3 and Supplemental Fig. 4). Therefore, the data supported the in vivo association of the two proteins.

To test whether the distinct localization of TPXL3 was dependent on dynamic microtubules, we challenged cells with low doses of the microtubule-depolymerizing agent oryzalin. The TPXL3-GFP signal became largely overlapped with that of microtubules and associated with microtubules in the spindle midzone during late anaphase and phragmoplast (Supplemental Fig. 3). Therefore, we concluded that the polarized localization pattern of TPXL3 was dependent on the dynamic microtubule arrays during mitosis.

**Compromised TPXL3 expression leads to delocalization of α Aurora and γ-tubulin**

Because of the colocalization of TPXL3 and AUR1, we asked whether compromised expression of TPXL3 would affect the localization of AUR1. To do so, we had the GFP-AUR1 fusion protein expressed in the amiR-TPXL3 mutant and compared its localization to that in the control cells. In the control cells, GFP-AUR1 prominently decorated spindle microtubules but was absent from the microtubule segments in the vicinity of chromosomes (Fig. 4A). In the amiR-TPXL3 mutant, however, the GFP-AUR1 signal became largely diffuse in the cytoplasm and its signal on spindle microtubules did not stand out significantly (Fig. 4B). The contrasted difference was obvious when the spindle-associated signal was referenced to the cytoplasmic one (Fig. 4C). Therefore, we concluded that TPXL3 is required for AUR1 localization on spindle microtubules in *A. thaliana*.

Conversely, we asked whether compromised α Aurora function would impact TPXL3 localization. We compared the TPXL3-GFP signal in the control cells of the complemented line and in the *aur1 aur2* double mutant background. The localization in the prophase spindle on the nuclear envelope, mitotic spindle, and the phragmoplast-devoid region was comparable in both genetic backgrounds (Supplemental Fig. 4). Therefore, we concluded that TPXL3 likely achieves its localization independently to α Aurora, and in turn dictates the localization of α Aurora.

The spindle pole-biased localization of TPXL3 and α Aurora resembled that of microtubule-nucleating factors represented by γ-tubulin in *A. thaliana* 16. To discern the relationship between α Aurora/TPXL3 and γ-tubulin, we first carried out dual localization experiments by detecting TPXL3-GFP and γ-tubulin with respective antibodies (Fig. 4D). Prior to nuclear envelope breakdown in prophase, TPXL3 prominently accumulated on the nuclear envelope when γ-tubulin was barely detectable (Fig. 4D). Starting from late prophase, the TPXL3 signal was largely overlapping with γ-tubulin on kinetochore fibers of the mitotic spindle (Fig. 4E). Striking differences were discovered in the spindle midzone and developing phragmoplasts where γ-tubulin prominently decorated microtubule minus ends (Fig. 4F, G). TPXL3, however, was barely detected and later accumulated at the distal edges of the two groups of γ-tubulin signals (Fig. 4F, G). This finding was surprising because γ-tubulin is generally thought to be at the minus end of phragmoplast microtubules. Therefore, α Aurora/TPXL3 are associated with subcellular structures that flank the phragmoplast microtubules.
Because TPXL3 appeared earlier than γ-tubulin on mitotic arrays, we then examined γ-tubulin localization in amiR-TPXL3 mutant cells. Compared to the conspicuous localization of γ-tubulin on prophase spindle poles and developing spindles in the control cells, such polarized pattern was largely lost and replaced by weak signals clouding on microtubules (Fig. 4H, I). The γ-tubulin signal associated with spindle microtubules were seriously reduced while the diffuse signal in the cytoplasm became more noticeable (Fig. 4J). Therefore, the results support the notion that TPXL3 plays a critical role in regulating the localization of αAurora and perhaps consequently γ-tubulin complex on the spindle microtubule arrays.

**TPXL3 directly binds to microtubules in vivo and is phosphorylated by αAurora**

Because the TPXL3 polypeptide exhibits significant sequence divergence from the canonical TPX2 protein\(^{15}\), we dissected the structure-function relationship by transiently expressing truncated versions in tobacco cells (Fig. 5). To do so, we divided TPXL3 into the following five segments (I-V): the N-terminal Aurora-binding motif (I), first novel domain (II), domain III with the first predicted nuclear localization signal, and domain IV with the second nuclear localization signal and importin-binding site, and the C-terminal novel domain (V) (Fig. 5A). When the full length TPXL3 was expressed in fusion with GFP under the control of the constitutive viral 35S promoter, the fusion protein was nuclear with stronger signals in the nucleolus (Fig. 5B). The deletion of either domain V or IV did not alter such a localization pattern (Fig. 5B). The removal of domains III to V, however, resulted in the fusion protein decorating cortical microtubule-like network, and domain II was sufficient for this localization pattern (Fig. 5B). To ascertain whether the cytoskeletal or nuclear localization would dominate when both features were included, domains II-V were expressed and exhibited nuclear localization like others seen above (Fig. 5B). In fact, domains IV plus V, III plus IV, or I plus III-V also rendered similar localization patterns (Fig. 5B). Finally, we tested whether domain V had a localization determinant and found its GFP fusion was uniformly diffuse in the cytoplasm and nucleus (Fig. 5B). To ascertain whether domain II interacted with cortical microtubules, we expressed the truncated protein together with a microtubule marker of CKL6 and detected completely overlapping patterns either with domains I and II or domain II alone (Fig. 5C). Therefore, we concluded that domain II constitutes a microtubule-binding site in TPXL3 while domains III and IV have nuclear localization activities. The essential function of TPXL3 is perhaps brought about by these domains that make respective contributions.

Both αAurora and TPXL3 exhibited nuclear localization in interphase cells\(^ {15}\). When co-expressed, the full length TPXL3 fused with GFP had exclusive nuclear localization with an emphasis in the nucleolus, as the TagRFP-AUR1 fusion protein (Fig. 6A). To test whether αAurora's nuclear localization was dependent on TPXL3, we had TagRFP-AUR1 co-expressed with truncated TPXL3 containing only domains I and II corresponding to the Aurora-binding motif and microtubule-binding domain, respectively. Consequently, TagRFP-AUR1 decorated cortical microtubules and completely overlapped with TPXL3\(^ {I+II}\)-GFP (Fig. 6B). Such microtubule localization was completely dependent on the domain I as the loss of this AUR-binding site abolished the microtubule-association (Fig. 6C).
The AUR1-TPXL3 interaction was further tested \textit{in vitro} using fusion proteins expressed in and purified from bacterial hosts. When compared to the interaction between the full-length GST-TPXL3 and 6xHis-AUR1, it was lost when the GST-TPXL3$^{II-IV}$ fusion protein was used (Supplemental Fig. 5). The deletion of domains III to V did not affect the interaction (Supplemental Fig. 5).

We then tested how specific domain(s) of TPXL3 contributed to the kinase activity of α Aurora. AUR1 exhibited some autophosphorylation activities, while full-length TPXL3, TPXL3$^{II-IV}$, or TPXL3$^{I+II}$ alone did not (Fig. 6D). The addition of full-length TPXL3 enhanced AUR1 phosphorylation and had TPXL3 also phosphorylated, and such an activity was completely dependent on the Aurora-binding domain at the N-terminus (Fig. 6D). Furthermore, the results also showed that the phosphorylation sites were largely included in the Aurora- and microtubule-binding sites within the first two domains (Fig. 6D).

**TPXL3 regulates spindle morphogenesis and axial growth**

To link TPXL3 function to microtubule remodeling during mitosis, we delivered a GFP-TUB6 marker of microtubules into the amiR-TPXL3 mutant to monitor mitotic arrays and compared them to those in control cells by live-cell imaging (Supplemental Movies 2 and 3). Microtubules are assembled into a bipolar prophase spindle around the timing of nuclear envelope breakdown in the control cell expressing TPXL3 (times 00:00 to 01:00, Fig. 7A). This array was followed by the spindle arrays of prominent kinetochore fibers converged towards obvious poles (02:00 to 06:00, Fig. 7A). Following the anaphase onset, converged kinetochore fibers shortened and microtubules in the spindle midzone emerged and coalesced before the appearance of two mirrored microtubule sets joined by the fluorescently dark midline (06:00 to 08:30, Fig. 7A). Such a dynamic pattern was largely altered in the amiR-TPXL3 mutant cell although microtubules continued to undergo rapid reorganization (Fig. 7B). Unlike the fusiform appearance of spindle microtubule in the control cells, mutant cells assembled discrete microtubule bundles running in parallel to each other upon nuclear envelope breakdown (00:00, Fig. 7B). These microtubule bundles were not integrated into a spindle with converged spindle poles when bundles freely splayed outwards (01:00 to 03:00, Fig. 7B). Although a bipolar spindle array was formed later with obvious kinetochore fibers terminating at the metaphase plate, it lacked converging poles (04:00 to 07:00, Fig. 7B). Anaphase onset was delayed and sometimes anaphase spindle elongation was minimized, and microtubules in the spindle midzone were developed into robust bundles that later coalesced into the phragmoplast array (09:00 to 13:00, Fig. 7B).

To quantitatively evaluate convergence of spindle microtubules, we measured spindle solidity, which is defined as the ratio of the spindle area to the convex hull area (Fig. 7C, Supplemental Fig. 6A, B). Converged and splayed microtubules result in high and low ratios, respectively. Repression of \textit{TPXL3} expression significantly decreased solidity, leading to splayed organization of spindle microtubules (Fig. 7C). To quantitatively evaluate spindle elongation, we also measured spindle aspect ratio, which is a morphometric parameter that is defined as the ratio of the major axis length to the minor axis length of the fitted ellipse (Fig. 7D, Supplemental Fig. 6A, C). Knockdown of \textit{TPXL3} significantly decreased aspect ratio, leading to decreased spindle elongation (Fig. 7D).
Although the amiRNA-TPXL3 mutant cell formed distorted spindle microtubule arrays, it produced a bipolar phragmoplast array that underwent robust expansion towards the cell cortex during cytokinesis, similar to what was observed in the control cell (09:30 to 16:30 in the control and 13:00 to 22:00 in amiR-TPXL3, Fig. 7A, B). Therefore, we concluded that down regulation of TPXL3 expression had greater impacts on spindle microtubule remodeling than on the phragmoplast array.

To explore the functionality of different TPXL3 domains in spindle morphogenesis, we expressed different derivatives of TPXL3 under the control of the native TPXL3 promoter in the amiR-TPXL3 mutant plants. The amiR-TPXL3 (5) mutant line as the host for the expression of different TPXL3 derivatives. First, we used anti-tubulin immunofluorescence to examine metaphase spindle microtubule arrays and detected disorganized poles in mutant cells, similar to what was observed by live-cell imaging (Fig. 8A). When the full-length microRNA-resistant form of TPXL3 (mTPXL3) was expressed, metaphase cells restored typical spindles with converged poles (Fig. 8A). Concomitantly, the transgene suppressed the stunted growth phenotype of the host plant and rendered adult plants that resembled the wild-type control (Fig. 8B). Then, we had the AUR-binding motif (domain I) or the microtubule-binding site (domain II) removed and found that the truncated TPXL3\textsuperscript{II-V} and TPXL3\textsuperscript{ΔII} proteins did not restore the spindle morphology, neither did they improve seedling growth (Fig. 8A, B). These results supported the notion that TPXL3’s function was inseparable from its interactions with α Aurora or microtubules. Surprisingly, the mTPXL3\textsuperscript{I-IV} derivative with the C-terminal domain V removed was able to restore the converged spindle microtubule arrays in metaphase cells and growth and reproduction almost as robust as the wild-type control (Fig. 8A, B). The derivative with domain IV removed, however, enhanced spindle defects with kinetochore microtubule fibers arranged in a palisade-like fashion and resulted in great retardation of plant growth (Fig. 8A, B). Similarly, expression of a truncated version of TPXL3 containing only domains I and II resulted in a similar if not more exaggerated negative impact in both spindle morphogenesis and seedling growth as mTPXL3\textsuperscript{I-IV} (Fig. 8A, B). Collectively, these results affirmed that defects in spindle microtubule organization are translated into deficiencies in overall growth besides demonstrating the indispensability of domains I (AUR-binding), II (microtubule-binding), and IV (importin binding) for TPXL3 function.

Because the mTPXL3\textsuperscript{ΔII+II} protein had a stronger negative impact in spindle morphogenesis and plant growth when compared to the deletion of either domain, we asked whether TPXL3’s function was completely aligned with α Aurora. To do so, we introduced amiR-TPXL3 into the aur1 aur2 double mutant. Down regulation of TPXL3 in the aur mutant further enhanced the growth defects (Fig. 8C). Therefore, TPXL3 may possess novel function(s) in addition to targeting and activating the α Aurora kinase.

Discussion

The dissection of the essential TPXL3 function indicated that TPXL3 not only plays an essential role in targeting α Aurora to spindle microtubules and activating its kinase activity but also governs the localization of the microtubule nucleating factor γ-tubulin (complex). TPXL3 is required for spindle morphogenesis that directly contributes to robust axial growth in A. thaliana.
The TPXL3 but not TPX2-regulated function for α Aurora is essential in A. thaliana

The evolutionarily conserved TPX2 protein, first identified as an interacting/targeting protein of the Kinesin-12 motor XKLP2 in frog cells, is one of the most visible and important microtubule-associated proteins (MAP) for mitotic spindle assembly. The utmost noticeable function of TPX2 is to target Aurora A to spindle microtubules towards spindle poles in order to phosphorylate proteins like microtubule-based motors and MAPs that often play critical roles in the organization of spindle poles. Recently, TPX2 was also identified as a microtubule nucleating factor that works with the augmin complex for activating the γ-tubulin ring complex in order to produce branching microtubules on the wall of extant microtubules. TPX2 interacts with importin and is released when importin binds to Ran-GTP generated by the RanGEF RCC1 associated with chromatin. TPX2 interacts with motors of Kinesin-5 and Kinesin-12 for their localization and spindle pole organization. These features determine how important TPX2 is for spindle assembly.

A. thaliana, representing flowering plants, produces a single TPX2 and eight TPXL proteins. Among the eight TPXL proteins, four contain the Aurora-binding motif so that perhaps their functions all are linked to the Aurora kinase. Because TPXL proteins all miss one or more domains found in the canonical TPX2, TPX2 could have had the most versatile or perhaps most critical function in mitosis. Indeed, AtTPX2 not only exhibits a typical spindle pole biased localization pattern but also is competent to induce ectopic microtubule production upon over expression. Therefore, it was surprising to discover that in A. thaliana TPXL3 becomes essential for mitosis while the canonical TPX2 is dispensable although both interact with the α Aurora kinase in vivo. When considering that TPXL3 misses the extended C-terminal domain that accounts for kinesin interaction, one would expect that the canonical TPX2 possesses functions that are likely missing in TPXL3. This phenomenon may be attributed to the specification of substrates brought about by the difference between the two proteins. Besides TPX2, the Kinesin-5 motor Eg5, Polo-like kinase, CDK5RAP2, and the TACC (transforming acidic coiled-coil) protein are among the most noticeable or perhaps most important substrates of the vertebrate TPX2. In plants, there are no obvious homologs of Polo, CDK5RAP2, or TACC. Although a temperature sensitive mutation in a Kinesin-5 gene RSW7 causes spindle collapse at restrictive temperatures, null mutations do not seem to cause a noticeable phenotype. Therefore, if AtTPX2 functions in regulation of Kinesin-5 in A. thaliana, such a function assigned to TPX2 may not be essential, just like RSW7 being dispensable (Fig. 8D).

Conversely, TPXL3 likely bears features that are not shared by TPX2, e.g. specifying selective substrates that are not recognized by TPX2. Based on the phenotype of compromised localization of the γTuRC, it is likely that certain subunits of the complex or its regulatory proteins may be substrates of α Aurora-TPXL3 (Fig. 8D). It is known that the γTuRC has most subunits like GCP6 heavily phosphorylated. Recently, we showed that in A. thaliana GCP6 also plays a critical role in the spindle localization of γ-tubulin. It would be interesting to test whether AtGCP6 shows a cell cycle-dependent phosphorylation pattern linked to its function in spindle morphogenesis. Studies in vertebrates also identified the MAP subunit of the
augmin complex as a substrate of Aurora A and showed the phosphorylation down-regulate its microtubule binding and spindle localization. Although plants also produce the 8-subunit augmin complex, the polypeptide sequences of its subunits often are very divergent from the animal counterparts. In *A. thaliana*, EDE1 serves as the M phase specific microtubule-associated protein (MAP) subunit of augmin and is required for the complex to decorate spindle microtubule array. Again, it remains to be tested whether EDE1 is recognized by the α Aurora-TPXL3 complex and has its function regulated by Aurora phosphorylation.

**TPXL3-dependent spindle morphogenesis**

Angiosperms have lost the centriole-based centrosome structure so that their cells form acen
trosomal spindle microtubule arrays during mitosis, unlike fungal and typical animal cells that have spindle poles focused on the centrosomes. However, mitotic plant cells have spindle microtubules converged towards spindle poles despite the lack of the centrosome structure. These so-called microtubule-converging centers are likely established by minus end-directed motors. In animal cells, spindle pole focusing is largely brought about by the microtubule minus end-directed motor cytoplasmic dynein together with assistance from Kinesin-14 motors. Plants lack cytoplasmic dynein and produce an expanded subfamily of Kinesin-14 motors with 21 members in *A. thaliana* that are predicted to be minus end-directed. Among them, the KatA/ATK1 and ATK5 perhaps play dominant role in spindle morphogenesis as the loss of either motor leads to mitotic spindles with widened poles. Single *atk1* or *atk5* mutant does not exhibit obvious vegetative growth phenotype but the simultaneous loss of both leads to lethality, suggesting that enhanced challenges to spindle morphogenesis may have caused spindle malfunctioning. The question is whether KatA/ATK1 and ATK5 are substrates of α Aurora-TPXL3 and their functions in spindle assembly are regulated by phosphorylation. In other words, the next step would be to test whether the function of TPXL3 in spindle assembly is mediated by KatA/ATK1 and ATK5. This task is challenged by the lethality of the *atk1 atk5* double mutant so that a conditional mutation would be useful.

On the other hand, defects in spindle microtubule arrays in the amiR-TPXL3 cells might be caused by lack of γTuRC activity associated with spindle microtubules. The vertebrate Aurora A-TPX2 complex recruits the γTuRC to spindle poles by a direct interaction between TPX2 and a protein called XHRAMM which in turn bring in the γTuRC-targeting factor NEDD1. However, there is no obvious homolog of XHRAMM in angiosperms based on amino acid sequence alignment. Therefore, plant must have evolved a different mechanism that regulates the activity of γTuRC on spindles, especially for pole organization.

**Translating spindle morphogenesis to plant axial growth**

It is often enigmatic how morphologically “defective” spindles might affect vegetative growth or whether the spindle pole convergence is linked to plant wellness. This seemingly straightforward question may not be answered when mutations that affect fundamental behaviors of microtubules are employed, like those directly impact microtubule assembly and disassembly. On the other hand, the Arabidopsis...
Kinesin-14 motor KatA/ATK1 plays a critical role in microtubule accumulation at spindle poles but such a seemingly defective spindle does not compromise mitotic progression. Interestingly, the loss of the paralogous ATK5 leads to the production of mitotic spindles with splayed poles. Again, the atk5 mutant does not seem to noticeably affect mitosis and vegetative growth. It is suggested that the function of these redundant motors perhaps was dosage dependent. The correlated phenotypes summarized here provide a direct evidence showing that morphologically compromised spindles significantly affected axial growth because of the reduced function of αAurora and TPXL3. Our results demonstrated that the more severely detorted spindles were associated with more retarded axial growth. Therefore, the αAurora/TPXL3-associated mitotic function is an important factor to promote robust vegetative growth in angiosperms.

**Methods**

**Plant Materials and Growth Conditions**

Mutant seeds of *A. thaliana* are obtained from the Arabidopsis Biological Research Center (ABRC). These include the SAIL_350_B08 and GABI_480B12 lines for the *TPXL3* (AT4G22860) locus, SALK_079098 for the *TPXL2* (At4g11990) locus, and the *aur1-2/aur2-2* mutant. All plants were grown under a 16-h-light and 8-h-dark cycle with 70% relative humidity at 22 °C. Live-cell imaging and immunolocalization experiments were carried out using young seedlings germinated on solid medium supplied with 1/2 Murashige Skoog salt mixture (Sigma).

**Plasmid construction**

Primers used in this study and their corresponding sequences are listed in Supplemental Table 1. Genomic fragments of TPXL2, TPXL3 and AUR1, which contain the promoter and coding sequences, were amplified using Phusion DNA polymerase (Thermo Fisher). The amplified fragments were cloned into the Gateway pENTR/D-TOPO vector (Thermo Fisher). To produce the GFP-AUR1 or Flag-AUR1 fusion construct, the entry vector containing genomic AUR1 was linearized by inverse PCR, then a EGFP or Flag fragment was inserted in front of the start codon via Gibson Assembly method (New England Biolabs). The resulting plasmids were recombined with pGWB4 or pGWB10 by LR recombination reactions.

Constructs for tobacco leaf infiltration experiments were produced as follows. The cDNA fragments of *TPXL3* or *AUR1* were amplified using the RAFL07-16-B14 or RAFL07-91-B16 plasmids (RIKEN, Japan) as the templates. The resulting products were cloned into the Gateway pENTR/D-TOPO vector. The entry vector containing TPXL3 CDS was linearized by reverse PCR and was ligated using a *BamH*I site to produce truncated TPXL3 vectors. The resulting entry clones were delivered into the destination vector pGWB605 or pGWB661.

**amiR-TPXL3 Construction and Complementation**

The artificial miRNA construct was designed according to published protocols. Briefly, the amiR-TPXL3 guide sequence was designed via P-SAMS (http://p-sams.carringtonlab.org/amiRNA/designer) and cloned.
into the pENTR-AtMIR390a vector (Carbonell et al, 2014), followed by an LR reaction with the pGW602Ω destination vector. The resulting amiR-TPXL3 plasmid was introduced into Arabidopsis wild-type (Col) plants and transgenic T1 plants were selected by spraying Finale (AgrEvo) for Basta resistance. To analyze the silencing efficiency, RNA samples were prepared from young seedlings of 3-day-old amiR-TPXL3 lines and wild-type control plants, followed by real-time quantitative RT-PCR according to a previous study (Miao et al, 2019).

To make a construct that contains an amiR-TPXL3 resistant version of TPXL3, eight silent mutations were introduced into the TPXL3 entry clone using Gibson Assembly method. Specifically, the amiR-TPXL3 target site 5’-CGGAAAAAGAGCACTCCGAAA-3’ was replaced by 5’-CGtAAgAAatcCACaCCaAAg-3’, with base changes indicated by lowercase letters. This entry clone was introduced into a binary vector pGWB4 for generating a GFP fusion by the LR reaction and transformed into the amiR-TPXL3 lines #5 and #3.

**Transient Expression in Nicotiana benthamiana**

Agrobacterium GV3101 carrying each constructed plasmid was cultured overnight at 28°C and resuspended in an infiltration buffer containing 10 mM MES (pH 5.7), 10 mM MgCl₂ and 150 µM acetosyringone to a final OD600 of 1.0, and equal volumes of cultures carrying different constructs were mixed for co-infiltration. These cells were then mixed with Agrobacterium C58C1 (pCH32-35S:p19) in a 1:1 ratio. After incubation for 3 h at room temperature, the resulting cultures were infiltrated into leaves of 4-week-old N. benthamiana using a syringe. Localization of expressed proteins were observed under fluorescence microscopy three days after infiltration.

**Production of Recombinant Proteins and In Vitro Kinase Assay**

The coding sequences of TPXL3 or AUR1 were cloned into pGEX-KG at the EcoRI & NcoI sites or pET28a at the BamHI & SalI sites. The recombinant plasmids rendered the expression of GST or His tagged proteins in bacteria host BL21. The fusion proteins were purified using Glutathione HiCap matrix (Qiagen) or TALON® Metal Affinity Resins (Takara Bio) according to the manufacturer’s instructions. For the pull-down assay, GST-fused TPXL3 or TPXL3 truncations and His-fused AUR1 were mixed with glutathione beads and incubated at 4°C for 2 h. Beads were washed five times and proteins were eluted from beads by boiling in SDS loading buffer then separated by SDS-PAGE.

For in vitro kinase assay, GST, GST-TPXL3 and truncated GST-TPXL3 were incubated with GST-AUR1 in 40 µL of kinase buffer [10 mM HEPES (pH 7.5), 20 mM MgCl₂, 1 mM DTT, 5 mM EGTA, 0.1 mM ATP]. After incubation at 37°C for 60 min, the reactions were stopped by adding 10 µL of 5×SDS loading buffer and boiling for 10 min. Proteins were resolved by SDS-PAGE and phosphorylation was detected by the Pro-Q™ Diamond Phosphoprotein Gel Staining Kit (Thermo Fisher).

**Immunolocalization and Fluorescence Microscopy**

For immunolocalization, meristematic cells from root tips were used according to previously described protocols. The primary antibodies used here include the affinity-purified polyclonal rabbit anti-GFP
antibodies, DM1A mouse anti-α-tubulin monoclonal antibody (Sigma-Aldrich), goat anti-tubulin polyclonal antibodies (Cytoskeleton, Inc), G9 mouse anti-γ-tubulin monoclonal antibody \(^{50}\), and C2 mouse anti-FLAG monoclonal antibody (Shanghai Genomics). Secondary antibodies are fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG, FITC-conjugated donkey anti-mouse IgG, Texas Red-conjugated donkey anti-mouse IgG (Rockland Immunochemicals). Stained cells were observed under an Eclipse 600 microscope equipped with 60X Plan-Apo and 100X Plan-Fluor objectives (Nikon). Images were acquired by an OptiMOS camera (Q Imaging) controlled by the µManager software package.

For live-cell observation, 5-day-old seedlings were transferred to glass slides and submerged in water. Meristem cells were observed under an Axio Observer inverted microscope equipped with the LSM710 laser scanning confocal module (Carl Zeiss) by using a 40X C-Plan (water) objective. Images were acquired using the ZEN software package (Carl Zeiss) and processed in ImageJ (www.imagej.nih.gov/ij).

**Image processing to quantify spindle morphology**

To quantitatively evaluate spindle morphology, we first obtain binary image from the time-lapse confocal images by Gaussian filter (Sigma = 2 pixels) and Otsu’s thresholding (Supplementary Fig. 5A). From the binary images, the segmented spindle solidity and aspect ratio were analyzed using the ‘Analyze Particles’ command in the ImageJ software \(^{51},^{52}\).

**Declarations**

**ACKNOWLEDGEMENTS**

This work was supported by the NSF grant MCB-1920358. BL is supported by from the U. S. Department of Agriculture (USDA)-the National Institute of Food and Agriculture (NIFA) under an Agricultural Experiment Station (AES) hatch project (CA-D-PLB-2536-H). We are grateful to Dr. D. Van Damme for sharing the Aurora double mutant and Dr. T. Horio for G9 antibody.

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**Figures**
Figure 1

Retarded growth caused by repressed TPXL3 expression. (A) A chimeric MiR390a-based construct forms a hairpin structure via the amiRNA-TPXL3 and amiRNA-TPXL3* sequences. (B) Growth phenotypes of 9-week-old plants of the wild-type control (Col-0) and five independent amiRNA-TPXL3 transformants. (C) TPXL3 expression was repressed at different degrees in four amiRNA-TPXL3 lines as determined by real-time RT-PCR.
Figure 2

TPXL3 but not TPXL2 is associated with mitotic spindles. (A) Root cells express TPXL3-GFP under the control of the TPXL3 promoter. Mitotic cells have the GFP signal associated with spindles (arrowheads). (B) In a root expressing TPXL2-GFP under the TPXL2 promoter, the fusion protein shows nuclear localization but no mitotic cells are highlighted by the GFP signal. (C) Snapshots of a time-lapse movie (Supplemental Movie 1) illustrate characteristic localization patterns from prophase to cytokinesis in a dividing cell. Scale bars, 20 µm (A and B) and 5 µm (C).
Figure 3

Colocalization of TPXL3 and Aurora1. Immunolocalization images of TPXL3-GFP (pseudocolored in green), FLAG-AUR1 (red), and DNA (blue) in mitotic cells at different stages of cell division. The merged images have the three colors composited together and the yellow signal represents colocalization of TPXL3 and AUR1. (A) At late prophase, both TPXL3 and AUR3 are concentrated at spindle poles appeared in the polar caps. (B) The metaphase cell has both signals decorate kinetochore fibers. (C) Both TPXL3
and AUR1 remain at the shortened kinetochore fibers at anaphase and the proteins are barely detectable in the central spindle region between two segregating sets of sister chromatids. (D) Upon the arrival of chromatids at the spindle poles in telophase, remnant signal can be detected at the polar regions while new signal appears in the nuclear region opposite to the poles. (E) When the daughter nuclei are formed during cytokinesis, both TPXL3 and AUR1 signals become concentrated in areas close to the nuclear surface and facing the opposite nucleus. (F) When cytokinesis progresses, scarce signals are detected in the phragmoplast region while the nucelar envelope becomes highlighted. Scale bar, 5 µm.
Figure 4

TPXL3 is required for the localization of Aurora and g-tubulin in the mitotic apparatus. The merged images have DNA in blue and corresponding green and red signals combined. (A) A functional GFP-AUR1 fusion protein (green) decorates spindle microtubules (red) with biases towards spindle poles, leaving prominent red signal near the metaphase plate in the merged image. (B) In the amiR-TPXL3 mutant cells at metaphase, the GFP-AUR1 signal becomes mostly diffuse in the cytosol and no longer concentrated along spindle microtubules. (C) Quantitative assessment shows that spindle-associated GFP-AUR1 signal is greatly compromised and replaced by diffuse cytoplasmic signals as revealed by the ratio of spindle and cytoplasm GFP-AUR1 signals assayed in 20 cells. (D-G) Comparative localizations of TPXL3-GFP and g-tubulin in mitotic cells. At prophase, TPXL3 is heavily associated with the nuclear envelope where g-tubulin begins to accumulate (D). Both TPXL3 and g-tubulin are loaded on kinetochore fibers as demonstrated in the anaphase cell (E). At telophase, TPXL3 and g-tubulin remain at polar regions while weak TPXL3 signal is detected proximal to the chromatid mass, but g-tubulin is abundantly detected in the central spindle region (F). During cytokinesis when g-tubulin is heavily associated with the phragmoplast, TPXL3 can only be detected near the reforming daughter nuclei (G). (H, J) At metaphase, g-tubulin localization on spindle MTs in the control cell is replaced by mostly diffuse signal in the cytoplasm. (J) Quantitative assessment shows that spindle-associated g-tubulin signal is compromised and replaced by diffuse cytoplasmic signals as revealed by the ratio of spindle and cytoplasm g-tubulin signals assayed in 45 cells. Scale bar, 5 µm.
Figure 5

Functional domains of TPXL3. (A) TPXL3 is divided into five domains (I to V). Full length and truncated versions of TPXL3 are used in later assays. (B) Transient expression of TPXL3 and its derivatives in interphase cells. The nuclear localization of the protein is retained when either domain III or IV is included. The removal of domains III, IV, and V renders the truncated protein on cortical MTs and domain II is sufficient for the association. Domain IV overrides domain II to drive the truncations to the nucleus.
novel C terminal domain V assumes a generic nucleocytoplasmic localization pattern. (C, D) Both the TPXL3I+II-GFP and TPXL3II-GFP fusion proteins decorate the cortical MT network marked by the CKL6-mCherry fusion protein. Scale bar, 10 µm.

Figure 6

TPXL3 governs y Aurora localization and activates its kinase activity. Co-localization can be visualized in yellow color in the merged images. (A) Full length TPXL3 and AUR1 exhibit nuclear localization upon
transient expression in tobacco cells. (B) Co-expression of TPXL3I+II-GFP and TagRFP-AUR1 relocalize AUR1 to cortical MTs. (C) TagRFP-AUR1 remains in the nucleus when it is co-expressed with TPXL3II-GFP which decorates cortical MTs. (D) AUR1 is associated with and activated by TPXL3 as assayed by the Pro-Q phosphoprotein assay. The same gel is stained by SYPRO Ruby to reveal total proteins. Weak autophosphorylation activity of AUR1 is enhanced upon binding to TPXL3 which becomes phosphorylated. Such an effect is dependent on domain I which renders AUR1-binding. The TPXL3I+II truncation is sufficient for both the binding and activation of AUR1. Scale bar, 10 µm.
Figure 7

Mitotic MT reorganization in the control and amiRNA-TPXL3 mutant cells. Snapshots are taken from Supplemental movies 2 and 3. (A) When the control cell enters mitosis, MTs are organized into a fusiform pattern at late prophase (00:00). Such an array is later replaced by metaphase spindle after the cell establishes all kinetochore fibers that are converged towards spindle poles (arrowheads) (01:00-03:00). Spindle MTs continuously converge towards poles throughout mitosis (4:30-7:30). Upon the completion of mitosis, MTs in the spindle midzone are assembled and arranged in a mirrored configuration with a fluorescently dark line in the middle as a phragmoplast MT array (8:30-9:30). These phragmoplast MTs are shortened at distal ends while the array expands towards the cell periphery concomitantly with the disappearance of MTs towards the center (10:00-16:30). (B) In the amiRNA-TPXL3 mutant cell, MTs appear in discrete bundles at late prophase and prometaphase (00:00). In the rest of prometaphase, these MTs do not form a converged array and point at various directions (arrowheads) (1:00-3:00). Kinetochore fibers are established prior to their shortening during anaphase (4:00-7:00). MTs in the spindle midzone coalesce following mitosis and are developed into a phragmoplast array that expands towards the cell periphery, similarly as in the control cell (9:00-22:00). (C) The mean solidity of amiRNA-TPXL3 spindles is significantly reduced when compared to the control based on 23 and 15 movies, respectively (P = 0.0004261 by U-test). (D) The mean aspect ratio of amiRNA-TPXL3 spindles is significantly reduced when compared to the control based on 23 and 15 movies, respectively (P = 0.001246 by U-test). Scale bar, 5 µm.
Compromised spindle apparatus is associated with retarded plant growth. (A) Spindle MT arrays (red) in metaphase cells indicated by the DNA staining (blue) from indicated transgenic lines. While the amiRNA-TPXL3 mutant cell produces a spindle with disorganized spindle poles, the expression of the full-length microRNA resistant TPXL3R restores the spindle morphology. So is the truncated protein lacking C-terminal domain (TPXL3I-IV). While the deletion of domains I (TPXL3II-V) or II (TPXL3DII) does not render
a toxic effect to the amiRNA-TPXL3 spindles, the deletion of domain IV (TPXL3DIV) or III-V (TPXL3I-II) greatly enhanced the deformation of spindles that have kinetochore MT fibers arranged in parallel bundles. (B) Growth phenotypes associated with the expression of various TPXL3 derivatives. While the expression of microRNA resistant TPXL3R greatly suppresses the growth phenotype of the amiRNA-TPXL3 plant, the expression of TPXL3II-V or TPXL3DII does not significantly alter the growth of the mutant. However, the expression of TPXL3DIV or TPXL3I-II further enhances growth defects as indicated by severe inhibition of axial growth and production of inflorescence. (C) Down regulation of TPXL3 and a Aurora leads to enhanced growth retardation. While both the amiRNA-TPXL3 and aur1 aur2 mutants produce dwarf plants, the expression of amiRNA-TPXL3 in the aur1 aur2 mutant background further enhances defects in growth. (D) Hypothesized actions of a Aurora and TPX2/TPXL3 in spindle MT organization. a Aurora targets at proteins like Kinesin-5 via TPX2 for the stabilization of spindle MTs. Its association with TPXL3 leads to the activation of g-tubulin complex for MT nucleation in spindles. Scale bar, 5 µm.

Supplementary Files

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