NIMA-related kinases 6, 4, and 5 interact with each other to regulate microtubule organization during epidermal cell expansion in *Arabidopsis thaliana*

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SUMMARY

NimA-related kinase 6 (NEK6) has been implicated in microtubule regulation to suppress the ectopic outgrowth of epidermal cells; however, its molecular functions remain to be elucidated. Here, we analyze the function of NEK6 and other members of the NEK family regarding epidermal cell expansion and cortical microtubule organization. The functional NEK6-green fluorescent protein fusion localizes to cortical microtubules, predominantly in particles that exhibit dynamic movement along microtubules. The kinase-dead mutant of NEK6 (*ibo1-1*) exhibits a disturbance of the cortical microtubule array at the site of ectopic protrusions in epidermal cells. Pharmacological studies with microtubule inhibitors and quantitative analysis of microtubule dynamics indicate excessive stabilization of cortical microtubules in *ibo1*/*nek6* mutants. In addition, NEK6 directly binds to microtubules in vitro and phosphorylates β-tubulin. NEK6 interacts and co-localizes with NEK4 and NEK5 in a transient expression assay. The *ibo1-3* mutation markedly reduces the interaction between NEK6 and NEK4 and increases the interaction between NEK6 and NEK5. NEK4 and NEK5 are required for the *ibo1*/*nek6* ectopic outgrowth phenotype in epidermal cells. These results demonstrate that NEK6 homodimerizes and forms heterodimers with NEK4 and NEK5 to regulate cortical microtubule organization possibly through the phosphorylation of β-tubulins.

INTRODUCTION

Development relies on the coordinated control of cellular growth leading to proper spatiotemporal morphological patterns. Previous genetic and physiological studies demonstrated that cortical microtubules are essential for plant cell morphogenesis and are regulated by phytohormones and microtubule-associated proteins (MAPs) (reviewed in Shibaoka, 1994; Lloyd and Chan, 2004; Hashimoto and Kato, 2005; Sedbrook and Kaloriti, 2008; Wasteneys and Ambrose, 2009), whereas the mechanism of microtubule regulation during plant cell growth remains to be elucidated.

Pharmacological analyses suggested the involvement of protein phosphorylation in the regulation of microtubule organization and cell expansion (Shibaoka, 1994; Baskin and Wilson, 1997). Genetic analysis identified several components of phosphorylation-dependent cortical microtubule regulation. PROPYZAMIDE HYPERSENSITIVE1 (PHS1) functions as a mitogen-activated protein kinase (MAPK) phosphatase and regulates directional cell growth through stabilizing cortical microtubule organization in *Arabidopsis thaliana* (Naoi and Hashimoto, 2004; Walia *et al.*, 2009). TONNEAU2 of *A. thaliana* and DISCORDIA1 and ALTERNATIVE DISCORDIA1 of *Zea mays* are nearly identical proteins with significant similarities to a regulatory subunit of protein phosphatase 2A and participate in the control of cortical microtubules, preprophase bands, and organ development (Camilleri *et al.*, 2002; Wright *et al.*, 2009). Recently, casein kinase 1-like 6 (CKL6) has been shown to associate with microtubules and to phosphorylate β-tubulin (Ben-Nissan *et al.*, 2008). Overexpression of CKL6 affects the morphology of leaf pavement cells (Ben-Nissan *et al.*, 2008). These reports suggest that signaling cascades involving protein phosphorylation participate in the control of microtubule organization.

NIMA-related kinases (NEKs) comprise a family of mitotic kinases conserved in eukaryotes and regulate various mitotic events including mitotic entry, centrosome separation, spindle formation, and cytokinesis (O'Connell *et al.*, 2003; O'Regan *et al.*, 2007). Mammalian Nek2 localizes to the centrosomes and initiates the separation of duplicated centrosomes through the phosphorylation of structural linker proteins that bridge them (Fry *et al.*, 1998;

Bahe *et al.*, 2005; reviewed in Fry, 2002). Nercc1/Nek9 associates with centrosomes and participates in bipolar spindle formation through the activation of two other centrosomal NEKs, NEK6 and NEK7 (Roig *et al.*, 2002; 2005; Belham *et al.*, 2003; Kim *et al.*, 2007; Rapley *et al.*, 2008; O'Regan and Fry, 2009; Richards *et al.*, 2009). In addition, several NEKs have been shown to regulate microtubule function in cilia and flagella (Mahjoub *et al.*., 2002; Bradley *et al.*, 2005; Quarmby and Mahjoub, 2005). These studies show that NEKs are essential signaling components for centrosome function and microtubule organization.

Although previous studies have identified multiple members of the plant NEK family implicated in plant development, molecular and cellular functions of the plant NEKs remain to be elucidated. A tomato (*Solanum lycopersicum*) NEK named SPAK has been identified as interacting with SELF PRUNING, an ortholog of TERMINAL FLOWER1 of *A. thaliana*, and antisense expression of *SPAK* affects the shape of the fruit (Pnueli *et al.*, 2001). Poplar (*Populus tremula* x *alba*) *NEK1* (*PNek1*) mRNA accumulates at the onset of mitosis (Cloutier *et al.*, 2005), and the *PNek1* promoter is preferentially activated at the site of auxin accumulation (Vigneault *et al.*, 2007). Overexpression of *PNek1* induces abnormal flowers in *A. thaliana* (Cloutier *et al.*, 2005). Comparative expression analysis of *NEKs* in poplar, rice, and *A. thaliana* suggests the involvement of *NEKs* in organ development and vascular formation (Vigneault *et al.*, 2007).

The genome of *A. thaliana* encodes seven members of the *NEK* family (Vigneault *et al.*, 2007), but their functions remain unknown with the exception of NEK6. We have previously shown that NEK6 controls epidermal cell expansion and morphogenesis (Motose *et al.*, 2008; Sakai *et al.*, 2008). The loss-of-function mutant of NEK6, *ibo1*/*nek6*, exhibits ectopic protuberances in epidermal cells of hypocotyls and petioles (Motose *et al.*, 2008; Sakai *et al.*, 2008), indicating that NEK6 suppresses ectopic outgrowth in epidermal cells. Detailed phenotypic analysis shows that a single ectopic protrusion is formed in the middle of the cell of the non-stomatal cell file and ethylene signaling promotes ectopic outgrowth in *ibo1* (Motose *et al.*, 2008). Genetic and biochemical analyses revealed that the kinase activity of NEK6 and the

association of NEK6 with microtubules are essential for suppressing ectopic outgrowth (Motose *et al.*, 2008). The *ibo1-1* mutation (E177R) in the kinase domain completely abolishes NEK6 kinase activity. The C-terminal region truncated in the *ibo1-2* allele (Q660stop) is required for the microtubule localization of NEK6. The *ibo1-3* mutation replaces Pro916 with Thr in the plant NEK C-terminal motif (PNC motif). The *ibo1-4*/*nek6-1* mutant is a T-DNA insertion mutant, in which *NEK6* mRNA is abolished (Sakai *et al.*, 2008).

These studies suggest that NEK6 regulates cellular growth and morphogenesis through microtubule function. However, the molecular basis of NEK6-dependent microtubule regulation remains unknown. In addition, the other six members of the NEK family remain to be characterized. Therefore, further analysis of NEK6 and other NEK members should provide new insight into the control of cellular morphogenesis and microtubule function. In this study, we show that NEK6 localizes to microtubules, phosphorylates β-tubulins, interacts with other NEK members, and participates in the cortical microtubule organization during epidermal cell expansion.

RESULTS

Dynamic expression and localization of NEK6

Our previous study demonstrated that green fluorescent protein (GFP)-tagged NEK6 transiently expressed in *Nicotiana benthamiana* leaves localizes to cortical microtubules (Motose *et al.*, 2008). To demonstrate the functional relevance of NEK6 to microtubule function, we analyzed the subcellular localization and dynamics of NEK6 in live cells of *A. thaliana*. For this experiment, GFP was fused to the C-terminus of NEK6 and expressed under the control of the native *NEK6* promoter in the *ibo1-4*/*nek6-1* mutant background. We used the mutant allele *ibo1-4*/*nek6-1* in which *NEK6* mRNA is absent (Sakai *et al.*, 2008). The *ibo1-4* mutants exhibited ectopic outgrowth of epidermal cells in the hypocotyls and petioles, while the *NEK6pro:NEK6-GFP* lines in the *ibo1-4* background had no ectopic outgrowth or protrusions (Figure S1). This result indicated that GFP-tagged NEK6 was functional.

NEK6-GFP localized to filamentous structures and concentrated in particles along the filaments (Figure 1a, b, c, d). We investigated the pattern of NEK6-GFP localization by double immunofluorescence microscopy using antibodies against α-tubulin and GFP. As shown in Figure 1a, NEK6-GFP localized to cortical microtubules. To confirm the association of NEK6-GFP with microtubules, we treated NEK6-GFP lines with a microtubule-depolymerizing drug oryzalin and a microtubule-stabilizing drug taxol. In the presence of oryzalin, NEK6-GFP signal was dispersed throughout the cytosol, whereas the addition of taxol stabilized the association of NEK6-GFP with cortical microtubules (Figure 1b). The localization pattern of NEK6-GFP was almost identical to that of transiently expressed GFP-NEK6 in our previous study (Motose *et al.*., 2008). These results demonstrated that NEK6 associates with cortical microtubules.

Time-lapse imaging with spinning disc confocal microscopy revealed the dynamic behavior of NEK6-GFP particles on the microtubules (Figure 1c, d, Movie S1, S2). When one moving particle labeled with NEK6-GFP encountered another, these particles were collided and repulsed from each other (upper panels in Figure 1c, Movie S1). Some particles collided and fused with

each other and then divided into small size particles (lower panels in Figure 1c, Movie S1). The NEK6-GFP signals were sometimes concentrated at the ends of filaments labeled with NEK6-GFP, which exhibited growth and shrinkage (boxed region in Figure 1c, d, Movie S1, S2). When the NEK6-GFP particle encountered another preexisting microtubule labeled by NEK6-GFP, this NEK6-GFP particle was repulsed (Figure 1d, Movie S2). During cytokinesis, NEK6-GFP localized to phragmoplasts and mitotic spindles (Figure 1e, f). From these results, NEK6 predominantly associates with microtubules and the particles labeled with NEK6 exhibit dynamic movement along microtubules.

To determine whether NEK6 directly binds to microtubules, we performed an in vitro microtubule co-sedimentation assay. Taxol-stabilized microtubules were incubated with or without the full-length NEK6 fused at the N-terminus with glutathione S-transferase (GST-NEK6) and proteins were pelleted by centrifugation. When GST-NEK6 was incubated and centrifuged in the absence of microtubules, GST-NEK6 existed in the supernatant. When GST-NEK6 was incubated with microtubules, GST-NEK6 was recovered in the pellet (Figure S2). This result indicates the direct interaction of NEK6 with microtubules.

To investigate the expression pattern of NEK6 protein during development, β-glucuronidase (GUS) was fused to the C-terminus of NEK6 and expressed under the native *NEK6* promoter in the *ibo1-1* mutant. The *NEK6pro:NEK6-GUS* construct restored the wild-type phenotype to the *ibo1-1* mutant, indicating that the NEK6-GUS fusion protein was functional (Figure S1). NEK6-GUS was preferentially accumulated in dividing or expanding cells of embryos and seedlings (Figure S3). In addition, NEK6-GUS was accumulated in the root apical meristem, lateral root meristem, shoot apical meristem, leaf primodia, and vascular tissue (Figure S3). During reproductive development, NEK6-GUS was preferentially expressed in inflorescent stems, flower buds, and carpels. These expression patterns suggested that NEK6 is involved in cellular division and expansion during the development of various organs and tissues.

Involvement of NEK6 in microtubule organization

To investigate the possible involvement of NEK6 in microtubule function, we

monitored cortical microtubule organization in the wild-type and *ibo1-1* mutant by using GFP-tagged β-tubulin 6 (GFP-TUB6). We used seedlings grown in the long-day condition (16 h light and 8 h dark) or in continuous light (24 h light) because the ectopic protrusions are formed in these conditions but suppressed in the dark condition (Motose *et al.*, 2008). In the wild-type epidermal cells, cortical microtubules are arranged in a homogeneous array perpendicular or slightly oblique to the longitudinal axis of cells (Figure 2a, b). In the epidermal cells of the *ibo1-1* mutant before ectopic outgrowth, cortical microtubules were arranged more randomly and irregularly than those of the wild-type (Figure 2c). At the first stage of outgrowth, cortical microtubules appeared to curve around the outgrowth point (Figure 2d). During ectopic outgrowth in the epidermal cells, cortical microtubule arrays became more irregular and gradually exhibited a whirled pattern in the early outgrowth point (Figure 2e, f). Finally, cortical microtubules oriented perpendicular to the growth axis of ectopic protuberance in the *ibo1-1* mutant (Figure 2g, h). Figure 2i illustrates the stages of ectopic outgrowth in Figure 2c to 2h. This result suggested that NEK6 is involved in the organization of cortical microtubule arrays.

Our previous immunofluorescence analysis could not detect cortical microtubule defects in *ibo1* mutants (Figure S3 in Motose *et al.*, 2008). This might be due to technical problems. In our previous analysis, it was difficult to stain cortical microtubules of hypocotyl epidermal cells and cortical microtubules were detected in the relatively small number of hypocotyl cells. Therefore, this might make it difficult to investigate microtubules specifically focusing on morphologically affected cells in hypocotyls. The previous immunofluorescence analysis only allowed limited cells to be studied whereas our current study make it possible to study more cells at different stages of protrusion formation.

To address the role of NEK6 in microtubule organization, changes of microtubule dynamics in the *ibo1-1* mutant were analyzed. First, we analyzed the effect of a microtubule-depolymerizing drug, oryzalin, on cortical microtubules in the hypocotyl epidermal cells. In the wild-type, the majority of the cortical microtubules were depolymerized after treatment with oryzalin at 6 μM for 40 min (Figure 2j). In contrast to this, cortical microtubules in the *ibo1-1*

mutant were resistant to the same treatment with oryzalin (Figure 2j). In both the wild-type and *ibo1-1* mutant, treatment with 10 μM oryzalin for 15 min was sufficient to depolymerize all cortical microtubules. This result suggests increased stability of cortical microtubules in the hypocotyl epidermal cells of *ibo1-1* mutant.

Next, we analyzed microtubule dynamics in the wild type and *ibo1-1* mutant. The dynamics of the plus end of individual cortical microtubules was monitored in the epidermal cells of hypocotyls by using GFP-TUB6. Table S1 shows the velocities of growth and shrinkage, frequency of catastrophe and rescue, the microtubule dynamicity, and percentages of time microtubules spent in periods of growth, shrinkage, and pause. Most parameters of microtubule dynamics were not significantly different between the wild type and *ibo1-1* mutant, whereas the time spent in the pause state in *ibo1-1* was significantly increased than that in the wild type (Table S1). The plus-ends of microtubules in *ibo1-1* tended to spend more time in pause than did that of the wild type. These results indicate that microtubules were stabilized in the epidermal cells of the *ibo1-1* mutant.

Effects of microtubule inhibitors on the *ibo1* phenotype

To further investigate the involvement of NEK6 in microtubule regulation, we analyzed the effects of a microtubule-depolymerizing drug propyzamide and a microtubule-stabilizing drug taxol on ectopic outgrowth in *ibo1* mutants (Figure 3). Propyzamide induced protrusions in hypocotyl epidermal cells in the wild-type at a low frequency (0.7 protrusions per seedling) as described by Sakai *et al.* (2008). Interestingly, propyzamide significantly suppressed protuberance formation of the hypocotyl epidermal cells in *ibo1* mutants (Figure 3). On the contrary, the addition of taxol strongly enhanced protuberance formation in *ibo1* mutants (Figure 3). These results suggest that microtubule stabilization is involved in the *ibo1* ectopic outgrowth phenotype.

In the previous study, the *nek6* phenotype was slightly enhanced but not suppressed by propyzamide (Sakai et al., 2008). Here, we find the suppression by propyzamide of the *nek6* phenotype. This discrepancy might be due to differences in the growth conditions including the growth media and light conditions. Seedlings were grown on the Okada and Shimura medium in Sakai

et al. (2008) while seedlings are grown on the modified half-strength Murashige and Skoog (MS) medium in this study. Because our previous study indicates that the *nek6* phenotype is enhanced by ethylene but is suppressed by gibberellin and etiolation (Motose et al., 2008), growth condition and endogenous hormone levels may affect the *nek6* phenotype and effects of microtubule inhibitors.

NEK6 phosphorylates β-tubulin

Because of the association of NEK6 with microtubules and irregular cortical microtubules in *ibo1-1*, we hypothesized that NEK6 might phosphorylate MAPs and/or microtubules themselves. To examine this possibility, protein fractions that contained MAPs and microtubules (MAPs fractions) were purified from *Arabidopsis* suspension cells and fractionated on an anion-exchange column. Each fraction was then subjected to an in vitro kinase assay with or without the recombinant GST-NEK6 protein. Phosphorylation of a protein band (~50 kDa) was significantly enhanced in the addition of GST-NEK6 (Figure S4). This protein band was analyzed by matrix-assisted laser desorption/Ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis, which showed that the protein band was a mixture of α-tubulin and β-tubulin (Table S2).

To examine whether NEK6 could phosphorylate tubulin, we incubated purified Arabidopsis tubulin with or without GST-NEK6 in the presence of γ^{32}P-ATP. GST-NEK6 significantly phosphorylated purified tubulin (Figure 4a). Next, we incubated recombinant His-tagged β-tubulin 4 (TUB4) or β-tubulin 6 (TUB6) with or without GST-NEK6 in the presence of γ^{32}P-ATP; both TUB4 and TUB6 were strongly phosphorylated by GST-NEK6 (Figure 4b, c). The phosphorylated TUB4 and TUB6 were completely dephosphorylated by the incubation with λ protein phosphatase (λPP), and the dephosphorylation by λPP was overcome by the addition of phosphatase inhibitors (Figure 4d). These results demonstrated that β-tubulin is a bona fide NEK6 substrate.

Interaction of NEK6 with NEK4 and NEK5

To identify proteins that interact with NEK6, a yeast two-hybrid screen was conducted using full-length NEK6 as the bait. Three NEK genes, including

NEK6, NEK4, and NEK5, were repeatedly isolated from 11 positive colonies (Table S3). cDNA fragments of NEK6, NEK4, and NEK5 were recovered from the yeast and found to be truncated at the 5' end. These cDNAs encoded C-terminal regions that contained coiled-coil domains. These proteins bound to NEK6 and induced the *ADE2*, *HIS3*, *MEL1*, and *AUR1-C* reporter genes in yeast cells (Figure 5a), suggesting that NEK6 interacts with NEK4, NEK5, and itself through their C-terminal regions.

Next, we analyzed the interaction of NEK4 with NEK5, NEK6, and itself in a yeast two-hybrid assay. The NEK4 construct was used as bait and transformed into yeast cells together with the prey vectors. As shown in Figure 5b, NEK4 interacted with NEK6 and itself but not with NEK5.

To confirm the interaction of NEK6 with NEK4 and NEK5 in plant cells, the NEK6 C-terminal tail immediately following the kinase domain (from Q263 to S956) was fused with GFP at the N terminus (GFP-NEK6C) and coexpressed with hemagglutinin (HA)-tagged C-terminal tail immediately following the kinase domain of NEK4 or NEK5 [HA-NEK4C (from Q259 to V555) and HA-NEK5C (from Q269 to I427)] in the leaves of *N. benthamiana*. The protein complex containing GFP-NEK6C was immunoprecipitated using anti-GFP antibody. Both HA-NEK4C and HA-NEK5C were coimmunoprecipitated with GFP-NEK6C (Figure 5c), indicating the interaction of NEK6 with NEK4 and NEK5 in planta.

It has been previously shown that the *ibo1-3* mutation, in which a conserved Pro residue at amino acid position 916 in the plant NEK C-terminal (PNC) motif is substituted by Thr, induced ectopic outgrowth in epidermal cells, a loss-of-function phenotype of NEK6 (Motose *et al.*, 2008; Figure 3). Because the C-terminal region is required for the dimerization of NEKs (Pnueli *et al.*, 2001; Cloutier *et al.*, 2005), we investigated the effect of the *ibo1-3* mutation ($NEK6^{P916T}$) on the interaction of NEK6 with NEK4 and NEK5 in a yeast two-hybrid assay. The *ibo1-3* mutation was introduced into the NEK6 construct used as the bait and transformed into yeast cells together with the prey vectors. As shown in Figure 5d, the *ibo1-3* mutation markedly reduced the interaction between NEK6 and NEK4, whereas *ibo1-3* increased the interaction of NEK6 with NEK5 in yeast cells. However, the dimerization of NEK6 was not affected by the *ibo1-3* mutation (Figure 5d). These results suggest that the NEK6-NEK4

interaction might be required for NEK6 function, and the *ibo1-3* mutation could alter the affinity of NEK6 for NEK4 and NEK5.

Colocalization of NEKs

To address the subcellular localization of NEKs, NEK proteins tagged with GFP at the N-terminus were transiently expressed in the leaves of *N. benthamiana* under the control of the cauliflower mosaic virus (CaMV) 35S promoter. GFP-tagged NEK1, NEK2, NEK3, NEK5, and NEK7 localized to microtubules in a similar manner as GFP-NEK6 (Figure 6a). In contrast, GFP-NEK4 mainly accumulated in the cytoplasm (Figure 6a). Co-expression of GFP-NEK proteins with NEK6 tagged with red fluorescent protein (RFP) at the N-terminus (RFP-NEK6) demonstrated that all of the GFP-NEKs colocalized with RFP-NEK6 in a punctate filamentous pattern (Figure 6b). Interestingly, GFP-NEK4 exhibited a marked accumulation in punctate structures with RFP-NEK6. These results implied that NEK6 colocalizes with other NEK members and regulates the localization of NEK4.

Genetic interaction of *NEK6* with *NEK4* and *NEK5*

Based on the colocalization and interaction of NEK6 with NEK4 and NEK5, NEK6 might function with NEK4 and NEK5 in the regulation of cell expansion and morphogenesis. To test the genetic interaction of *NEK6* with *NEK4* and *NEK5*, we analyzed phenotypes of T-DNA insertion mutants of *NEK4* and *NEK5* and their double mutants with *ibo1*. We identified *nek4-1* and *nek5-1* mutants in Salk T-DNA insertion lines and generated the *ibo1 nek4* and *ibo1 nek5* double mutants. The *nek4-1* and *nek5-1* mutants did not express mRNA for *NEK4* and