Short Communication

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

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Abstract

NIMA-related kinase 6 (NEK6) regulates cellular expansion and morphogenesis through microtubule organizaiton 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 beta-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.

TEXT

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 *nim*A 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 beta-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 beta-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 beta-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 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 phosphoryation of beta-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 expansion[9-11] 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 beta-tubulin and ARKs, we identified novel 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.

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References

1. Barlow PW, Baluška F. Cytoskeletal perspectives on root growth and morphogenesis. Ann Rev Plant Physiol Plant Mol Biol 2000; 51:289-322; http://dx.doi.org/10.1146/annurev.arplant.51.1.289.

2. Sedbrook JC, Kaloriti D. Microtubules, MAPs and plant directional cell expansion. Trends Plant Sci 2008; 13:303-310; http://dx.doi.org/10.1016/j.tplants.2008.04.002.

3. Wasteneys GO, Ambrose JC. Spatial organization of plant cortical microtubules: close encounters of the 2D kind. Trends Cell Biol 2009; 19:62-71; http://dx.doi.org/10.1016/j.tcb.2008.11.004.

4. Camilleri C, Azimzadeh J, Pastuglia M, Bellini C, Grandjean O, Bouchez, D. The Arabidopsis TONNEAU2 gene encodes a putative novel protein phosphatase 2A regulatory subunit essential for the control of the cortical cytoskeleton. Plant Cell 2002; 14:833-845; http://dx.doi.org/10.1105/tpc.010402.

5. Naoi K, Hashimoto T. A semidominant mutation in an Arabidopsis mitogen-activated protein kinase phosphatase-like gene compromises cortical microtubule organization. Plant Cell 2004; 16:1841-1853; http://dx.doi.org/10.1105/tpc.021865.

6. Ben-Nissan G, Cui W, Kim DJ, Yang Y, Yoo BC, Lee JY. Arabidopsis casein kinase 1-like 6 contains a microtubule-binding domain and affects the organization of cortical microtubules. Plant Physiol 2008; 148:1897-1907; http://dx.doi.org/10.1104/pp.108.129346.

7. O'Connell MJ, Krien MJE, Hunter, T. Never say never. The NIMA-related protein kinases in mitotic control. Trends Cell Biol 2003; 13:221-228; http://dx.doi.org/10.1016/S0962-8924(03)00056-4.

8. O'Regan L, Blot J, Fry AM. Mitotic regulation by NIMA-related kinases. Cell Div 2007; 2:25-36; http://dx.doi.org/10.1186/1747-1028-2-25.

9. Sakai T, van der Honing H, Nishioka M, Uehara Y, Takahashi M, Fujisawa N, Saji K, Seki M, Shinozaki K, Jones MA, Smirnoff N, Okada K, Wasteneys GO. Armadillo repeat-containing kinesins and a NIMA-related kinase are required for epidermal-cell morphogenesis in Arabidopsis. Plant J 2008; 53:157-171;

http://dx.doi.org/10.1111/j.1365-313X.2007.03327.x.

10. Motose H, Tominaga R, Wada T, Sugiyama M, Watanabe Y. A NIMA-related protein kinase suppresses ectopic outgrowth of epidermal cells through its kinase activity and the association with microtubules. Plant J 2008; 58:829-844; http://dx.doi.org/10.1111/j.1365-313X.2008.03445.x.

11. Motose H, Hamada T, Yoshimoto K, Murata T, Hasebe M, Watanabe Y, Hashimoto T, Sakai T, Takahashi T. NIMA-related kinases 6, 4, and 5 interact with each other to regulate microtubule organization during epidermal cell expansion in *Arabidopsis thaliana*. Plant J 2011; 67:993-1005; http://dx.doi.org/10.1111/j.1365-313X.2011.04652.x.

12. Zhang B, Chen HW, Mu RL, Zhang WK, Zhao MY, Wei W, Wang F, Yu H, Lei G, Zou HF, Ma B, Chen SY, Zhang JS. NIMA-related kinase NEK6 affects plant growth and stress response in Arabidopsis. Plant J 2011; 68:830-843; http://dx.doi.org/10.1111/j.1365-313X.2011.04733.x.

13. Agueci F, Rutten T, Demidov D, Houben A. Arabidopsis AtNek2 kinase is essential and associates with microtubules. Plant Mol Biol Rep 2012; 30:339-348; http://dx.doi.org/10.1007/s11105-011-0342-1.

14. Lee SJ, Cho DL, Kang JY, Kim MD, Kim SY. AtNEK6 interacts with ARIA and is involved in ABA response during seed germination. Mol Cells 2010; 29:559-566; http://dx.doi.org/10.1007/s10059-010-0070-7.

15. De Veylder L, Larkin JC, Schnittger A. Molecular control and function of endoreplication in development and physiology. Trends Plant Sci 2011; 16:624-634; http://dx.doi.org/10.1016/j.tplants.2011.07.001.

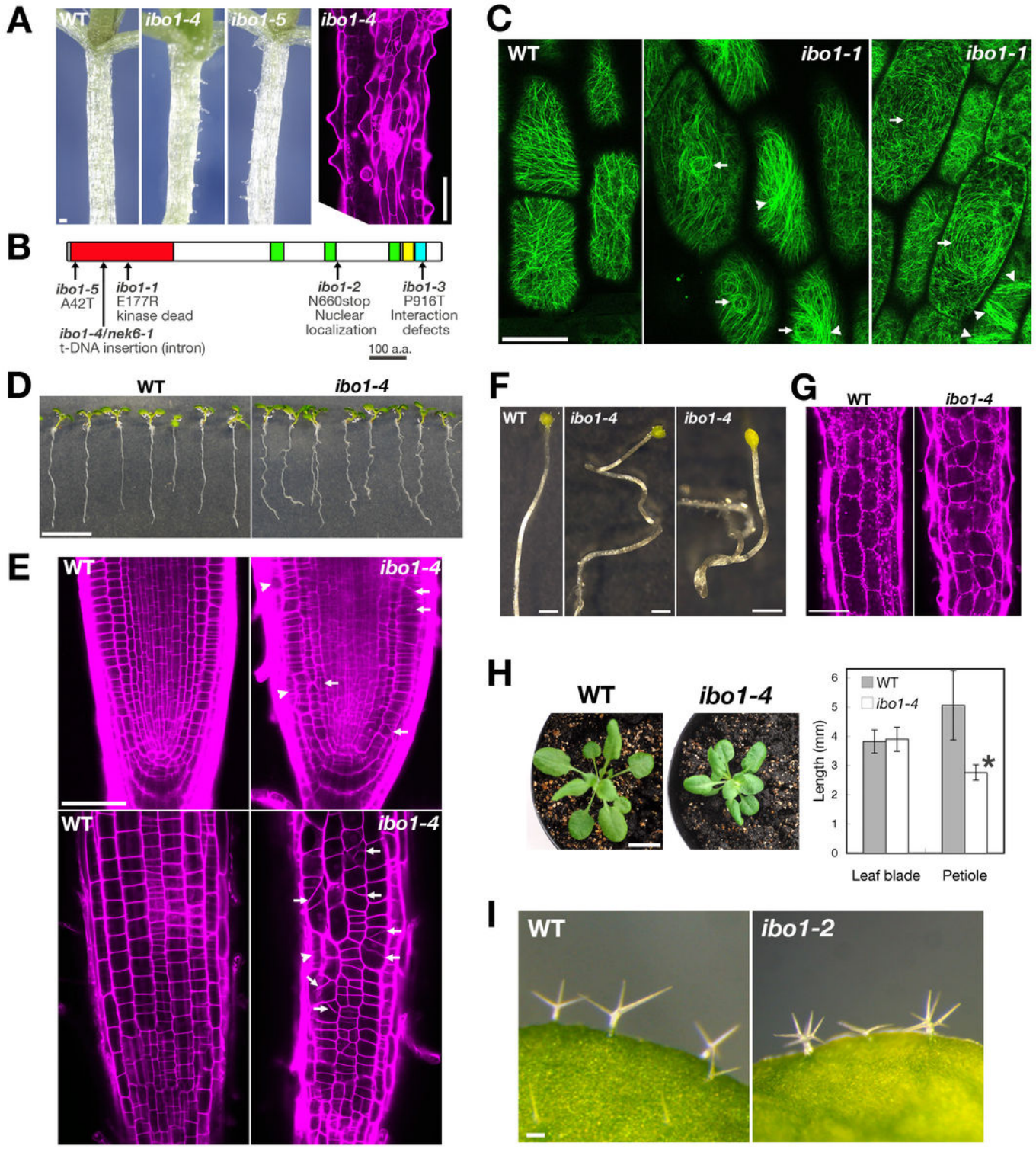
16. Komaki S, Sugimoto K. Control of the plant cell cycle by developmental and environmental cues. Plant Cell Physiol 2012; 53:953-964; http://dx.doi.org/10.1093/pcp/pcs070.

Figure Legends

Figure 1. The *nek6/ibo1* mutants exhibit disorganized cell growth. (A) Morphology of hypocotyls of 10-day-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 days. (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 days. (G) Hypocotyl cortex of the wild type (WT) and *ibo1-4*. Seedlings grown in the light for 7 days 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-day-old seedlings (right parts). Values are means ± 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 μm (A,

G and I), 50 μm (C and E), 10 mm (D and H) or 1 mm (F).

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 days on the MS agar medium in the absence (Mock) or presence of 3 μM propyzamide (+PPM) or 1 μM taxol (+TAX). Bar = 10 mm. (B) Root length of the wild type (WT) and *nek* mutants grown for 10 days in the absence (Mock) or presence of 3 μM propyzamide (PPM) or 1 μM taxol (TAX). (C) Root slanting angles (θ) of 8-day-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 ± SE ($n \geq 22$). Asterisks indicate significant difference from the wild type (Student *t*-test, $P < 0.02$).


A
WT
ibo1-4
ibo1-5
ibo1-4
B
ibo1-5
A42T
ibo1-1
E177R
kinase dead
ibo1-4/nek6-1
t-DNA insertion (intron)
ibo1-2
N660stop
Nuclear
localization
ibo1-3
P916T
Interaction
defects
100 a.a.
C
WT
ibo1-1
ibo1-1
D
WT
ibo1-4
E
WT
ibo1-4
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G
WT
ibo1-4
H
WT
ibo1-4
WT
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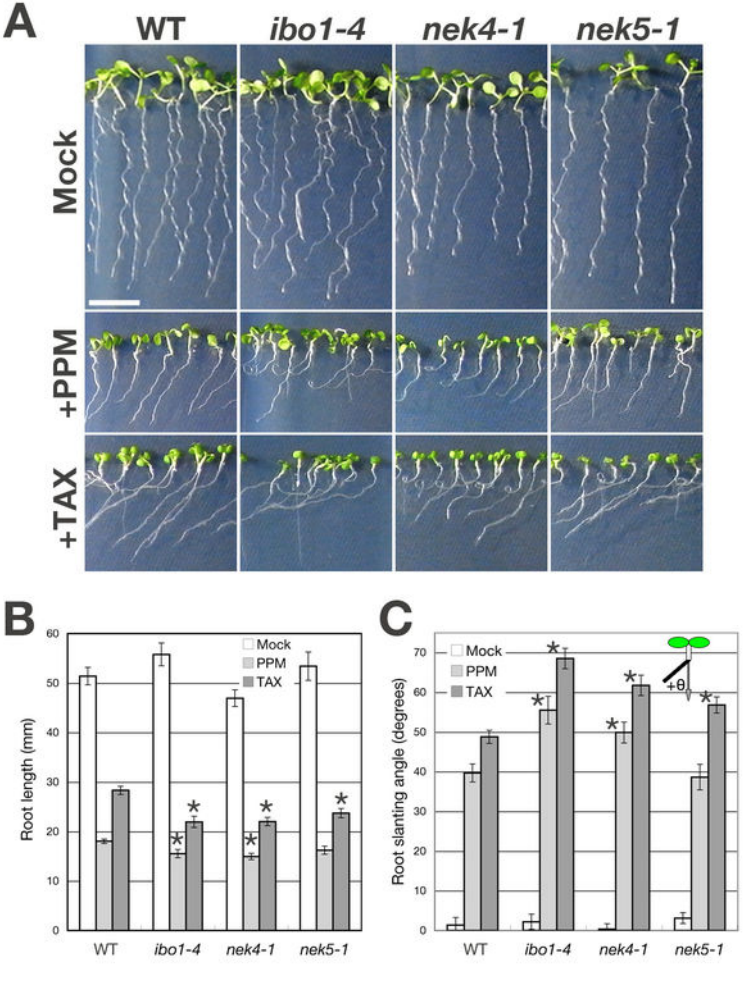
3
4
5
6
Leaf blade
Petiole
*
I
WT
ibo1-2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WT
ibo1-4
nek4-1
nek5-1
Mock
+PPM
+TAX
B
Root length (mm)
Mock
PPM
TAX
WT
ibo1-4
nek4-1
nek5-1
C
Root slanting angle (degrees)
Mock
PPM
TAX
+θ
WT
ibo1-4
nek4-1
nek5-1