

NIMA-related kinases regulate directional cell growth and organ development through microtubule function in *Arabidopsis thaliana*

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NIMA-related kinase 6 (NEK6) regulates cellular expansion and morphogenesis through microtubule organization in *Arabidopsis thaliana*. Loss-of-function mutations in *NEK6* (*nek6/ibo1*) cause ectopic outgrowth and microtubule disorganization in epidermal cells. We recently found that NEK6 forms homodimers and heterodimers with NEK4 and NEK5 to destabilize cortical microtubules possibly by direct binding to microtubules and the β -tubulin phosphorylation. Here, we identified a new allele of *NEK6* and further analyzed the morphological phenotypes of *nek6/ibo1* mutants, along with alleles of *nek4* and *nek5* mutants. Phenotypic analysis demonstrated that NEK6 is required for the directional growth of roots and hypocotyls, petiole elongation, cell file formation, and trichome morphogenesis. In addition, *nek4*, *nek5*, and *nek6/ibo1* mutants were hypersensitive to microtubule inhibitors such as propyzamide and taxol. These results suggest that plant NEKs function in directional cell growth and organ development through the regulation of microtubule organization.

The development of multicellular organisms depends on cellular growth and morphogenesis. The directional growth of plant cells depends on the cortical array of microtubules.^{1–3} However, the mechanism of microtubule regulation remains to be elucidated. Recent genetic analyses suggested the involvement of protein phosphorylation in microtubule organization and directional cell expansion.^{4–6}

Never in mitosis A (NIMA) is a Ser/Thr protein kinase, which was first discovered from a mitotic mutant *nimA* of *Aspergillus nidulans*.^{7,8} NIMA-related kinases (NEKs) have been found in various fungi and animals, and they comprise a family of mitotic kinases conserved in eukaryotes. In fungi and animals, NEKs regulate various mitotic events including mitotic initiation, centrosome separation, spindle formation, and cytokinesis.^{7,8} Plants have 6–9 *NEK* genes but their function is not clearly understood. NEK6 has been found to function in epidermal cell expansion and morphogenesis in *Arabidopsis thaliana*.^{9,10} NEK6 has also been identified as an interacting protein with armadillo repeat-containing kinesins (ARKs).⁹ The loss-of-function mutant of *NEK6*, *ibo1/nek6*, exhibits ectopic protuberances in epidermal cells of hypocotyls and petioles (Fig. 1A and B), indicating that NEK6 suppresses ectopic outgrowth in epidermal cells.^{9,10} A single ectopic protrusion is formed in the middle of the cell of the non-stomatal cell file in hypocotyls, suggesting that the protrusion might be a trichome-like structure.¹⁰ The ectopic outgrowth of *ibo1/nek6* mutants is strongly promoted by ethylene signaling.¹⁰ Genetic and biochemical analyses revealed that the kinase

activity of NEK6 and the microtubule localization of NEK6 are essential for suppressing ectopic outgrowth.¹⁰

Recently, we showed that NEK6 interacts with other NEK members, directly binds to microtubules, phosphorylates β -tubulins, and regulates cortical microtubule organization during epidermal cell expansion.¹¹ The functional NEK6–green fluorescent protein fusion was concentrated in particles exhibiting dynamic movement along microtubules. The *nek6/ibo1* mutants showed disturbance in the cortical microtubule array at the site of ectopic protrusions in epidermal cells (Fig. 1C). The quantitative analysis of microtubule dynamics indicated excessive stabilization of cortical microtubules in *ibo1/nek6*. In addition, NEK6 directly bound to microtubules and phosphorylated β -tubulin in vitro. The interaction of NEK6 with NEK4 and NEK5, which is affected by the *ibo1-3* mutation, was shown to be required for the ectopic outgrowth phenotype of *ibo1/nek6*.¹¹ These results suggest that NEK6 regulates cortical microtubule organization by interacting with other NEKs and the phosphorylation of β -tubulin.

Here, we identified a new allele of *NEK6* (*ibo1-5*), which had a point mutation in the kinase domain (Ala to Thr substitution at position 42). The *ibo1-5* mutant exhibited ectopic protrusions from epidermal cells, which were identical to those of other *nek6/ibo1* mutants (Fig. 1A). This result supports that the kinase domain is essential for the function of NEK6. We further analyzed the developmental phenotypes of *nek6* mutants. The *nek6-1/ibo1-4* seedlings showed aberrant root waving or

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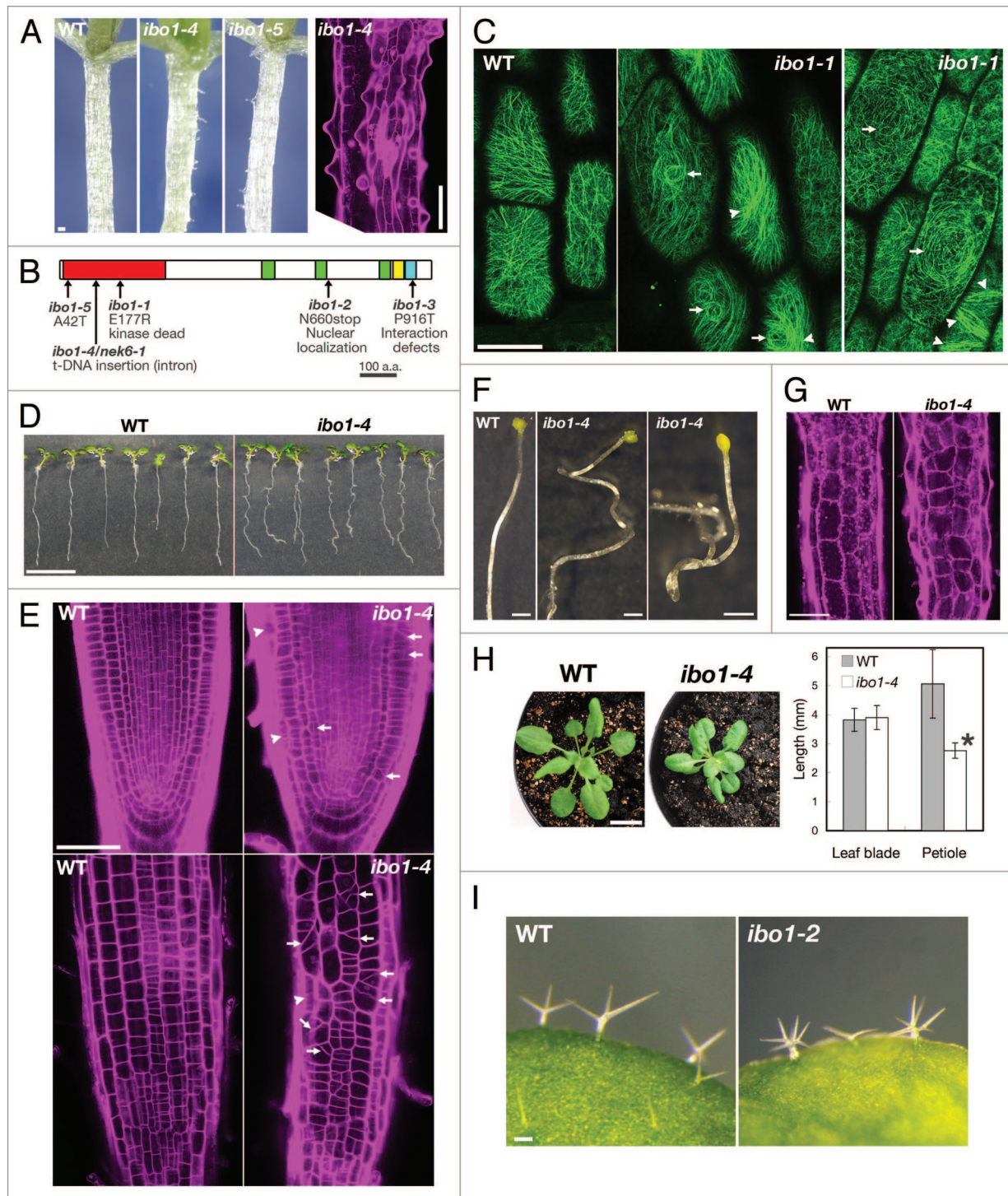


Figure 1. The *nek6/ibo1* mutants exhibit disorganized cell growth. (A) Morphology of hypocotyls of 10-d-old seedlings of the wild type (WT) and *ibo1*. The right part shows a hypocotyl of *ibo1-4* stained with propidium iodide. (B) Structure of NEK6. PEST sequence (green), coiled-coil domain (yellow), plant NEK C-terminal motif (blue) and mutation sites are shown. (C) Cortical microtubules were visualized with GFP-TUB6 in hypocotyl epidermal cells of the wild type (WT) and *ibo1-1*. Arrows and arrowheads in (C) indicate whirled arrays of microtubules and microtubule bundles, respectively. (D and E) Morphology of the wild type (WT) and *ibo1-4* seedlings grown vertically for 7 d. (E) Seedlings were stained with propidium iodide and root tips were observed under a confocal microscope. Median longitudinal optical section (upper parts in E) and epidermis (lower parts in E) of root tip were shown. Arrows and arrowheads indicate aberrant cell plates and irregular cell files, respectively. (F) Morphology of hypocotyls of the wild type (WT) and *ibo1-4* grown in the dark for 7 d. (G) Hypocotyl cortex of the wild type (WT) and *ibo1-4*. Seedlings grown in the light for 7 d were stained with propidium iodide and observed under a confocal microscope. (H) Morphology of 4-week-old wild type (WT) and *ibo1-4* plants (left parts) and quantification of lengths of leaf blades and petioles of 14-d-old seedlings (right parts). Values are means \pm SD ($n = 10$). Asterisk indicates significant difference from the wild type (Student t-test, $p < 0.01$). (I) Trichomes of the wild type (WT) and *ibo1-4*. Bars = 100 μ m (A, G and I), 50 μ m (C and E), 10 mm (D and H) or 1 mm (F).

skewing pattern when grown vertically (Fig. 1D). In the root tip of the *nek6-1/ibo1-4* mutant, cell files were disorganized and abnormal cell plates were formed (Fig. 1E), indicating that NEK6 is required for organized cell division and expansion, leading to regular cell file formation. The hypocotyl of a dark-grown *nek6-1/ibo1-4* seedling exhibited twisted growth (Fig. 1F). In the hypocotyl cortex of the *ibo1-4* mutant, reduced cell length and less organized cell files were observed (Fig. 1G). In addition, the *nek6-1/ibo1-4* mutant had short petioles (Fig. 1H). Furthermore, trichomes in *ibo1-2* had more branches than in the wild type (Fig. 1I). These results indicate that NEK6 is required for directional growth, organized cell division and expansion, petiole elongation and trichome branching.

To study the involvement of NEK4, NEK5 and NEK6 in the process of microtubule-dependent growth, we analyzed the effects of a microtubule-depolymerizing drug, propyzamide, and a microtubule-stabilizing drug, taxol, on the root growth in *nek4*, *nek5* and *nek6/ibo1* mutants. The *nek4-1*, *nek5-1*, and *nek6-1/ibo1-4* mutants were hypersensitive to propyzamide and taxol (Fig. 2A-C). In the presence of microtubule inhibitors, the mutant roots were shorter and slanted rightward more severely than those of the wild type. This result implies that NEK4, NEK5, and NEK6 are involved in the regulation of microtubule organization during root growth.

Taken together, the findings of the present study reveals that NEK6 regulates multiple developmental processes, including the directional growth of roots and hypocotyls, cell file formation, petiole elongation, and epidermal cell morphogenesis. Because NEK6 participates in the destabilization of microtubules, possibly through the phosphorylation of β -tubulin,¹¹ the regulation of microtubule organization by NEK6 may be important for directional cell growth and an organized pattern of cell division during organ development. The hypersensitivity of *nek4*, *nek5* and *nek6* mutants to microtubule inhibitors indicates that NEK4 and NEK5 also regulate microtubule-dependent cellular growth in concert with NEK6. The pleiotropic phenotype of *nek6/ibo1*, together with the mild phenotypes of *nek4* and *nek5*, suggests that NEK6 plays central roles in NEK-related regulatory pathways to control cell growth. In consonance with this, our previous study implied that NEK6 regulates the activity and localization of NEK4 and NEK5.¹¹ This is also consistent with other reports of multiple functions of NEK6.⁹⁻¹² In addition, we demonstrated the involvement of other NEK members in microtubule function, as was recently reported for another NEK member, namely, AtNek2 in *A. thaliana*.¹³ Fungal and animal NEKs mainly regulate mitotic cell division^{7,8} whereas plant NEKs control directional cell expansion⁹⁻¹¹ and also participate in stress response and seed germination.^{12,14} This might reflect that plant NEKs have evolved to acquire novel functions for the adaptation of plants to changing environmental conditions. Recent genetic analyses suggest that plant mitotic regulators might be recruited for the regulation of cell growth (e.g., endocycle) and environmental responses.^{15,16} Besides β -tubulin and ARKs, we identified novel

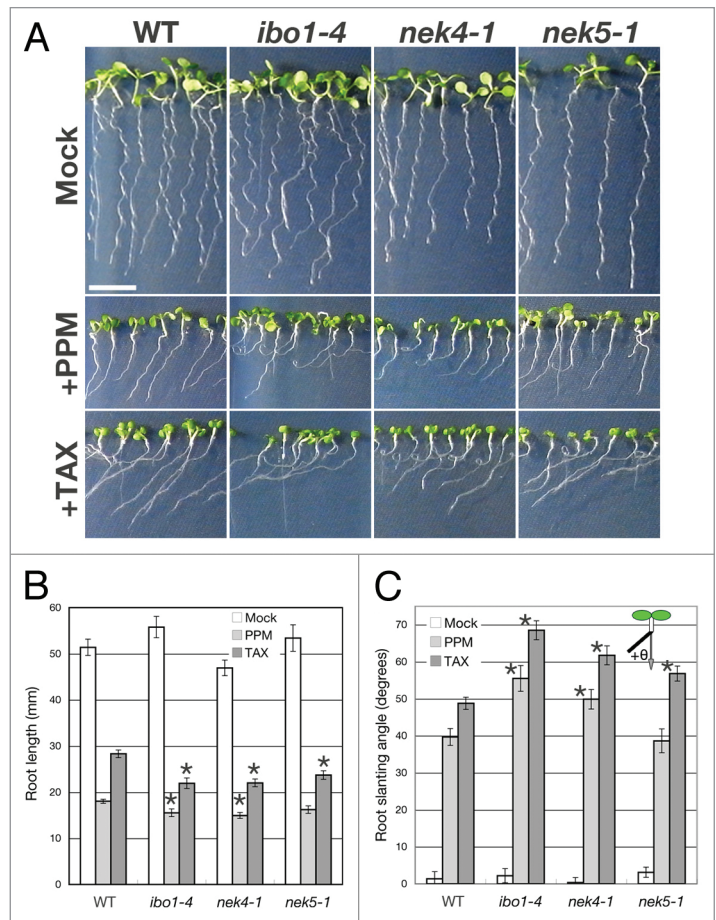


Figure 2. The *nek4*, *nek5* and *nek6/ibo1* mutants are hypersensitive to microtubule inhibitors. (A) Morphology of the wild type (WT) and *nek* mutant seedlings grown for 8 d on the MS agar medium in the absence (Mock) or presence of 3 μ M propyzamide (+PPM) or 1 μ M taxol (+TAX). Bar = 10 mm. (B) Root length of the wild type (WT) and *nek* mutants grown for 10 d in the absence (Mock) or presence of 3 μ M propyzamide (PPM) or 1 μ M taxol (TAX). (C) Root slanting angles (θ) of 8-d-old seedlings of the wild type (WT) and *nek* mutants grown as described above. Rightward- and leftward-slanting angles (viewed from the shoot apex) are expressed as positive and negative values, respectively. In (B) and (C), values are means \pm SE ($n \geq 22$). Asterisks indicate significant difference from the wild type (Student t-test, $p < 0.02$).

proteins interacting with NEK6 and substrates phosphorylated by NEK6. Further analysis of the plant NEK family and its downstream factors will provide novel insights into the regulation of organized cell growth and the underlying microtubule functions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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