A guide to plant TPX2-like and WAVE-DAMPENED2-like proteins

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Abstract

TPX2 proteins were first identified in vertebrates as a key mitotic spindle assembly factor. Subsequent studies demonstrated that TPX2 is an intricate protein, with functionally and structurally distinct domains and motifs including Aurora kinase binding, importin-binding, central microtubule binding, C-terminal TPX2 conserved domain, among others. The first plant TPX2-like protein, WAVE-DAMPENED2, was identified in *Arabidopsis* as a dominant mutation responsible for reducing the waviness of roots grown on slanted agar plates. Each plant genome encodes at least one “canonical” protein with all TPX2 domains and a family of proteins (20 in *Arabidopsis*) that diversified to contain only some of the domains. Although, all plant TPX2-family proteins to date bind microtubules, they function in distinct processes such as cell division, regulation of hypocotyl cell elongation by hormones and light signals, vascular development, or abiotic stress tolerance. Consequently, their expression patterns, regulation, and functions have diverged considerably. Here we summarize the current body of knowledge surrounding plant TPX2-family proteins.

Summary statement

TPX2 protein family consists of WAVE-DAMPENED2-like (WDL) and TPX2-like (TPXL) groups. WDLs govern cell expansion in response to ethylene, light, brassinosteroids, and abiotic stresses. TPXLs function in mitotic spindle assembly and activating Aurora kinase.

Introduction

Microtubules in plants direct intracellular trafficking and govern spatial distribution of organelles. Organization of microtubules changes to suit any developmental or physiological

situation that a plant could encounter throughout its life cycle. Of the most spectacular examples is assembly of structurally and functionally distinct microtubule arrays during mitosis: the pre-prophase band, mitotic spindle, anaphase spindle, the phragmoplast, and cortical microtubule array. The pre-prophase band forms during transition from G2 to prophase and marks the position of the division plane; the metaphase spindle ensures equal inheritance of information between daughter cells; the anaphase spindle separates daughter chromatids; and the phragmoplast constructs partition between daughter cells (reviewed in [1]). The cortical microtubule array forms during interphase and amongst many roles determines the direction of cell expansion. Typically, cells expand perpendicular to the orientation of cellulose microfibrils in the cell wall, which in turn mimics orientation of cortical microtubules [2-4]. Ability to direct orientation of cellulose microfibrils makes microtubules a key effector of signalling pathways that regulate plant morphogenesis [5,6].

Organization of microtubules is governed by an ensemble of microtubule-associated proteins (MAPs) that facilitate formation of new microtubules; stabilize, destabilize, or sever microtubules; link microtubules together or to other structures; and facilitate trafficking along microtubules (reviewed in [7]). Some MAPs are conserved across all eukaryotes, e.g. microtubule nucleating gamma-tubulin ring complex [8], microtubule severing protein katanin [9], or microtubule polymerization factor MOR1/GEM1 [10,11]. Other MAPs are plant-specific e.g. microtubule-stabilizing proteins MAP70 [12,13]. Several groups of MAPs are partially conserved. One of them are TPX2-like (TPXL) and WAVE-DAMPENED2-like proteins that combine evolutionarily conserved and plant-specific domains [14,15]. Here we overview the current body of knowledge about this family of proteins and highlight the key knowledge gaps.

Discovery of plant TPX2 proteins

TPX2 was originally identified in vertebrates as a Targeting Protein for *Xenopus laevis* kinesin-like protein 2 [TPX2; 16,17]. Depletion of TPX2 from *Xenopus* egg total protein extracts using TPX2 antibody abrogated targeting of *Xenopus laevis* kinesin-like protein 2 (Xklp2) to the spindle poles and resulted in mitotic failure. Subsequent studies revealed that TPX2 is an evolutionarily conserved microtubule-binding protein in vertebrates and invertebrates [18-21]. The list of TPX2 functions during mitosis in vertebrates encompasses targeting Aurora A protein kinase to the spindle poles; activating Aurora A [18,22]; and promoting branching microtubule nucleation by increasing the efficiency of microtubule nucleation in concert with the γ-tubulin ring complex (γ-TuRC) [23,24]. TPX2 can also promote microtubule nucleation by stabilizing early nucleation intermediates [25-27].

Each of the above TPX2 functions is undertaken by specialized domains (**Figure 1**). The N-terminus contains an Aurora-binding and activation domain [28]. The centrally positioned importin-binding domain, called the TPX2/Importin domain, mediates the localization of Xklp2 to the mitotic spindle [29-31]. A nuclear localization signal (NLS) keeps TPX2 in the nucleus during interphase [32]. During mitosis activity of TPX2 is inhibited through associates with α/β Importin complex TPX2 [19,33]. A high concentration of RanGTP, produced by the chromatin component Ran-GEF RCC1, sequesters importin β causing dissociation of importin α from TPX2. Then TPX2 can contribute to microtubule nucleation, and Aurora A targeting and activation [17,34]. γ-TuRC activation is facilitated by a motif near the C-terminus of the protein [24]. The kinesin-interacting domain and a highly conserved, but poorly characterized, TPX2-C domain (PF6886) are located on the very C-terminus (**Figure 1**).

The first TPX2-family protein in plants, WAVE-DAMPENED 2 (WVD2) was identified in *Arabidopsis thaliana* activation tag screen for altered root growth on slanted agar plates [35]. Over-expression of WVD2 resulted in altered anisotropic cell expansion and, consequently, shorter and thicker roots and etiolated hypocotyls, with shorter and wider leaves [35]. Analysis of the *A. thaliana* reference genome revealed one TPX2-family protein, *At*TPX2, showing ca. 40% amino acid sequence similarity with human TPX2 and containing all conserved domains (**Figure 1**) [36,37]. In addition, a TPX2 signature motif composed of 26-28 amino acids was identified within the TPX2/Importin binding domain of this protein (PF12214) of *At*TPX2 [36].

All angiosperm genomes sequenced to date contain at least one TPX2 protein with all conserved domains and a family of proteins lacking certain domains [14,15,38-40]. For example, *A. thaliana* genome has 20 TPX2-family genes (**Table 1; Figure 2; Table S1**) with highly diverse sequences and functions. The functions of TPX2-family proteins extend beyond cell division into facilitating anisotropic cell expansion in response to developmental and environmental cues. Below we summarize the current knowledge on plant TPX2-family proteins.

Diversity of TPX2 proteins in plants

TPX2-family proteins were first classified according to the TPX2-C sequence of WVD2 [35,38,39,41]. TPX2-C of *A. thaliana* WVD2 has been noted to have a second haplotype containing a plant-specific “KLEEK” motif [35], hereinafter referred to as TPX2-C^{KLEEK}. Four major clades of WAVE-DAMPENED2-like (WDL) proteins were identified in *Arabidopsis*, aspen, *Eucalyptus,* cotton, moss and others (**Figure 3**) [14,15,38-40,42]. Proteins with TPX2-C^{KLEEK} form two clades, WDLA and WDLB **(Figure 2, Figure 3)**, whereas proteins lacking the TPX2-

C^{KLEEK} constitute the MAP20 clade [39,40]. Currently, no information is available on what protein sequence features contribute to segregation of proteins into WDLA and WDLB clades.

Although analysis of 574 TPX2-like plant protein sequences in the Gene Bank identified TPX2-C^{KLEEK} in representatives of WDLC clade, *Arabidopsis* members of this clade, with exception of *At*WDL7, lack the KLEEK motif (**Figure 2**). Most likely, these sequences group for the conserved N-terminal WAVE-DAMPENED2-like New Domain (WAND) with yet unknown functions (**Figure 2, Figure 3**). One member of this clade, MDP40, lacks any TPX2-domains meaning MDP40 *de-facto* is not a TPX2-family protein.

Several TPX2-family proteins contain the N-terminal Aurora binding and central TPX2/Importin binding domains, but lack the TPX2-C [43-45]. These proteins were named TPX2-like proteins (TPXL). The latest phylogenetic study in *A. thaliana* with the sequences of the N-terminal Aurora binding domain and central TPX2-signature domain identifies a novel clade consisting of TPXL proteins TPXL2/3/4/8 (**Figure 2, Figure 3; Table S1**) [44].

TPX2-family genes have a complex non-overlapping transcription pattern. The most ubiquitous is WDLB clade followed by WDLA and TPX2 [39,40]. The TPX2 clade exhibits the highest expression in roots of *Eucalyptus* [39], whereas expression of WDLA and WDLB is the highest in developing cotton fibres [40]. Members of WDLA clade in cotton are strongly upregulated 20 days post anthesis [40]. *MAP20* expresses mostly during late differentiation stages of phloem and xylem in poplar and Brachypodium distachyon [38,46].

In summary, the hodgepodge of domain architecture within members of TPX2-family proteins taken together with the transcriptomics data indicates that functions of these proteins were evolved to fine-tune microtubule dynamics in the context of distinct, but highly specialized cellular processes (**Supplemental Table 2**).

Functions of TPX2-family proteins during cell division

Activation of Aurora kinase and targeting of Aurora kinase to microtubules is an essential activity of animal TPX2. Animal Aurora kinase regulates mitotic spindle assembly and karyokinesis [18,22]. *A. thaliana* Aurora kinase in addition to the above functions also contributes to establishing new tissues by orienting formative cell divisions [47]. Animal Aurora kinase requires activation and one mechanism is autophosphorylation of the activation loop [Reviewed in 48]. Using ancestor sequence reconstruction, it was shown that TPX2 is the second mechanism to evolve for allosteric activation of Aurora A in human [49].

Activation of Aurora kinase by TPX2 family proteins in plants is supported by several observations in *A. thaliana*. First, TPXL2 and TPXL3 interact with Aurora 1 and 2 under high-

stringency tandem affinity tag purification and yeast two-hybrid analyses, and TPX2 interacts with both kinases only under the least stringent wash conditions [45]. Fluorescent lifetime microscopy confirms stronger interaction of Aurora 1-RFP with GFP fusions of TPXL2 or TPXL3 than with TPX2-GFP [45]. Second, either full-length *A. thaliana* TPX2 or its first 100 amino acids fragment which includes the Aurora-binding domain enhance both Aurora 1 auto-phosphorylation activity and the downstream histone 3 phosphorylation activity [43]. N-terminal domains of TPXL2 and TPXL3 also activate Aurora 1 kinase [45].

Notably, Aurora 1 phosphorylates both full-length TPX2 and TPX2 lacking the Aurora-binding domain [43]. Candidate Aurora phosphorylation sites were also identified in TPXL2 and TPXL3 [45]. Currently, the role of TPXL phosphorylation by Aurora remains unknown. Thus, plant TPXL can bind and activate Aurora 1 through the conserved Aurora-binding domain on the N-terminus and Aurora 1 can phosphorylate TPX2 independently of the binding domain.

TPXLs exhibit localization that is typical for microtubule-binding proteins. Antibody against human TPX2 labelled nuclei prior to nuclear envelope breakdown (NEB), mitotic spindle and spindle poles, but not the phragmoplast in *Nicotiana tabacum* BY-2 cells [36]. Similar localization during mitosis was reported in *Arabidopsis* root cells stably expressing GFP-TPX2 [45,50,51]. TPXL2 and TPXL3 localise to the nuclear envelope during prophase, associate with mitotic spindle microtubules, and concentrate around daughter nuclei after anaphase. In addition, TPXL3 localizes to the phragmoplast distal zone [45].

TPXL proteins play an essential role in mitosis. Microinjecting the anti-human TPX2 into *Tradescantia* stamen hair cells prevented the onset of mitosis and NEB [36]. Knockout of *TPXL3* in *Arabidopsis* resulted in embryo-lethality [45]. On the contrary, *TPXL2* loss-of-function allele *tpxl2* and two TPX2 loss-of-function alleles *tpx2-3* and *tpx2-4* exhibited no discernible phenotype [45]. These observations suggest functional redundancy between TPX2 and TPXL proteins, but a unique role for TPXL3 (**Figure 4**).

The role of TPXLs in Aurora targeting was thus far addressed only in the interphase cells. These experiments were facilitated by the discovery that ectopic expression of GFP-TPX2 or GFP-TPXL3 in *Nicotiana benthamiana* leaf pavement cells results in labelling of the intra-nuclear microtubules, whereas TPXL2 and Aurora 1 localize in the nucleoplasm [37,45]. Co-expression of TPXL3 with Aurora 1 was sufficient for targeting Aurora 1 to the intranuclear microtubules; co-expression of TPXL2 and Aurora 1 resulted in both proteins associated with microtubules; and somewhat surprisingly, Aurora 1 caused dissociation of TPX2 from intranuclear microtubules [45]. Transient over-expression experiments in the interphase cells showed microtubule and nuclear localization of TPXL1, TPXL2, TPXL3, TPX4 and TPX8;

microtubule only localization of TPXL5 and TPXL6; and nuclear envelope only localization of TPXL7 [44]. Co-expression of these proteins with Aurora kinase relocated TPXL7 into nucleus and caused stronger nuclear labelling of other proteins with exception of TPXL1 [44]. This outcome suggests involvement of many TPXLs in Aurora localization and activation (**Figure 4**; **Supplemental Table 2**).

The origin of intranuclear microtubules and their function remain unclear. Although plant nuclei reportedly contain tubulin [52], microtubules were not reported using conventional live-cell imaging probes. Plausibly, ectopic TPXL expression stimulates microtubule assembly from the nuclear tubulin pool. The next crucial step will be determining how the above findings *in vitro* and in interphase cells relate to Aurora activation and targeting during mitosis.

TPX2 binds microtubules in *aur1/aur2* demonstrating that TPX2 can associate to spindle microtubules independently of Aurora (corroborating findings of studies in animal systems). Aurora 1 localizes to spindle MTs in *tpx2-3* mutant cells further supporting functional redundancy of canonical TPX2 in plants [45].

In conclusion, of many known TPX2 functions in animals, only activation of Aurora kinase has been examined in plants thus far. Furthermore, at least three plant TPXL proteins appear to be responsible for the mitotic activities of single animal TPX2. Considering profound differences of spindle construction between plants and animals, one of which is lacking structurally-defined spindle poles, TPXL proteins might play many unique roles in plant mitosis.

Functions of WDLs in anisotropic cell expansion and signalling

Orientation of cortical microtubules governs the direction of cell expansion by influencing orientation of cellulose microfibril deposition in the cell wall [53,54]. In expanding cells, the expansion axis is generally perpendicular to the overall orientation of microtubules, and consequentially the cellulose microfibrils [55]. Cortical microtubules influence orientation of cellulose microfibrils by serving as tracks for movement of cellulose synthase complexes [56,57]. As cell expansion drives plant morphogenesis, microtubule organization appears to be a key effector in many hormonal pathways [5,58,59], though mechanistic understanding of this phenomenon remains limited. Several *A. thaliana* WDLs, WDL3, WDL5, MDP60, and MDP40, contribute to regulation of microtubule orientation, cell expansion, and hypocotyl elongation in response to ethylene, brassinosteroids, and light (**Figure 5; Supplemental Table 2**).

The idea that WDLs govern cell expansion was originally proposed by Yuen and co-authors based on reduction of cell length in roots of *A. thaliana* plants overexpressing *A. thaliana* WVD2 or WDL1 [35]. Roots in these plants were shorter and leaves, siliques, and

etiolated hypocotyls were smaller. Furthermore, the epidermal cells of etiolated hypocotyls exhibited right-handed circumferential rotation and rosette leaves exhibited left-handed circumferential rotation. The knockout phenotype of *WVD2* and *WDL1* was not reported thus far, but analysis of mutants in other *WDL* genes in *A. thaliana* support importance of these proteins in cell expansion.

Biochemical properties of WDLs.

All *A. thaliana* WDLs studied thus far bind microtubules *in vivo* or *in vitro*. *Eucalyptus* WDL3-YFP and WDL3L-GFP localize to cortical microtubules in tobacco leaf pavement cells [39,40]. Cotton WDLA2 and WDLA7 also bind cortical microtubules in tobacco leaf pavement cells and interact with α-tubulin TUA2 in yeast two-hybrid assay, whereas WDLA4 and WDLA9 show diffused localization in the cytoplasm and nucleus [40].

A. thaliana WVD2, WDL3 and WDL5 stabilize microtubules. For example, WDL3 and WDL5 reduce dilution-induced depolymerization of microtubules *in vitro* and cortical microtubules in *WDL5* knockout or *WDL3* knockdown are more sensitive to the inhibitor of microtubule polymerization oryzalin [60,61]. Over-expression of WDL3 in *A. thaliana* increases microtubule tolerance to oryzalin [61]. Furthermore, these proteins can also bundle microtubules. Structural illumination microscopy reveals reduced microtubule bundling in cells of *A. thaliana wdl5-1* etiolated hypocotyls relative to the wild-type control [62]. All three proteins produce microtubule bundles *in vitro*, but electron microscopy shows microtubules in bundles induced by WDL3 and WVD2 are "glued" together without an apparent linker [41,60,61]. Over-expression of WVD2 in *A. thaliana* seedlings increases bundling of microtubules (zippering) and reduces frequency of catastrophe events compared with controls [41].

On the contrary, *A. thaliana* MDP60 and MDP40 destabilize microtubules [63,64]. MDP60 binds microtubules *in vitro* and *in vivo* [64]. Incubation of microtubules with MDP60 *in vitro* generates somewhat shorter microtubules and over-expression of MDP60 increases microtubule sensitivity to oryzalin. *MDP60* knockout makes microtubules more tolerant to oryzalin treatment implying MDP60 acts through direct interaction with the microtubule lattice.

Knockdown of *MDP40* in *A. thaliana* also makes microtubules more tolerant to oryzalin treatment [63]. Although MDP40-GFP binds microtubules *in vivo*, direct interaction between MDP40 and microtubules has not yet been examined [63]. This indicates MDP40 could destabilize microtubules by affecting activity of known microtubule-severing factors or through interaction with other proteins. If the WAND domain functions as a protein interface, MDP40 could localize to microtubules through interaction with this domain of WDL7, WDL8, or WDL9.

The smallest family member, MAP20, was reported in aspen as a target of the cell-wall synthesis herbicide 2,6-dichlorobenzonitrile [38]. GFP fusions of MAP20 from aspen, *Eucalyptus,* and *B. distachyon* were shown to bind microtubules in the interphase cells [38,39,46]. Furthermore, aspen and *B. distachyon* MAP20 can directly bind and stabilize microtubules *in vitro* [38,46]. *B. distachyon* MAP20 suppresses microtubule depolymerization by reducing tubulin loss from microtubule ends [46]. Over-expression of aspen or *Eucalyptus* MAP20 in *Arabidopsis* perturbs normal cell expansion and causes deformation and twisting of cotyledon and hypocotyl similarly to the *wvd2* phenotype [35,39]. Comparison of *B. distachyon* MAP20 activity with that of *Xenopus* TPX2 in the *Xenopus* egg total protein extract demonstrated that MAP20 does not facilitate microtubule nucleation by γ-TuRC, but promotes microtubule elongation [46]. Collectively, MAP20, WVD2, WDL3 and WDL5 act as microtubule stabilization factors *in vitro* and *in vivo*.

Ethylene and light signalling

Hypocotyl length is controlled by light and phytohormone signals, which modulate cell length and microtubule orientation [65,66]. Dark-induced hypocotyl cell elongation is accompanied by transverse orientation of microtubules, whereas suppression of cell elongation by light results in oblique and longitudinal microtubules [67]. Ethylene plays an important role in this process; exposure to ethylene promotes formation of longitudinal microtubules and inhibits cell elongation resulting in a short, thick, curled plant phenotype [68]. Ethylene signalling occurs through a well-studied pathway consisting of endoplasmic-reticulum membrane bound protein EIN2 (Ethylene Insensitive 2). The C-terminal region of EIN2 is cleaved and localizes to the nucleus where it activates transcription factors EIN3 and EIL1 that drive the expression of ethylene-responsive genes [reviewed in 69]. Out of these two transcription factors, EIN3 plays an essential role in regulating expression of several TPX2-family proteins.

One of EIN3 targets in *A. thaliana* is *WDL5* (**Figure 5B**). EIN3 binds to *WDL5* promoter through three conserved motifs and promotes *WDL5* transcription in response to ethylene [60]. Several observations demonstrate that ethylene promotes microtubule stability through WDL5. First, pre-treatment of seedlings with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) makes microtubules more stable resulting in lower susceptibility to oryzalin in control but not in *wdl5-1* plants [60]. Second, Sun et al. showed that microtubules are less stable in ethylene unresponsive mutants *ein2-5* and *ein3eil1* [70,71] and more stable in constitutively activated ethylene pathway mutant *ctr1-1* [62].

Impaired microtubule stability in *A. thaliana wdl5-1* knockout allele correlates with abrogation of microtubule response to ethylene. Microtubules in hypocotyl epidermal cells of both *ein2-5* and *wdl5-1* alleles stay predominantly transverse even after ACC treatment [62], whereas microtubules in *ctr1-1* are mostly longitudinal, oblique, or random even without ACC treatment [62]. Epidermis cells of etiolated hypocotyls of *wdl5-1* were longer than control plants either with or without ACC treatment [62]. Consequently, etiolated hypocotyls of *wdl5-1* were longer indicating WDL5 functions in ethylene-dependent inhibition of cell elongation.

Another target of ethylene is phytochrome-interacting factor 3 (PIF3) [72]. Light suppresses hypocotyl elongation by inducing degradation of PIF3 whereas ethylene promotes hypocotyl elongation by up-regulating *PIF3* expression. PIF3, in turn, binds the *MDP60* promoter [64]. Consequently, both ethylene and PIF3 upregulate *MDP60* transcription (**Figure 5B**). MDP60 promotes assembly of transverse microtubules [64]. Consequently, the frequency of transverse microtubules in response to light or ACC depends on the *MDP60* gene-dosage. Hypocotyl epidermal cells and hypocotyls of light-grown *mdp60* knockout allele are shorter than in control seedlings, whereas over-expression of *MDP60* in wild-type background causes longer hypocotyl epidermal cells and longer hypocotyls [64]. Furthermore, *MDP60* over-expression can rescue shorter hypocotyl phenotype in the *pif3* or ethylene-insensitive *ein2* mutant backgrounds. Thus, MDP60 mediates ethylene-induced transverse orientation of microtubules and hypocotyl elongation in response to light (**Figure 5A**).

COP1-dependent signaling

Another important regulator of light signaling is an ubiquitin E3 ligase CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1)[73,74]. COP1 represses photomorphogenic responses, such as hypocotyl elongation, by conjugating ubiquitin with positive regulators of photomorphogenesis for degradation by the 26S proteasome [75,76]. In dark-grown plants, COP1 primarily localizes to the nucleus, however some is also present in the cytoplasm [reviewed in 74]. Cytoplasmic COP1 was shown to interact with *A. thaliana* WDL3 in pull-down, yeast-two-hybrid, firefly luciferase complementation imaging, and co-immunoprecipitation assays [77]. Bimolecular fluorescence complementation and *in vitro* fluorescent microtubule assays showed interaction between WDL3 and COP1 at the cortical microtubules [77].

WDL3 transcript was detected in both light and dark-grown *A. thaliana* seedlings, however WDL3-GFP was only observed in light-grown seedlings. Treatment with 26S proteasome inhibitor restored WDL3-GFP in dark-grown seedlings suggesting that 26S

proteasome is responsible for WDL3 degradation [61,77]. COP1 is responsible for ubiquitination of WDL3 prior the degradation (**Figure 5B**).

WDL3 promotes formation of longitudinal microtubule arrays in the cells of light-grown seedlings (**Figure 5A**). Consequently, transverse to longitudinal switch of microtubules in hypocotyl cells during light response was partially abrogated in *WDL3* RNAi plants. The light-grown *WDL3* RNAi seedlings had longer hypocotyl epidermal cells and longer hypocotyls, while over-expression of WDL3 resulted in shorter cells and shorter hypocotyls. Furthermore, *WDL3* knockdown can partially rescue reduced dark-induced hypocotyl elongation in *cop1-6* mutants [77]. Thus, WDL3 is a downstream factor of COP1-mediated hypocotyl cell elongation (**Figure 5A,B**).

Brassinosteroids signaling

Brassinosteroids regulate almost all aspects of plant life including cell elongation and hypocotyl growth through transcription factor BRASSINAZOLE-RESISTANT1 (BZR1)[reviewed in 78]. Although several upstream components of brassinosteroid-signaling have been linked to regulation of hypocotyl growth through BZR1 phosphorylation, the downstream effectors remain less understood [reviewed in 78].

BZR1 binds to the *MDP40* promoter and up-regulates *MDP40* transcription in *A. thaliana* (**Figure 5B**)[63]. Treatment of seedlings with the brassinosteroid, brassinolide, induces formation of transverse cortical microtubule arrays in cotyledon epidermal cells of the wild-type but not in the *MDP40* knockdown seedlings grown in the dark. Etiolated hypocotyls of *MDP40* RNAi knockdown lines were shorter than in wild-type seedlings [63]. Overexpression of *MDP40* rescues the short cell phenotype in the hypocotyl of brassinosteroid synthesis-deficient mutant, *det2* [79]. This indicates that MDP40 contributes to brassinosteroids-induced hypocotyl elongation in the dark.

Stress adaptation

WDLs play an essential role in response and adaptation to environmental stresses. Salt stress was found to activate *WDL5* transcription through the ethylene pathway in *A. thaliana* seedlings [80]. One of the salt stress phenotypes is depolymerization of microtubules and plant survival depends on the ability of cells to re-polymerize microtubules [reviewed in 81]. Several observations highlight importance of ethylene in this process. First, microtubules in *ein2-5* hypocotyl and cotyledon epidermal cells are hypersensitive to depolymerization under NaCl treatment [80]. Second, *ein3eil1* double knockouts failed to reassemble microtubules in

cotyledon pavement cells under salt stress. WDL5 appears to be the ethylene effector responsible for re-polymerization of microtubules. *wdl5-1* cells frequently failed to reassemble microtubules after 30-48 hours of salt stress, whereas over-expression of *WDL5* resulted in faster regeneration of microtubule network and nearly doubled plant survival in medium with 200 mM NaCl. Furthermore, *WDL5* overexpression rescues microtubule reassembly defects and salt-susceptibility phenotype of *ein3eil1* double knockouts [80].

MDP60 contributes to hypocotyl elongation in response to submergence stress in *A. thaliana*. Submergence causes microtubule orientation switch from longitudinal to transverse [82]. Two facts suggest MDP60 is essential for this response: *MDP60* transcription increases after 2 hours of seedling submergence, and *MDP60* knockout partially abrogates this microtubule reorientation. Upregulation of *MDP60* transcription and microtubule re-orientation is suppressed in the ethylene-insensitive mutant *ein2*, suggesting similarities between regulation of microtubule orientation in response to light and stress [82].

MAP20 appears to be essential for adaptation to drought in *B. distachyon*. MAP20 localizes to the edges of vascular pits in developing xylem cells [46]. Pits play a dual role in facilitating transport of solutions through the xylem during the rainy season and preventing the spread of air pockets (embolisms) under drought [83-85]. Knockdown of *MAP20* in *B. distachyon* resulted in larger pits with thinner pit membranes and greater drought susceptibility [46]. However, the cell wall thickness and cellulose content in the mutant was reduced only slightly, suggesting non-linear relationship between MAP20 expression level and cell wall synthesis [46]. Determining pit architecture in xylem cells is likely to be one of many MAP20 functions.

Concluding remarks

TPX2-family proteins deserve attention for their exciting evolutionarily history and functional diversity. Gaining insight into the activity of these proteins will advance our knowledge about regulation of microtubules in the context of different processes while enabling the development of predictive models of plant responses to environmental and developmental cues. Sustaining progress in analysis of these proteins requires addressing the following challenges:

- Characterize functions of all members of TPX2-family proteins (**Supplemental Table 2**).
- Determine how TPX2-family proteins promote microtubule polymerization and organization both *in vivo* and *in vitro*; how MDP60 and MDP40 destabilize microtubules.
- Advance understanding in the relationships between Aurora kinase and TPXL proteins; determine why some TPXL have lost TPX2-C; what is special about functions of TPXL3;

what are the functions of TPXL phosphorylation by Aurora. In addition, understanding the role of plant TPX2 protein if it is not the main targeting factors of Aurora to spindle microtubules.

- Determine whether TPXL are regulated by importins, can interact with kinesins, activate γ-TuRC, and can nucleate microtubules.
- Characterize WAND domain and find out why MDP40 contains WAND domain but lost all TPX2 domains.

Another intriguing question is whether WDL proteins have activities beyond regulation of microtubule stability during interphase; for instance activation and targeting to microtubules of proteins kinases or signalling factors.

Data availability

All protein sequence data and alignment using in the review are available freely upon request.

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Author contribution

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Figure legends

Figure 1. Conserved domains in human and *Arabidopsis* TPX2.

Figure 2. Domain organization of *Arabidopsis* TPX2-family proteins.

A. TPX2 has all conserved domains whereas other members of TPX2-family keep only some domains. In addition, several members have a plant-specific WAND domain. Position of the domains is listed in **Table S1**.

B. Alignment of the N-terminal region of TPX2-family proteins containing WAND domain with the corresponding region of TPX2. The WAND domain is highlighted in red.

Figure 3. The grouping of TPX2-family proteins.

Phylodendrogram of *Arabidopsis* (blue font) and *Eucalyptus* (red font) TPX2-family proteins. The bootstrap values were calculated from 1000 repeats. Branches with the bootstrap values below 60% were collapsed. TPX2, TPX2-like, MAP20, WDLA, WDLB and WDLC clades constitute TPX2-like and WDL2-like groups.

Figure 4. Role of TPXL proteins in cell division.

TPX2, TPXL2, and TPXL3 localize to the nuclear envelope, but not to the microtubules of the pre-prophase band during prophase. Aurora kinase also localizes to the nuclear envelope membrane. It is currently not known whether TPXLs and Aurora interact on the nuclear envelope and require each other for this localization. TPXL function redundantly in promoting nuclear envelope breakdown and mitotic spindle assembly. In the mitotic spindle, TPXLs target Aurora kinase to microtubules and activate it.

Figure 5. Antagonistic interactions between WDLs modulate hypocotyl elongation.

A. WDL3 and WDL5 stabilize microtubules, facilitate longitudinal orientation of microtubules and reduce cells elongation whereas MDP40 and MDP60 destabilize microtubules, promote transverse microtubule arrays, and stimulate cell elongation. The balance between activity of these proteins could fine-tune hypocotyl length under either light or dark conditions.

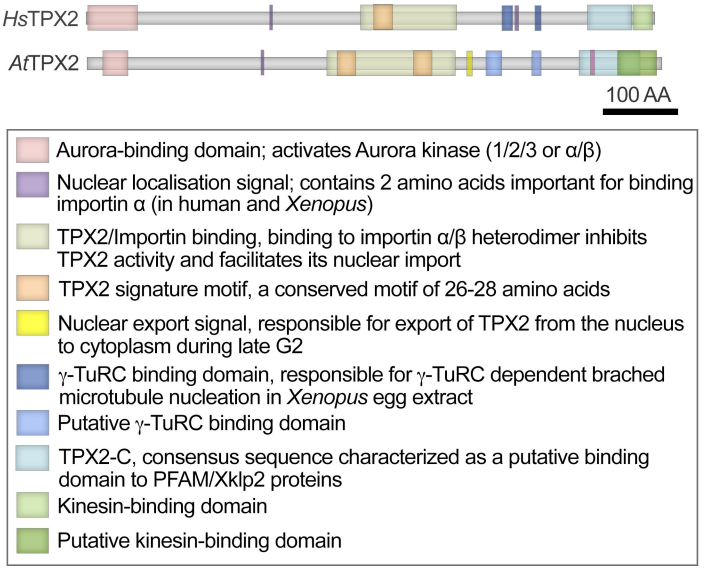
B. Regulation of WDL3, WDL5, MDP40 and MDP60 by light and hormones. Ethylene binding to ETR1 in the endoplasmic reticulum (ER) causes inactivation of kinase CTR1. This leads to cleavage of EIN2 and release of peptide CEND that moves to the nucleus, where it activates

transcription factor EIN3. Under light conditions EIN3 promotes transcription of *PIF3*; PIF3 promotes transcription of *MDP60*; MDP60 destabilizes microtubules. *WDL3* expression under light promotes microtubule stability. It remains unknown which pathway regulates transcription of *WDL3*. WDL3 could counteract MDP60. In the dark, the cytoplasmic COP1 ubiquitinates WDL3 and triggers its degradation. In parallel, EIN3 promotes transcription of *WDL5* and WDL5 stabilizes microtubules. Brassinosteroids bind BRI1-BAK1 receptor complex that through several cytoplasmic proteins including BSU1 activates BZR1. BZR1 promotes transcription of *MDP40*. Destabilization of microtubules by MDP40 could counteract WDL5.

Activating interactions are shown in red and inhibiting interactions are shown in red.

Table 1. Accession numbers, properties, and published nomenclature of *Arabidopsis* TPX2-family proteins.

Accession Number	Length (AA)	MW	pI	Yuen et al., 2003	Perrin et al., 2007	Rajangam et al., 2008	Vos et al., 2009	Wang et al., 2012	Tomstikova et al., 2015	Ma et al., 2018	Unpublished
At3g04630	287	32.1	10.1	WDL1							
At5g28646	202	23.3	9.1	WVD2							
At1g54460	338	37.4	10.3	WDL2							
At3g23090	338	37.8	9.6		WDL3						
At2g35880	432	46.7	10.5		WDL4						
At4g32330	437	47.5	9.0		WDL5						
At2g25480	404	44.3	9.3		WDL6						
At1g70950	478	53.2	7.8		WDL7						
At3g01710	391	43.9	10.3								WDL8
At3g26050	533	58.9	10.8								WDL9
At5g44270	309	35.9	11.0						TPXL7		
At1g03780	758	86.5	10.2				TPX2				
At3g01015	488	56.2	9.9						TPXL1	MDP60	
At5g15510	497	56.5	10.0						TPXL5		
At5g37478	178	20.4	9.92			MAP20			TPXL6		
At4g22860	509	58.1	10.1						TPXL3		
At4g11990	501	57.2	9.5						TPXL2		
At5g07170	397	45.1	9.7						TPXL4		
At5g62240	377	43.2	10.2						TPXL8		
At1g23060	361	41.0	10.6					MDP40			



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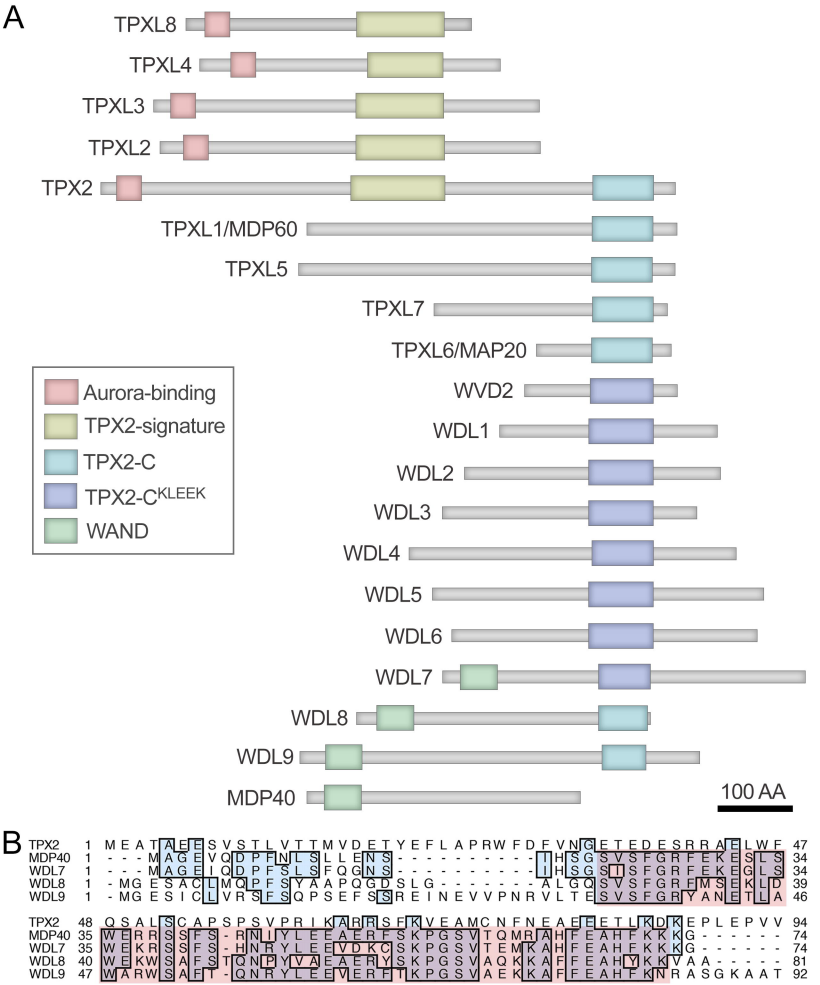

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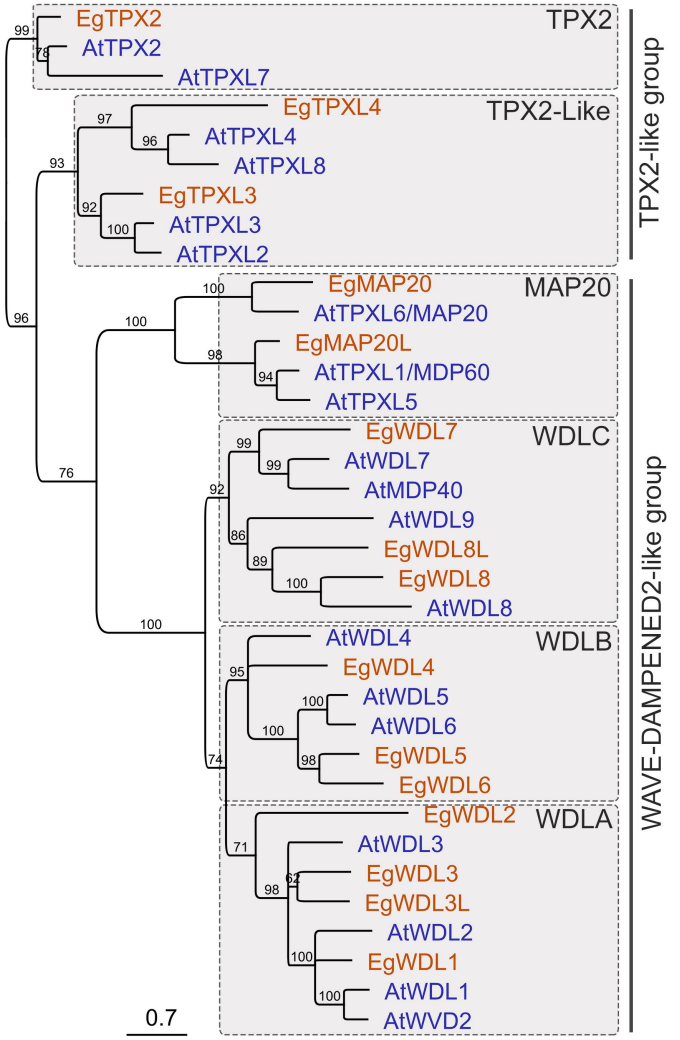

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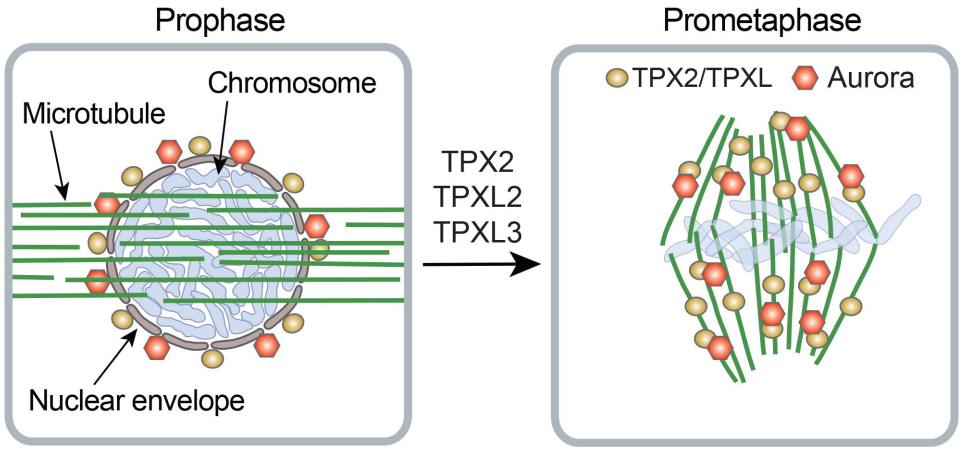

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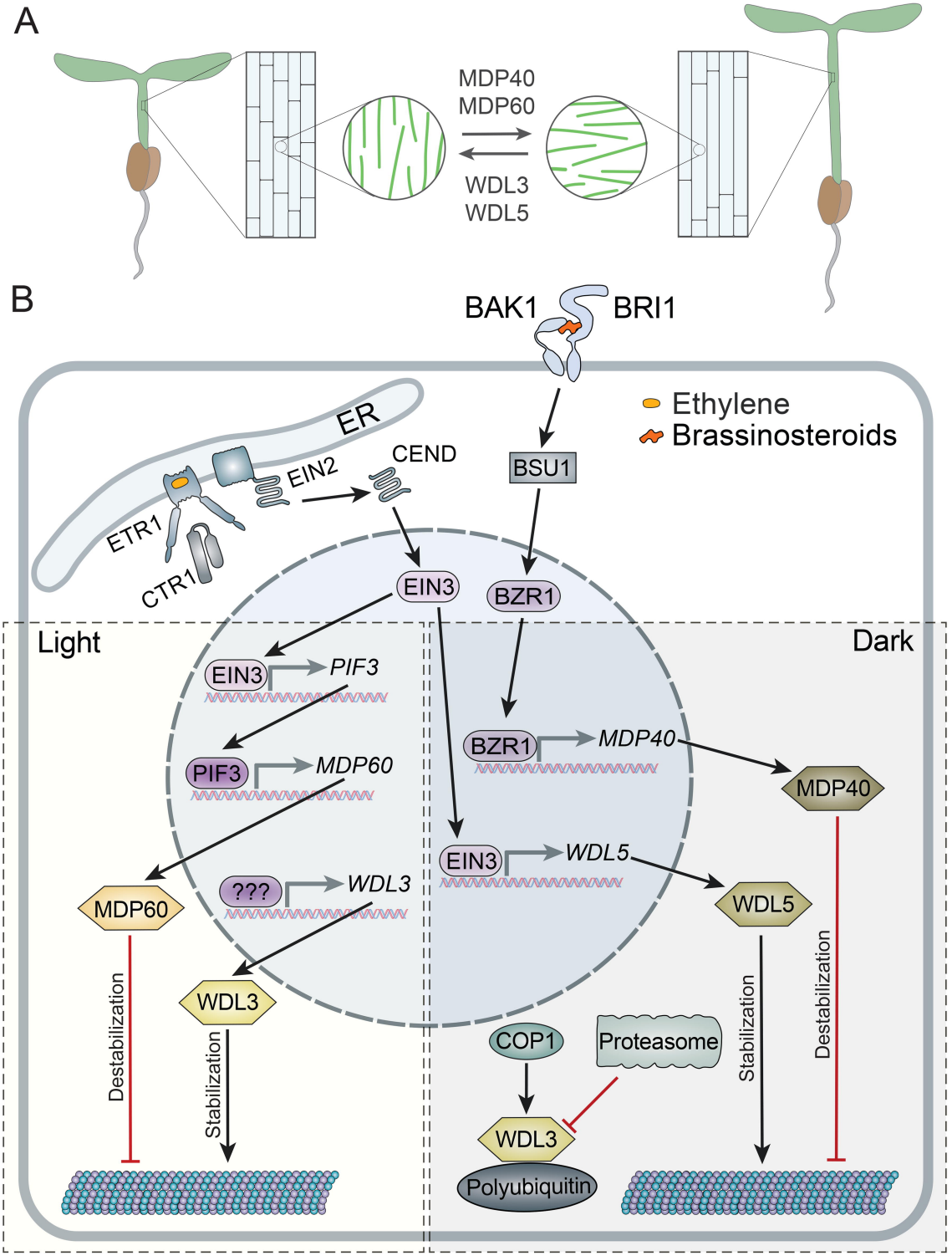

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