Structure, function, and evolution of plant NIMA-related kinases: Implication for phosphorylation-dependent microtubule regulation

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Structure, function, and evolution of plant NIMA-related kinases: Implication for phosphorylation-dependent microtubule regulation

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Abstract Microtubules are highly dynamic structures that control the spatiotemporal pattern of cell growth and division. Microtubule dynamics are regulated by reversible protein phosphorylation involving both protein kinases and phosphatases. Never in mitosis A (NIMA)-related kinases (NEKs) are a family of serine/threonine kinases that regulate microtubule-related mitotic events in fungi and animal cells (e. g. centrosome separation and spindle formation). Although plants contain multiple members of the NEK family, their functions remain elusive. Recent studies revealed that NEK6 of *Arabidopsis thaliana* regulates cell expansion and morphogenesis through β-tubulin phosphorylation and microtubule destabilization. In addition, plant NEK members participate in organ development and stress responses. The present phylogenetic analysis indicates that plant *NEK* genes are diverged from a single *NEK6*-like gene, which may share a common ancestor with other kinases involved in the control of microtubule organization. On the contrary, another mitotic kinase, polo-like kinase, might have been lost during the evolution of land plants. We propose that plant NEK members have acquired novel functions to regulate cell growth,

microtubule organization, and stress responses.

Keywords: NIMA-related kinase, phosphorylation, tubulin, microtubule, cell expansion, cell division

Introduction

The growth and morphogenesis of plant cells relies on the orientation of cellulose microfibrils and cortical microtubules. Microtubules are cytoskeletal polymers composed of α- and β-tubulin heterodimers. Microtubules are polarized with a fast growing plus end and a slow growing minus end, and exhibit dynamic behaviors such as rapid growth and shrinkage both in vivo and in vitro (Mitchison and Kirschner 1984; Horio and Hotani 1986; Sammak and Borisy 1988; Shaw et al. 2003; Nakamura et al. 2004). Cortical microtubules are specifically found in plant cells during interphase and are localized close to the cell cortex (Ledbetter and Porter 1963). Cortical microtubules align perpendicularly to the growth direction and regulate anisotropic growth and morphogenesis of rapidly expanding cells (Green 1962; Shibaoka 1994; Wasteneys 2002; Fig. 1). Findings from genetic studies of *Arabidopsis thaliana* mutants strongly support the essential roles of cortical microtubule arrays on directional cell growth (Whittington et al. 2001; Thitamadee et al. 2002; Abe et al. 2004; Ishida et al. 2007a; Ishida et al. 2007b; Sedbrook and Kaloriti 2008; Wasteneys and Ambrose 2009). In addition, microtubules regulate cell division and chromosome segregation. In the mitotic phase, microtubules form a series of arrays; a preprophase band that determines the future cell division plane, mitotic spindle that segregate chromosomes, and a phragmoplast that constructs the new cell plate (Fig. 1; Wasteneys 2002).

The parallel alignment of cortical microtubules and cellulose microfibrils together with isotropic growth induced by microtubule depolymerization supported the alignment hypothesis. This hypothesis states that cortical microtubules guide the movement of cellulose synthase complexes and determine the orientation of cellulose microfibrils at the innermost layer of the cell wall to control the direction of cell growth (Green 1962; Shibaoka 1994; Baskin 2001). Live cell imaging of cortical microtubules and cellulose synthase A6 (CESA6) demonstrated that cellulose synthase complexes move along the trajectories associated with cortical microtubules (Paredez et al. 2006). Note that cortical microtubule disruption can induce cell swelling without affecting the parallel alignment of cellulose microfibrils, suggesting that cortical microtubules could also regulate directional cell growth independently of cellulose microfibrils (Sugimoto et al. 2003). Fujita et al. (2011) have shown that cortical microtubule abundance affects cellulose crystallinity to promote directional cell growth. Microtubules might regulate the mobility and stability of cellulose synthase complexes to affect physical properties of cellulose microfibrils. ~~Because it is beyond the scope of this review,~~ Interested readers could consult the recent literature and references therein (Bringmann et al. 2012; Fujita et al. 2012; Lei et al. 2014). In this review, we will summarize recent findings on microtubule regulation with focus on phosphorylation-dependent regulatory mechanisms.

Microtubule regulation

Microtubule-associated proteins (MAPs) play pivotal roles in the regulation of microtubule dynamics (Hamada 2014). MAPs affect microtubule assembly and bundling and regulate their geometry and organization. Because the function and regulation of MAPs have been well described in detail, we show here a few examples from a cellular and developmental context [interested readers could consult Hamada (2014) and references therein].

Microtubules are nucleated from the γ-tubulin ring complex (γTuRC), which is a lockwasher-like ring structure containing 13 γ-tubulin proteins, which functions as a microtubule template (Moritz et al. 2000; Kollman et al. 2011). In animal cells, γTuRC localizes to the centrosome, which is a conspicuous microtubule organizing center. Although plant cells have no centrosome, the γ-tubulin complexes are localized on the side of microtubules, on the nuclear envelope, and on the plastid surface to initiate microtubule nucleation (Liu et al. 1994; Kumagai et al. 2003; Shimamura et al. 2004; Murata et al. 2005). The γ-tubulin complex of *A. thaliana* contains the same set of proteins as that of animals; γ-tubulin complex protein 2 (GCP2), GCP3, GCP4, GCP5, GCP6 and NEDD1/GCP-WD (Nakamura et al. 2010). Furthermore, several proteins that regulate the localization and activity of the γ-tubulin complex are also conserved in plants (Zeng et al. 2009; Ho et al. 2011; Hotta et al. 2012; Nakamura et al. 2012). The γ-tubulin and γ-tubulin complex proteins are essential for microtubule organization, cell expansion and cell division (Binarová et al. 2006; Pastuglia et al. 2006; Nakamura and Hashimoto 2009; Zeng et al. 2009; Kong et al. 2010). The γ-tubulin complex is recruited onto pre-existing microtubules and microtubule nucleation could occur by branching from extant microtubules (Murata et al. 2005; Murata and Hasebe 2007; Murata et al. 2013). Live cell imaging of the γ-tubulin complex revealed that microtubule nucleation is promoted by the association of the γ-tubulin complex with microtubules and newly formed daughter microtubules are dissected from the nucleation complex probably through the activity of katanin (Nakamura et al. 2010).

Katanin is an ATP-dependent microtubule-severing MAP composed of a 60 kDa catalytic subunit and an 80 kDa WD40-repeat regulatory protein (Hartman et al. 1998; Bouquin et al.

2003). Arabidopsis mutants of katanin-p60 catalytic subunit (KATANIN1, KTN1) exhibit defects in cell division and elongation, root development, stem elongation, orientation of microtubules and cellulose microfibrils, and response to phytohormones (Bichet et al. 2001; Burk et al. 2002; Webb et al. 2002; Bouquin et al. 2003).

Detailed analysis of microtubule organization and cell growth in the shoot apical meristem revealed that cortical microtubules are oriented toward the direction of maximal tension generated by tissue growth (Hamant et al. 2008), and that local mechanical forces amplify the variability of cell growth during the apical morphogenesis (Uyttewaal et al. 2012). KTN1 is required for growth heterogeneity in the shoot apical meristem and for the response to mechanical stress via the reorientation of cortical microtubules (Uyttewaal et al. 2012). A recent study revealed that blue light photoreceptors (phototropins) activate katanin-mediated microtubule severing at microtubule crossovers to drive microtubule reorientation during phototropism (Lindeboom et al. 2013). These results suggest that katanin plays an integrative role in the transduction of different signals. In *Xenopus laevis*, phosphorylation of katanin-p60 suppressed its microtubule-severing activity and increased spindle length (Loughlin et al. 2011). Although phosphorylation of plant katanin remains to be elucidated, such a regulatory mechanism may participate in the signal integration via katanin.

Pavement cells in the leaf epidermis have a unique jigsaw puzzle shape and represent a useful model system to analyze the mechanism of cell morphogenesis. Pavement cells undergo interdigitating growth: outgrowth of lobe regions and growth suppression at neck regions. Interdigitating growth is regulated by two counteracting signaling pathways (Fu et al. 2005): lobe outgrowth by Rho Of Plant 2 (ROP2) GTPase-induced F-actin formation and outgrowth suppression by microtubule bundling mediated by ROP-Interactive CRIB motif-containing protein 1 (RIC1). Following activation by ROP6, RIC1 promotes the microtubule severing activity of katanin (Fu et al. 2009; Lin et al. 2013). Interestingly, ROP11 GTPase and its binding partner MIcrotubule Depletion Domain1 (MIDD1) has been shown to regulate microtubule-mediated secondary cell wall patterning in xylem vessel elements (Oda et al. 2010; Oda and Fukuda 2012; Oda and Fukuda 2013). Locally activated ROP11 recruits MIDD1, which subsequently promotes the microtubule depolymerizing activity of kinesin-13A. These studies demonstrated the essential role of ROP-MAP signaling pathway on microtubule patterning.

Other MAPs, including CLASP, also regulates pavement cell morphogenesis via cortical microtubule organization (Ambrose et al. 2007; Kirik et al. 2007). CLASP promotes microtubule geometry in dividing cells by overcoming cell-edge-induced microtubule depolymerization (Ambrose et al. 2011). CLASP also interacts with sorting nexin 1 (SNX1) and promotes PIN2 recycling to regulate auxin transport (Ambrose et al. 2013). These results clearly indicate that CLASP is an important MAP that regulates microtubule organization and cell growth.

The organization and dynamics of microtubules are also regulated by post-translational modifications (PTMs) of tubulin, which include detyrosination/tyrosination, glutamylation, glycylation, acetylation, palmitoylation, and phosphorylation (Westermann and Weber 2003; Verhey and Gaertig 2007; Hammond et al. 2008; Wloga and Gaertig 2010; Wehenkel and Janke 2014). Of note, the C-terminal tail of α- and β-tubulin is a structurally variable region and is susceptible to PTMs. These modifications affect the microtubule dynamics, MAP binding affinity to microtubules, and microtubule motor activity. Sirajuddin et al. (2014) analyzed the motility of kinesins on microtubules, which were composed of homogeneous tubulin species with specific PTMs. These authors revealed that tubulin isoforms and PTMs could regulate the activity of kinesins. Recently, Barisic et al. (2015) demonstrated that the pattern of tubulin detyrosination at the mitotic spindle guides kinesin-7-mediated chromosome movement toward the cell equator during metaphase chromosome alignment.

Tubulin phosphorylation is found ubiquitously in eukaryotes, but in most cases, its functional significance remains to be elucidated (Westermann and Weber 2003; Verhey and Gaertig 2007; Hammond et al. 2008; Wloga and Gaertig 2010). During mitosis of animal cells, cyclin-dependent kinase 1 (Cdk1) phosphorylates Ser-172 on β-tubulin in the cytosolic free tubulin dimer and suppresses incorporation of the tubulin dimer into microtubules (Fourest-Lieuvin et al. 2006). Because Ser-172 is located in the binding site of GTP/GDP, its phosphorylation may interfere GTP binding and GTP/GDP exchange. Phosphorylation of the C-terminal tail of yeast γ-tubulin (Tyr-445) could promote microtubule number and assembly (Vogel et al. 2001). Phosphoproteomic analysis of yeast spindle pole body indicated that γ-tubulin and γ-tubulin complex proteins are extensively phosphorylated during mitosis (Lin et al. 2011; Keck et al. 2011). Interestingly, one phosphorylation site, Ser-360 on γ-tubulin is conserved in all eukaryotes, and phosphomimetic mutation of Ser-360 potently affects microtubule organization and spindle formation. In mammalian cells, centrosomal SADB kinase has been shown to phosphorylate Ser-131 on γ-tubulin and to regulate centrosome duplication (Alvarado-Kristensson et al. 2009).

Plant tubulin phosphorylation has been detected via the phosphoproteomics and immunological studies (Sugiyama et al. 2008; Blume et al. 2008; Nakagami et al. 2010). Although the biological roles of tubulin phosphorylation are not clear, identification of several tubulin kinases, as well as stress-inducible phosphorylation of α-tubulin, shed new light on the function of tubulin phosphorylation (Ben-Nissan et al. 2008; Motose et al. 2011; Ban et al. 2013; Fujita et al. 2013). ~~In this review,~~ We aim to provide an overview of phosphorylation-dependent microtubule regulation with special emphasis on Never in mitosis A (NIMA)-related kinases (NEKs).

Phosphorylation-dependent microtubule regulation in mitosis

Several protein kinases regulate the dynamic reorganization of microtubules during mitosis (Fig. 1). A series of studies using tobacco BY-2 (*Nicotiana tabacum* cv. Bright Yellow 2) cell line revealed that the MAP kinase cascade (NACK-PQR pathway) is required for phragmoplast expansion and for cell plate formation (Nishihama et al. 2001; Nishihama et al. 2002; Soyano et al. 2003; Sasabe et al. 2006). NACK kinesins activate the MAP kinase cascade, which consists of NPK1 mitogen-activated kinase kinase kinase (MAPKKK), NQK1 MAP kinase kinase (MAPKK), and NRK1 MAP kinase (MAPK). NRK1 phosphorylates microtubule-associated protein 65-1 (MAP65-1), suppresses microtubule bundling by MAP65-1, and promotes the microtubule turnover and centrifugal expansion of the phragmoplast (Sasabe et al. 2006). Recently, CDKs have been shown to regulate the timing of cytokinesis via phosphorylation of both NACK kinesins and NPK1, which suppresses the interaction between NACK kinesins and NPK1 (Sasabe et al. 2011a). The NACK-PQR pathway is also conserved in *A. thaliana* and regulates phragmoplast expansion and cortical microtubules (Strompen et al. 2002; Takahashi et al. 2010; Kosetsu et al. 2010; Beck et al. 2010; Sasabe et al. 2011b).

Other kinases, as well as a kinase-like protein, participate in microtubule regulation during mitosis. Aurora kinases are mitotic kinases that are involved in the spindle checkpoint and in faithful chromosomal segregation. In the genome of *A. thaliana*, there are three members of Aurora kinase family; AtAurora1 (AtAUR1), AtAUR2, and AtAUR3 (Kawabe et al. 2005; Demidov et al. 2005). AtAUR1 and AtAUR2 localize to the spindle microtubules whereas AtAUR3 localizes to the centromere. AtAUR kinases phosphorylate histone H3 on Ser-10 (H3S10). An inhibitor of aurora kinase, hesperadin, suppresses the phosphorylation of Ser-10 and leads to defects in chromosomal segregation (lagging chromosomes and micronuclei), indicating that aurora kinases are required for faithful chromosomal segregation and cohesion (Kurihara et al. 2006; 2008; Demidov et al. 2009).

TWO IN ONE (*TIO*) of *A. thaliana* encodes an ortholog of FUSED (FU) kinase from *Drosophila melanogaster* (Oh et al. 2005). TIO localizes to the midzone of the phragmoplast and is required for cytokinesis in the apical meristems and asymmetric cell division during male gametophyte formation. TIO kinase interacts with Kinesin-12 and participates in phragmoplast expansion (Oh et al. 2012).

RUNKEL (RUK)/EMB3013 of *A. thaliana* encodes a large kinase-like protein, which contains a putative kinase domain at the N-terminus and a long C-terminal tail (Krupnova et al. 2009). RUK is required for cytokinesis during embryogenesis. RUK binds to microtubules via its C-terminal tail and localizes to the preprophase band, mitotic spindle, and phragmoplast. RUK does not have kinase activity and RUK with kinase-dead mutations ~~could~~ rescues the lethality of *ruk* mutant, suggesting that the kinase activity is dispensable for its function (Krupnova et al. 2009). The downstream pathway of AtAURs, TIO, and RUK, and their functional interaction with NACK-PQR pathway remain to be elucidated.

Phosphorylation-dependent regulation of cortical microtubule dynamics

Cortical microtubule arrays are highly dynamic, self-organizing structures (Wasteneys 2002; Wasteneys and Ambrose 2009). Pharmacological and genetic analyses indicate that protein phosphorylation is involved in the regulation of cortical microtubule organization and cell expansion (Shibaoka, 1994; Baskin and Wilson 1997; Fig. 1). PROPYZAMIDE HYPERSENSITIVE1 (PHS1) is a MAPK phosphatase that regulates directional cell growth through cortical microtubule organization in *A. thaliana* (Naoi and Hashimoto 2004; Walia et al. 2009). Recently, Fujita et al. (2013) showed that PHS1 has an atypical kinase domain, whose activity is suppressed by its phosphatase domain. PHS1 kinase is activated by osmotic stress and phosphorylates Thr-359 on α-tubulin. This phosphorylation suppresses incorporation of the α/β-tubulin heterodimer into the microtubule, resulting in the depolymerization of cortical microtubules under osmotic stress in both *A. thaliana* and rice (Fujita et al. 2013; Ban et al. 2013). Because PHS1 localizes to the cytosol and microtubule depolymerization promotes phosphorylation of α-tubulin, PHS1 mainly phosphorylates cytosolic free tubulin.

Casein kinase 1-like 6 (CKL6) from *A. thaliana* associates with cortical microtubules and phosphorylates β-tubulin (Ben-Nissan et al. 2008). CKL6-GFP fusion protein associates with cortical microtubules via its C-terminal non-catalytic domain. The C-terminal domain directly binds to both α- and β-tubulin in vitro. CKL6 preferentially phosphorylates β-tubulin and also phosphorylates polymerized microtubules in vitro. Alanine substitution of predicted phosphorylation sites indicated that CKL6 phosphorylates both Ser-413 and Ser-420 of TUB3. Overexpression of CKL6 disorganizes the cortical microtubule array and reduces interdigitating growth in the epidermal pavement cells. These results demonstrate that CKL6 regulates directional cell expansion through β-tubulin phosphorylation.

The *tonneau1* (*ton1*) and *fass*/*ton2* mutants of *A. thaliana* exhibit a severe dwarf phenotype with the loss of preprophase bands and disorganized cortical microtubules (Torres-Ruiz and Jürgens 1994; Traas et al. 1995). TON1 protein shares sequence similarity with animal centrosomal proteins and interacts with a CENTRIN1 protein (Azimzadeh et al. 2008). FASS1/TON2 of *A. thaliana* and DISCORDIA1 and ALTERNATIVE DISCORDIA1 of *Zea mays* are nearly identical proteins that share significant similarity with a regulatory subunit of protein phosphatase 2A (PP2A) and participates in the control of cortical microtubules and preprophase bands (Camilleri et al. 2002; Wright et al. 2009; Kirik et al. 2012; Spinner et al. 2013). TON1-recruiting motif (TRM) proteins interact with TON1 and the PP2A complex containing FASS1/TON2 to form a TON1/TRM/PP2A (TTP) complex on microtubules (Drevensek et al. 2012; Spinner et al. 2013).

These reports indicate that signaling cascades involving protein kinases and phosphatases participate in the control of microtubule organization. However, target protein(s) of PP2A-FASS1, and functional interactions between kinases and PP2A, remain to be elucidated. The substrates and phosphorylation/dephosphorylation sites of kinases and phosphatases are summarized in Table S1.

Control of cell growth and microtubule organization by plant NEKs

NimA is a Ser/Thr protein kinase that was first discovered from a mitotic mutant *nimA* of *Aspergillus nidulans* (Osmani et al. 1987; Osmani et al. 1988; reviewed in O'Connell et al. 2003). NIMA-related kinases (NEKs) have been found in fungi, animals, and plants, and comprise a family of mitotic kinases in eukaryotes (Fig. 2). In fungi and animals, NEKs regulate various mitotic events including centrosome separation, spindle formation, and cytokinesis together with other kinase families, cyclin-dependent kinases (CDKs), polo-like kinases (PLKs), and Aurora kinases (O'Connell et al. 2003; O'Regan et al. 2007; Fry et al. 2012). The mitotic functions of NEKs may be due to their ability to regulate microtubule organization.

Although the function of plant NEKs is not well understood, recent studies have revealed that plant NEK members regulate cell expansion and morphogenesis through the regulation of cortical microtubule dynamics. The genome of *A. thaliana* encodes seven members of the *NEK* family (Vigneault et al. 2007), but their functions are largely unknown. We have previously shown that NEK6 of *A.thaliana* (AtNEK6) controls epidermal cell expansion and morphogenesis (Motose et al. 2008; 2011; Fig. 3). The loss-of-function mutant of AtNEK6, *ibo1*/*nek6*, exhibits ectopic protuberances in epidermal cells of hypocotyls and petioles (Motose et al. 2008; Sakai et al. 2008), indicating that AtNEK6 suppresses ectopic outgrowth in epidermal cells. A single ectopic protrusion is formed in the middle of the cell of the non-stomatal cell files in hypocotyls, which correspond to the atrichoblast cell files in roots. The homeobox gene *GLABRA2* (*GL2*) is specifically expressed in the non-stoma cell files and in trichomes, suggesting that the *ibo1* protrusion might be a trichome-like structure. The ectopic outgrowth of *ibo1*/*nek6* mutants is promoted by ethylene signaling and is suppressed by the promotion of cell elongation (e. g. etiolation and the addition of gibberellic acid).

Genetic and biochemical analyses have revealed that both the kinase activity and microtubule localization of AtNEK6 are essential for suppressing ectopic outgrowth (Motose et al. 2008). A single amino acid substitution in the activation loop of the kinase domain (*ibo1-1*) abolishes the kinase activity of AtNEK6 and induces ectopic outgrowth of epidermal cells. When transiently expressed in tobacco leaves, GFP-AtNEK6 associates with cortical microtubules. ~~whereas~~ A nonsense mutation in the C-terminal tail (*ibo1-2*, Fig. 3a) causes mislocalization of GFP-AtNEK6 to the nucleus. This suggests that the C-terminal region of AtNEK6 is required for its association with microtubules.

AtNEK6 has been shown to interact with armadillo repeat-containing kinesins (ARKs), namely ARK1, ARK2, and ARK3 (Sakai et al. 2008). The *ark1*/*mrh2* mutants exhibit root hair branching, indicating the involvement of ARK1 in root hair tip growth (Jones et al. 2006; Yang et al. 2007; Sakai et al. 2008). Microtubule imaging in the *ark1* mutant demonstrates that ARK1 may limit the assembly and distribution of endoplasmic microtubules during root hair formation (Sakai et al. 2008). Recently, Eng and Wasteneys (2014) have shown that ARK1 localizes to the microtubule plus ends and promotes microtubule catastrophe during root hair tip growth. These studies suggest that AtNEK6 regulates cellular growth and morphogenesis via microtubule function.

AtNEK6 regulates cortical microtubule organization and cell expansion via interaction with other NEK members and phosphorylation of β-tubulins (Motose et al. 2011; Fig. 4). The functional AtNEK6–GFP fusion associates with cortical microtubules, mitotic spindles, and phragmoplasts. AtNEK6-GFP is concentrated in particles that exhibit dynamic movement along microtubules. This is consistent with the direct binding of AtNEK6 to microtubules in vitro (Fig. S2 in Motose et al. 2011). The *nek6* mutants show disturbance in the cortical microtubule array at the site of ectopic protrusions in epidermal cells. Quantitative analysis of microtubule dynamics indicates that cortical microtubules are more stable in the *nek6* mutant. Microtubule stabilization by taxol enhances ectopic outgrowth in the *nek6* mutant, whereas microtubule destabilization by propyzamide suppresses the *nek6* phenotype, suggesting that the ectopic outgrowth is due to the microtubule stabilization. In addition, AtNEK6 phosphorylates β-tubulin in vitro. The interaction of AtNEK6 with AtNEK4 and AtNEK5 is affected by the *ibo1-3* mutation within the plant NEK C-terminal motif, and is required for the ectopic outgrowth phenotype of *nek6* (Motose et al. 2011). These results suggest that AtNEK6 phosphorylates β-tubulin and interacts with other NEKs to depolymerize~~s~~ cortical microtubules (Fig. 4). The localization and direct binding of AtNEK6 to microtubules suggests that AtNEK6 phosphorylates β-tubulin that is incorporated into microtubules.

We showed that AtNEK6 possibly regulate~~s~~ directional organ growth and regular cell file formation via the control of cell division and expansion (Motose et al. 2012). The *nek6* mutant exhibits short petioles, hypocotyls, and roots (Fig. 3). In the root meristem of the *nek6* mutant, cell files are disorganized and abnormal cell plates are formed, indicating the requirement of AtNEK6 for the organized cell division and expansion that is essential for regular cell file formation. In addition, the *nek4*, *nek5*, and *nek6* mutants are hypersensitive to microtubule inhibitors. This phenotype could be attributed to the defects of microtubule organization and dynamics in the *nek* mutants. Further analysis is required to determine whether the phenotype of *nek6* is completely due to the defect in microtubules, and to investigate the primary effect of NEKs on microtubule organization and function.

Agueci et al. (2011) showed that AtNEK2 is essential for plant development and that it associates with cortical microtubules. The homozygous progeny from a T-DNA insertion mutant of *AtNEK2* could not be isolated, suggesting an essential role of AtNEK2. However, homozygous mutants were isolated from other T-DNA insertion lines of *AtNEK2* (Vigneault et al. 2007; H. M. unpublished result). The lethality might be an allele-specific phenotype. Interestingly, *AtNEK2 RNAi* transgenic lines exhibit delayed growth, smaller leaves and stems, disorganized leaf tissues, and an altered epidermal cell pattern (Agueci et al. 2011). AtNEK2 fused with yellow fluorescence protein at the N-termini (YFP-AtNEK2) localized to the cortical microtubules. These results suggest that AtNEK2 is required for organ growth via microtubule function.

Functions of plant NEKs in flower development

Previous studies have shown that microtubules are involved in flower development and reproductive processes. Arabidopsis tubulin mutants and transgenic plants expressing modified α-tubulin showed helical growth of flower organs (Thitamadee et al. 2002; Abe et al. 2004; Abe and Hashimoto 2005). In shoot apical meristems, the cortical microtubule array is correlated with the polar localization of the auxin efflux carrier PIN1 (Heisler et al. 2010). A mechanical signal may coordinate the alignment of cortical microtubules and PIN1 polarity to regulate auxin distribution and positioning of flower primordia (Heisler et al. 2010). Auxin decreases anisotropy of cortical microtubule arrays to promote the formation of flower primordia (Sassi et al. 2014). Furthermore, microtubules are essential for gametogenesis (Berger and Twell 2011). Microtubule ORganization 1 (MOR1) is a member of the highly conserved eukaryotic XMAP215 family and promotes microtubule polymerization (Whittington et al. 2001; Twell et al. 2002; Hamada et al. 2004; Kawamura and Wasteneys 2008). MOR1 is required for spindle positioning and asymmetric cell division during male gametogenesis and the formation of spindles and phragmoplasts during somatic cell division (Twell et al. 2002; Kawamura et al. 2006). TIO Fused kinase, NACK kinesins and kinesin-12A/B also participate in asymmetric cell division during male gametogenesis (Lee et al. 2007; Berger and Twell 2011; Oh et al. 2012).

Recently, plant NEKs have been shown to interact with various proteins and to be involved in multiple biological processes including flower development and stress responses. A tomato (*Solanum lycopersicum*) NEK named SPAK was identified as to interact with SELF PRUNING (SP) (Pnueli et al. 2001). SP, TERMINAL FLOWER1 (TFL1) of *A. thaliana*, and CENTRORADIALIS (CEN) of *Antirrhinum majus* are negative regulators of floral initiation that act antagonistically with Flowering locus T (FT) (Kobayashi et al. 1999). The FT protein is a mobile floral inducer, termed florigen, that promotes floral initiation by forming a complex with a transcriptional regulator called Flowering locus D (FD) and 14-3-3 proteins (Abe et al. 2005; Notaguchi et al. 2008; 2009; Taoka et al. 2011). SP, TFL1, CEN, and FT are homologous proteins that share sequence similarity with phosphatidylethanolamine-binding proteins (PEBPs). PEBPs interact with phosphorylated proteins and participate in signaling events. SPAK also interacts with TFL1, FT, CEN, and 14-3-3 proteins. SP binds to one of two 14-3-3 protein-binding sites within the C-terminal tail of SPAK. *SPAK* mRNA accumulates in the shoot apical meristem and the antisense expression of *SPAK* induces the formation of oval-shaped fruits (Pnueli et al. 2001). Although the biological significance of the SPAK-SP interaction has not been clarified, SPAK might mediate signaling and/or transcriptional regulation during inflorescence development and flowering.

PNek1 from a hybrid poplar (*Populus tremula* × *P. alba*) was found to interact with a 14-3-3 protein. *PNek1* mRNA accumulates during mitosis (Cloutier et al. 2005) and the *PNek1* promoter is preferentially active in vascular tissues (Vigneault et al. 2007). Overexpression of *PNek1* induces abnormal flowers in *A. thaliana* (Cloutier et al. 2005). In addition, *O. sativa NEK3* (*OsNEK3*) is preferentially expressed in mature pollen, and overexpression of *OsNEK3* occasionally causes pollen grains to remain attached after cytokinesis, which resembles the *quartet* mutant of *A. thaliana* (Fujii et al., 2009). OsNek3 interacts with a LIM domain-containing protein that is specifically expressed in mature pollen. Taken together, these results indicate that plant NEKs participate in reproductive development, ~~but the cellular and molecular functions of NEKs remain to be elucidated. In particular,~~ although it remains to be shown that ~~is not clear whether or not the NEK~~ their function ~~during flower development~~ is mediated by microtubules.

Involvement of plant NEKs in stress responses

Plant microtubules respond to various stresses and may participate in stress tolerance (Nick 2013; Hardham 2013). Cortical microtubules participate in the response to salt stress (Shoji et al. 2006; Wang et al. 2007). Mutations in Salt Overly Sensitive 1 (SOS1), which is a sodium/proton antiporter required for salt tolerance, suppress right-handed helical growth of the *spiral1* (*spr1*) mutant (Shoji et al. 2006). *SPR1* encodes a plant-specific small MAP and SPR1-mediated microtubule function might be involved in the salt response. Furthermore, salt stress induces right-handed helical growth and transient depolymerization of cortical microtubules (Wang et al. 2007). Microtubule stabilization by taxol strongly reduces seedling survival under salt stress suggesting that microtubule depolymerization is required for salt tolerance (Wang et al. 2007). Microtubule disassembly in response to salt stress is mediated by the proteasome-dependent degradation of SPR1 (Wang et al. 2011).

Abscisic acid (ABA) is a representative phytohormone that is tightly associated with stress tolerance. ABA suppresses cell elongation and promotes longitudinal and oblique arrays of cortical microtubules in dwarf pea (Sakiyama and Shibaoka 1990; Sakiyama-Sogo and Shibaoka 1993) and cucumber (Ishida and Katsumi 1992). ABA decreases cortical microtubule abundance and suppresses seed germination and cell growth in *Coffea arabica*

(Da Silva et al. 2008). Recently, Takatani et al. (2015) found that ABA induces microtubule depolymerization and ectopic outgrowth of epidermal cells in *A. thaliana*. As described above, osmotic stress also depolymerizes cortical microtubules by PHS1-mediated α-tubulin phosphorylation (Fujita et al. 2013; Ban et al. 2013). Therefore, microtubule disassembly might be a common mechanism for sensing various types of stress.

NEK6 also participates in the ABA response and stress tolerance (Lee et al. 2010; Zhang et al. 2011). ABA binds to the PYRABACTIN RESISTANCE1/PYR1-LIKE (PYR/PYL) family of StAR-related lipid transfer (START) proteins and suppresses type 2C protein phosphatases (PP2C), which repress SnRK kinase activity (Cutler et al. 2010). The de-suppression of SnRKs stimulates intracellular signaling cascades leading to activation of the ABA responsive element binding factors/proteins (ABFs/AREBs), which are transcriptional regulators of ABA-responsive gene expression. Arm Repeat protein Interacting with ABF2 (ARIA) has been identified as a protein that interacts with ABF2/AREB1 and positively regulates the ABA response (Kim et al. 2004). AtNEK6 binds to ARIA in yeast two-hybrid analysis (Lee et al. 2010). Transgenic plants that overexpress *AtNEK6* exhibit slower growth and hypersensitivity to ABA, osmotic stress, and salt stress (Lee et al. 2010). The *nek6* mutant grows faster than the wild type and is partially insensitive to ABA, osmotic stress, and salt stress (Lee et al. 2010). These results suggest that NEK6 interacts with ARIA and positively regulates stress responses.

In contrast, Zhang et al. (2011) reported that overexpression of *AtNEK6* promotes the growth of rosette leaves, lateral root formation, seed yield, and stress tolerance. The *nek6-1* mutant has shorter leaves and fruits, a decreased number of lateral roots, and hypersensitivity to salt stress and osmotic stress (Zhang et al. 2011). Overexpression of *AtNEK6* increases the expression of cyclin genes such as *CYCB1;1* and *CYCA3;1*, while the *nek6-1* mutant has decreased cyclin expression. The *nek6-1* mutant exhibits increased expression of ethylene-related genes and ethylene accumulation. These results suggest that *AtNEK6* regulates plant growth and the stress response through activation of cyclin genes and suppression of ethylene. Because ethylene promotes AtNEK6 accumulation, *AtNEK6* participates in negative feedback regulation of ethylene biosynthesis and signaling (Zhang et al. 2011).

There are several discrepancies in these two reports, especially in the phenotype of *nek6* mutants and *NEK6* overexpressors. The *nek6* mutation enhanced stress tolerance whereas *NEK6* overexpression reduced stress tolerance in the study by Lee et al. (2010). In that by Zhang et al. (2011), the *nek6-1* mutation led to reduced stress tolerance whereas *NEK6* overexpression promoted stress tolerance. Although the reason for this discrepancy remains unknown, it could be due to differences in the growth conditions, stress treatments, and the *nek6* alleles used in two studies. Of note, it is difficult to establish stable transgenic lines that constitutively overexpress *NEKs* (Cloutier et al., 2005; Agueci et al. 2011, H. M. unpublished results). In our analyses, we found there are no obvious differences in the ABA response during seed germination, or in the expression of ethylene-related genes and cell cycle genes between the wild type and *nek6* mutants (H. M. unpublished results). Further analysis is required to clarify the role(s) of *AtNEK6* in stress responses.

A C3HC4 RING finger E3 ligase, *O. sativa* drought-induced SINA protein 1 (OsDIS1), participates in the drought stress response and interacts with OsNEK6 (Ning et al. 2011). When OsNEK6 is transiently expressed in tobacco leaves, it is degraded in an OsDIS1- and 26S proteasome-dependent manner. This result suggests that OsDIS1 may regulates the drought stress response via the degradation of OsNEK6.

Structure of NIMA-related kinases

In the next sections, we focus on the structure and evolution of the NEK family. NEK family members have a well-conserved kinase domain at the N-terminus and a regulatory tail at the C-terminus (Fig. 2). The X-ray crystal structure of the human Nek2 kinase domain demonstrates that Nek2 adopts an auto-inhibitory conformation similar to that of CDK2 (Rellos et al. 2007). The autoinhibitory α-helix (named αT) in the activation loop holds the catalytically important Glu-55 and prevents it from accessing the catalytic center. Phosphorylation of Ser/Thr residues within the activation loop (probably via autophosphorylation) releases this autoinhibition and stimulates the kinase activity of Nek2 (Rellos et al. 2007). Autophosphorylation sites within the activation loop are conserved in plant NEKs (Fig. S1a). Ala substitutions at the putative autophosphorylation sites of AtNEK6 result~~s~~ in a significant decrease in kinase activity (Motose et al. 2008), indicating that the similar autoinhibitory mechanism may regulate the activity of plant NEK family members.

The crystal structure of human Nek7 suggests a novel autoinhibitory mechanism (Richards et al. 2009). The inhibitory Tyr-97 residue in NEK7 points down to the catalytic center and prevents the formation of hydrophobic core, which is essential for the catalytic activity of Nek7. This “Tyr-Down” autoinhibitory conformation is released by the binding of the C-terminal domain of Nek9, which shifts the conformation from Tyr-Down to “Tyr-Up” (active form). Nek9 phosphorylates and activates Nek6 and Nek7 (Roig et al. 2002; Belham et al. 2003). The autoinhibitory mechanism might be common in at least three Neks, Nek6, Nek7, and Nek2 (Richards et al. 2009). Apart from AtNEK6, NEK proteins in *A. thaliana* also contain the conserved Tyr residue (Fig. S1b). In AtNEK6 and its orthologs in other plants, the inhibitory Tyr is substituted by Phe. This suggests that the Tyr autoinhibition and its release may regulate the activity of plant NEKs. Plant NEK6 may lose the inhibitory Tyr residue and be released from the autoinhibition.

Although the C-terminal tail is structurally divergent, it often contains a coiled-coil domain and PEST sequences (Fig. 2). The coiled-coil domain is required for the dimerization of NEKs. The PEST sequence is involved in the degradation and turnover of NEKs, probably via the ubiquitin-proteasome system. The degradation of animal Nek2 during early mitosis is dependent on the destruction box at the C-termini and is mediated by the anaphase promoting complex (APC/C)-Cdc20 ubiquitin ligase (Hames et al. 2001). Because plant NEKs interact with 14-3-3 proteins and 14-3-3 have been implicated in protein degradation (Yoon and Kieber 2013 and references therein), it is possible that 14-3-3 proteins regulate the degradation and stability of NEKs. However, the PEST sequence is not required for the binding of 14-3-3 proteins to plant NEKs (Pnueli et al. 2001; Cloutier et al. 2005). The biological function of 14-3-3 binding remains unknown.

The C-terminal tail also participates in the interaction with other proteins, direct binding to microtubules, subcellular localization, and autoinhibition. The autoinhibitory mechanism involves the C-terminal domains of vertebrate Nek9 and Nek11 suppressing their own kinase activity (Roig et al. 2002; Noguchi et al. 2004). Nek9 contains a Regulator of Chromosome Condensation 1 (RCC1)-like domain in the C-terminal tail. RCC1 is a guanine exchange factor for Ran GTPase. The RCC1-like domain may not have guanine exchange activity and self-inhibits Nek9 kinase activity (Roig et al. 2002). Human Nek2 directly binds microtubules via its C-termini (Hames et al. 2005). AtNEK6 also directly binds microtubules in vitro

(Motose et al. 2011) and its C-terminal region is required for the microtubule localization of AtNEK6 (Motose et al. 2008). In addition, AtNEK6 has been shown to interact with armadillo-repeat-containing kinesins (ARKs) via its C-termini (Sakai et al. 2008). Surprisingly, we found that the plant NEK C-terminal (PNC) motif that follows the coiled-coil domain and is conserved in plant NEKs (Fig. S1c), is required for the specificity of AtNEK6 binding to AtNEK4 and AtNEK5 (Motose et al. 2011). The substitution of a conserved Pro-916 of AtNEK6 within the PNC motif (*ibo1-3* mutation) reduces the interaction between AtNEK6 and AtNEK4 and increases the interaction between AtNEK6 and AtNEK5 (Motose et al. 2011). Therefore, the C-terminal domain is important for the NEK kinase activity, subcellular localization, interaction with substrates, and affinity to other NEK members.

Evolution of plant NEK family

Most fungal species including *A. nidulans*, *Neurospora crassa*, and yeasts have a single NIMA kinase, while most animals and plants have multiple members of the NEK family (Quarmby and Mahjoub 2005; Vigneault et al. 2007; Parker et al. 2007). Eleven *NEK* genes exist in the vertebrate genome and at least five of these (Nek2, Nek6, Nek7, Nek9, and Nek11) have been shown to regulate the mitotic cell cycle. Although the origin of the NEK family remains elusive, comprehensive protein kinase analysis of the human genome (the human kinome) demonstrated that the NEK family expanded from the bottom of the calmodulin (CaM)-regulated kinase (CAMK) clade (Manning et al. 2002). Comprehensive phylogenetic analysis of the NEK family indicates that at least five NEK subfamilies exist; HsNek1/3/5, HsNek2, HsNek4/11, HsNek6/7, and HsNek8/9 subfamilies (designated after the human Nek family described in Parker et al. 2007). This suggests that the five NEKs that were present in the last common ancestor of eukaryotes have been expanded or diminished in a lineage-specific manner (Parker et al. 2007).

In the *A. thaliana*, *Oryza sativa*, and *Populus trichocarpa* genomes, there are seven, six, and nine NEK genes, respectively (Vigneault et al. 2007; Table 1; Fig. 5). The genome of *Chlamydomonas reinhardtii* and *Volvox carteri* contains 13-14 members of NEKs, while no NEK is present in the green alga, *Ostreococcus tauri*, or in a primitive unicellular red alga, *Cyanidioschyzon merolae* (Quarmby and Mahjoub 2005; Parker et al. 2007; Table 1). The algal NEKs belong to the various NEK clades including the HsNek1/3/5, HsNek4/11, HsNek6/7, and HsNek8/9 clades, whereas all land plant NEKs belong to the HsNEK11 clade (Parker et al. 2007), suggesting that an HsNEK11-like gene may be a common ancestor for NEK genes in land plants (Fig. 6). Additional searches of plant genome databases identified novel members of the NEK family in algae, bryophytes, lycophyte, and angiosperms (Table 1, Fig. 5). Phylogenetic analysis revealed that plant NEKs can be divided into three NEK subfamilies; AtNEK1/2/3/4, AtNEK5/7, and AtNEK6 (Fig. 5). Intriguingly, we found that there is a single *NEK* gene in the genome of bryophytes such as *Physcomitrella patens* and *Marchantia polymorpha*, three *NEK* genes in the genome of the lycophyte *Selaginella moellendorffii*, and five to nine *NEK*s in the angiosperm species (Table 1, Fig. 5), suggesting that plant NEKs gradually diverged from a single NEK ancestor (Fig. 6). Because all *NEK* genes from basal land plants such as *P. patens*, *M. polymorpha*, and *S. moellendorffii* belong to the AtNEK6 clade (Fig. 5), the plant AtNEK6 subfamily may represent an origin of land plant NEKs (Fig. 6).

O. tauri and *C. merolae* (without NEKs) are small algae of about 1-μm length without cilia and flagella, whereas *C. reinhardtii* and *V. carteri* (with 13–14 NEKs) have a larger cell size (about 5–10 µm) and long flagella. Two NEK proteins from *C. reinhardtii,* Fa2p and Cnk2p, have been shown to regulate ciliary disassembly during cell cycle progression and flagellar length and cell size, respectively (Mahjoub et al. 2002; Bradley and Quarmby 2005). Therefore, conservation, diversification, and/or expansion of the NEK family might be correlated with ciliary function and cell size. Because several plant NEKs regulate cortical microtubule organization, which is essential for cell elongation and organ growth (Motose et al. 2008, 2011, Sakai et al. 2008), expansion of the HsNEK11 clade in land plants might be due to a requirement for different NEKs to control cortical microtubule organization during cell expansion and organ growth in complex life cycles.

To further investigate the evolution and origin of plant NEKs, we used the comparative genomics SALAD Database (Mihara et al. 2009; http://salad.dna.affrc.go.jp/salad/), which is useful for the genome-wide systematic comparison of protein sequences and domains. The SALAD phylogenetic tree indicates that plant NEKs are closely related to the FU/TIO kinase, AuTophaGy related 1/Unconditionated-51 (Unc-51)-like kinases (ATG1/ULKs), and a large kinase-like protein RUK/EMB3013 (Fig. S2). These kinases may share a common ancestor. In the human kinome, the NEK clade neighbors the ATG1/ULK clade (Manning et al. 2002). ATG1/ULKs regulate the initiation of autophagy and vesicle transport in yeast, mammals, and *A. thaliana* ((Mizushima and Komatsu 2011; Suttangkakul et al. 2011). Arabidopsis RUK contains an N-terminal kinase-like domain, which is similar to that found in NEKs, and a very long C-terminal region, which directly binds to microtubules (Krupnova et al. 2009). RUK and FU/TIO localize to the phragmoplast and regulate its expansion during cytokinesis (Krupnova et al. 2009; Oh et al. 2005; Oh et al. 2012). These characteristics closely resemble those of plant NEK kinases (e.g. domain composition, microtubule binding, and subcellular localization). In addition, the NEK/FU/ATG1/RUK clade is related to the MAPKKK family, which contains Arabidopsis NPK1-related kinase (ANP3) (Fig. S2). NPK1/ANPs are localized to the phragmoplast and regulate cell plate expansion through the NACK-PQR pathway (See above section). Our phylogenetic analysis suggests that the plant NEK family is derived from an ancestral kinase(s), which might be involved in the control of microtubule organization.

In land plants, the NEK family has expanded to form a multi-gene family as in the case of Aurora kinases and CDKs, whereas another mitotic kinase, polo-like kinase (PLK), seems to have been lost (Table 1). Interestingly, we found that at least one PLK exist in algae, such as *O. tauri*, *C. merolae*, *C. reinhardtii*, and *V. carteri*. Aurora kinases and CDKs are conserved in algae and land plants. This suggests that PLK was lost during the evolution of land plants. Because bryophytes, ferns, cycads, and *Ginkgo biloba* have flagellated sperm cells (Ikeno and Hirase 1897; Gifford and Foster 1989), the loss of PLK might not be due to the loss of flagella. Animal PLKs show partial sequence similarity (about 30–40%) to the SNF1-related kinases/CBL-interacting protein kinases (SnRKs/CIPKs). However, SnRKs/CIPKs might not be orthologs of PLKs because they represent a large family of plant kinases that are mainly involved in stress signaling and environmental responses. Considering that several NEKs have redundant and/or cooperative function with PLKs in yeast and vertebrates (e.g. Grallert and Hagan 2002; Rapley et al. 2005; Bertran et al. 2011; Mardin et al. 2011; Sdelci et al. 2012), it is likely that the functions of PLKs were substituted by NEKs and/or other mitotic kinases during the evolution of land plants. In summary, expansion of the NEK subfamily (NEK11 clade) in land plants might be related to the loss of PLK, the increased cell size, and the requirement for microtubule regulation during cell expansion.

Concluding remarks

In conclusion, NEKs mainly regulate microtubule organization and cell growth. However, the molecular function of NEKs might differ among organisms. NEKs diverged in a lineage specific manner and may have acquired novel functions following changes in the structure of the catalytic and C-terminal regulatory domains. Because NEKs cooperate with other kinases including PLKs and CDKs, to regulate mitotic events and microtubule organization, the kinase cascades and networks might be important for the function and evolution of NEK family.

In plants, NEK proteins regulate cell expansion, organ growth, and stress responses. These functions may have developed during the evolution of land plants, accompanied with the divergence of plant *NEK* genes and the loss of Polo-like kinase. Among plant NEK proteins, NEK6 is a central regulator that controls cell expansion, regular cell-file formation, and directional organ growth. NEK6 may control the activity and localization of other NEK members such as NEK4 and NEK5 to regulate cell expansion via tubulin phosphorylation and depolymerization of cortical microtubules.

Because single mutants of plant *NEK* members, other than *NEK6*, do not exhibit obvious morphological defects, it is difficult to analyze the function of plant NEKs. Our recent study suggested that plant NEKs redundantly control cell growth. Further cellular and molecular studies are required to reveal the redundant function of plant NEKs in cellular growth and morphogenesis. In addition, the roles of plant NEKs on stress responses remain elusive and some discrepancies have been noted, which should be addressed to clarify their molecular mechanisms. In particular, it would be of interest to determine whether plant NEKs regulate

DNA-damage checkpoints and/or spindle assembly checkpoints as observed in animal NEKs (Noguchi et al. 2002; Noguchi et al. 2004; Melixetian et al. 2009). It would also be of interest to determine whether tubulin phosphorylation by NEKs regulates cellular growth and morphogenesis in fungi and animals.

Another prospect is to identify upstream regulators and other downstream substrates of NEKs. The C-terminal domain of MOR1, a member of XMAP215 family, contains five putative phosphorylation sites (Twell et al. 2002) but their phosphorylation and function remains to be analyzed. In animal cells, phosphorylation of MAPs such as katanin and CLASP regulates microtubule organization (e. g. Kumar et al. 2009; Loughlin et al. 2011). Recently, Arabidopsis MPK6 was shown to phosphorylate End Binding protein1c (EB1c) and was suggested to maintain a normal cell division plane under the stress conditions (Kohoutová et al. 2015). It would be intriguing to investigate whether plant NEKs and other kinases phosphorylate MAPs to regulate microtubule organization. Further analysis of plant NEK function will provide new insight into microtubule regulation, cell growth, and plant development.

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Figure Legends

Fig. 1 Protein kinases and phosphatases involved in the microtubule regulation. See main text for further details. MT; microtubule.

Fig. 2 The structural conservation and diversification of NEKs. Kinase domain (red), dual kinase domain (DK, purple), PEST motif (PEST, green), coiled-coil domain (CC, yellow), plant NEK C-terminal motif (PNC, light blue), and RCC1 domain (RCC1, brown).

Fig. 3 AtNEK6 regulate organ growth and cell expansion. **a** Structure and mutation sites of AtNEK6. **b-c** The leaves, petioles, roots and hypocotyls of *nek6-1* mutant are shorter than those of the wild type (WT). Wild type (WT) and *nek6-1* mutant were grown on soil for four weeks (**b**). Wild type (WT) and *nek6-1* mutant were grown on agar medium for two weeks (**c**). The roots of *nek6-1* exhibit wavy growth (upper panels in **c**). **d-e** The *nek6-1* mutant exhibits ectopic outgrowth of epidermal cells. The hypocotyls of wild type (WT) and *nek6-1* mutant (**d**). **e** Scanning electron micrograph of hypocotyls (the upper panel in **e**) and petioles (the lower panel in **e**) of *nek6-1* mutant. Scale bars = 10 mm (**b**, upper panels in **c**), 1 mm (lower panels in **c**, **d**), and 100 μm (**e**).

Fig. 4 A schematic model of AtNEK6 function. AtNEK6 forms homodimers with AtNEK6 or heterodimers with AtNEK4 or AtNEK5 to regulate microtubule organization. The phosphorylation of β-tubulin depolymerizes microtubules and regulates microtubule organization. (Adapted from Kollman et al. 2011)

Fig. 5 Phylogenetic tree of plant NEK family. The alignment of the kinase domains was constructed by CLUSTAL W program and the phylogenetic tree is drawn by TreeView program.

Fig. 6 Putative evolutionary history of plant NEKs.

Fig. S1 Alignment of the deduced amino acid sequences of NEKs. **a-b** The activation loop (**a**) and the flanking sequences of inhibitory Tyr (**b**) within the kinase domain. **c** Plant NEK C-terminal motif. P-site is phosphorylation site.

Fig. S2 Phylogenetic tree of plant NEKs and other protein kinase family using SALAD database.

Table 1 Number of genes encoding mitotic kinase family. A mini phylogenetic tree outlines the evolutionary relationships among species (modified from Hori et al. 2014).

Species	NEK	Polo	Aurora
Cyanidioschyzon merolae	0	1	1
Ostreococcus tauri	0	1	1
Chlamydomonas reinhardtii	13	1	1
Volvox carteri	14	1	1
Klebsormidium flaccidum	5	0	1
Physcomitrella patens	1	0	2
Marchantia polymorpha	1	0	1
Selaginella moellendorffii	3	0	2
Arabidopsis thaliana	7	0	3
Oryza sativa	6	0	2
Populus trichocarpa	9	0	3

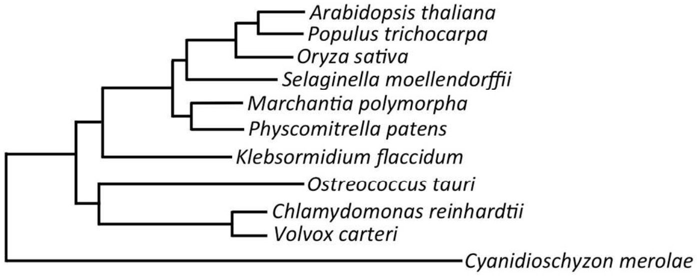
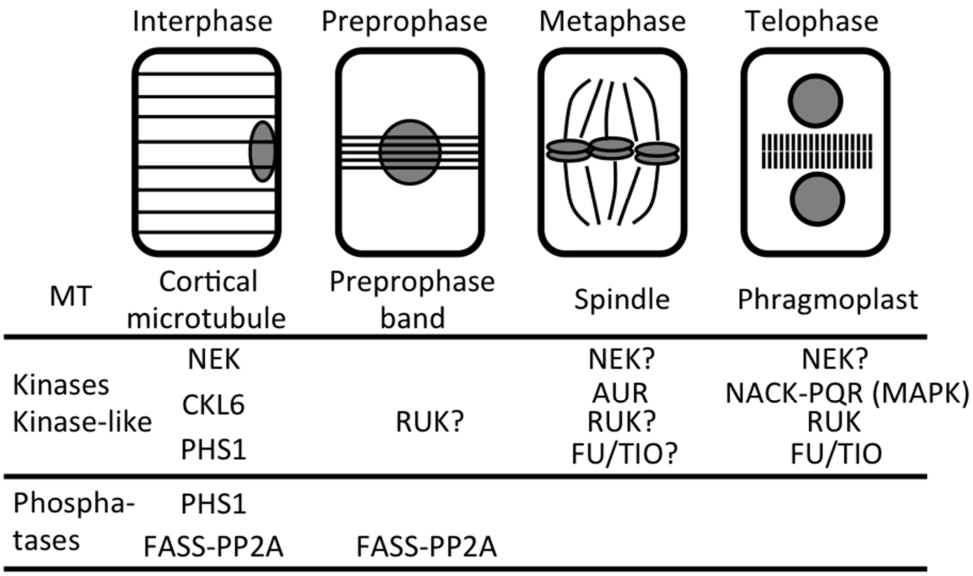


Table S1 Summary of kinases and their substrates

Kinases/ phosphatases	Target (substrate)	Phosphorylation/ de-phosphorylation site	Reference
NRK1	NtMAP65-1	T579	Sasabe et al. 2006
CDK	NtMAP65-1	T501, T503	Sasabe et al. 2006
CDK	NACK1	T675, T690, T836	Sasabe et al. 2011
CDK	NPK1	S575, S669, S687	Sasabe et al. 2011
Aurora	Histone H3	S10	Kawabe et al. 2005; Demidov et al. 2005
TIO1/FUSED	Kinesin-12?	unknown	Oh et al. 2012
PHS1 (kinase)	α-Tubulin	T359	Fujita et al. 2013
PHS1 (phosphatase)	MPK18	unknown	Walia et al. 2009
CKL6	β-Tubulin (TUB3)	S413, S420	Ben-Nissan et al. 2008
NEK6	β-Tubulin (TUB4, 6)	unknown	Motose et al. 2011
MPK6	EB1c	unknown	Kohoutová et al. 2015



MT	Cortical microtubule	Preprophase band	Spindle	Phragmoplast
Kinases Kinase-like	NEK CKL6 PHS1	RUK?	NEK? AUR RUK? FU/TIO?	NEK? NACK-PQR (MAPK) RUK FU/TIO
Phospha-tases	PHS1 FASS-PP2A	FASS-PP2A		

Fig 1
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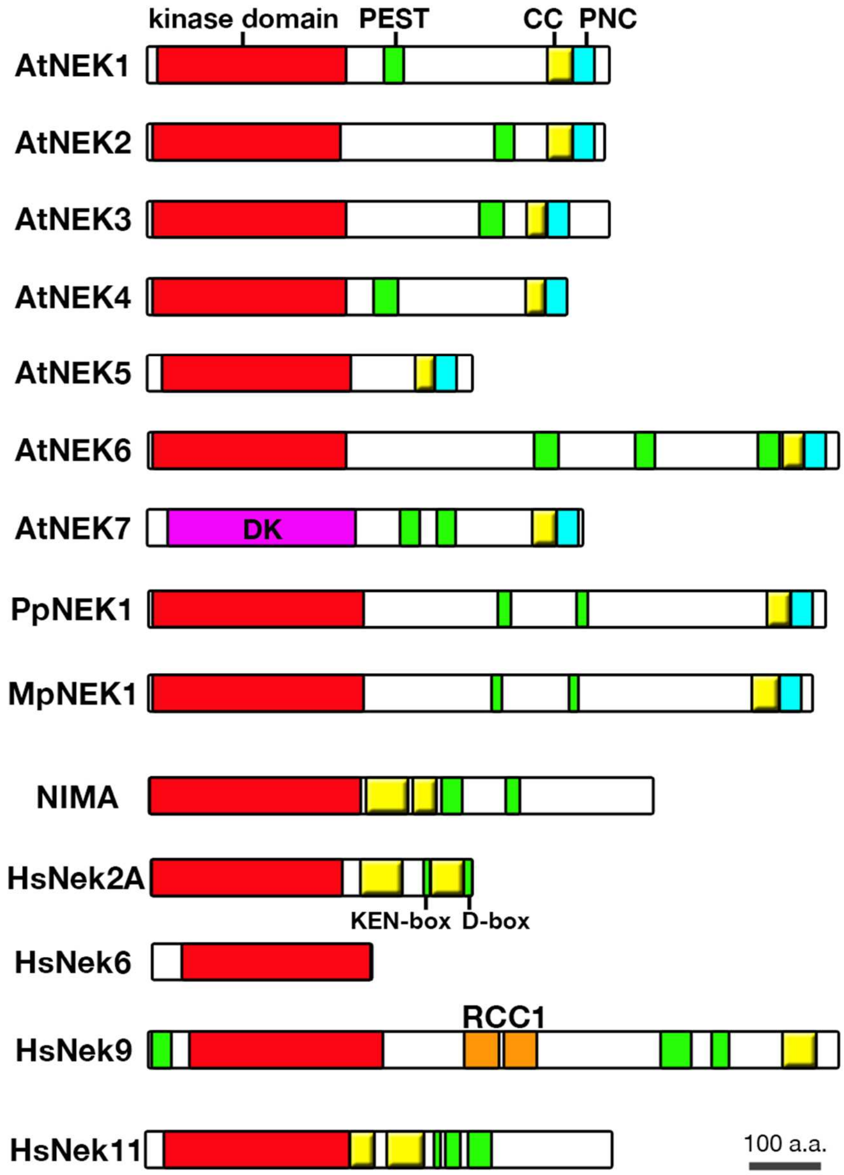

Fig 2
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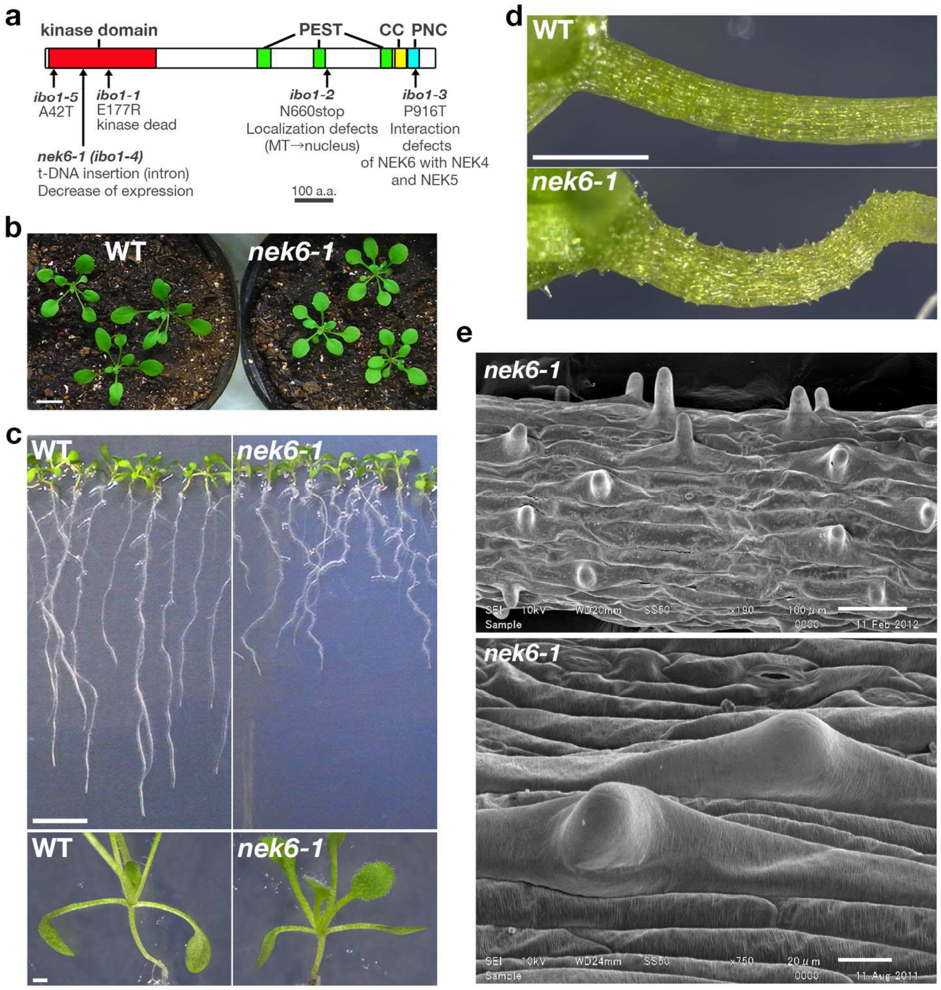

Fig 3
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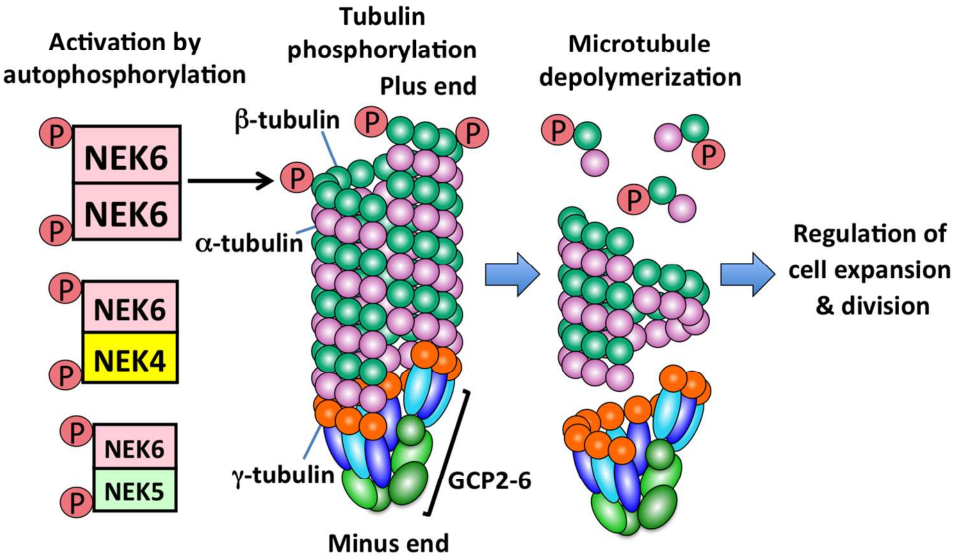

Fig 4
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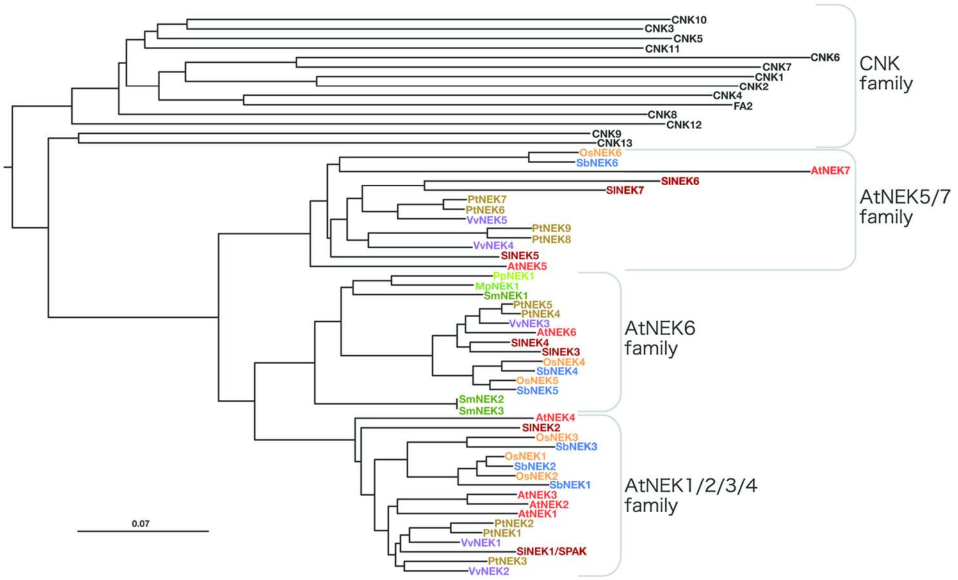

Fig 5

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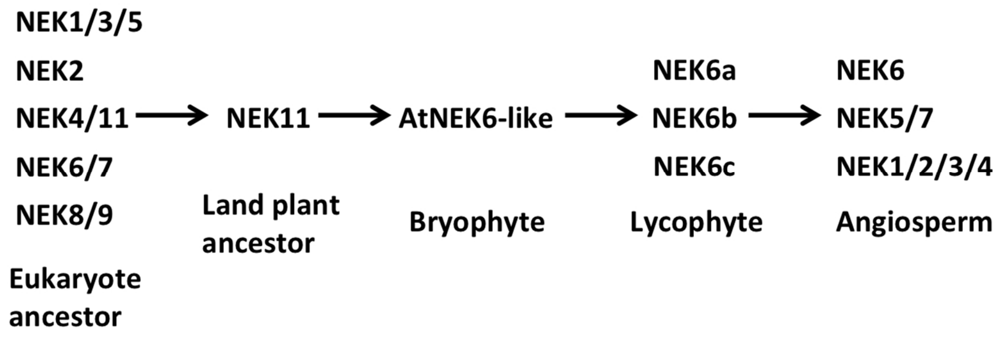

Fig 6
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Fig S1
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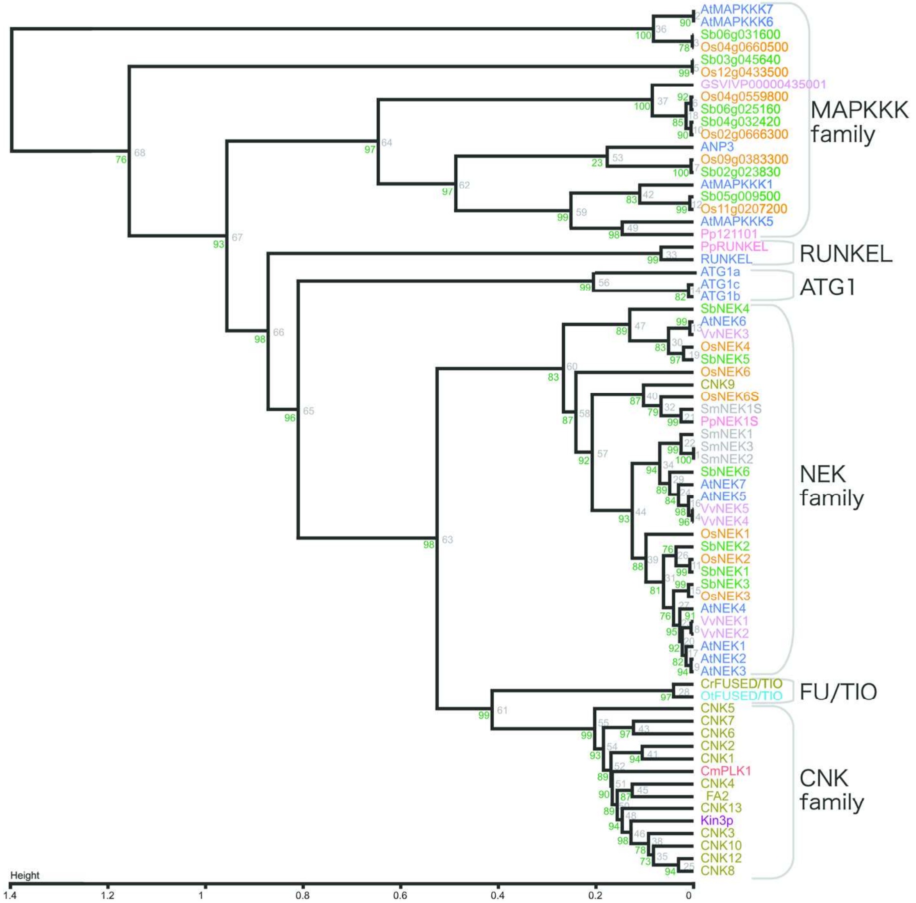

Fig S2
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