

The carboxyl-terminal tail of the stalk of Arabidopsis NACK1/HINKEL kinesin is required for its localization to the cell plate formation site

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Abstract Plant cytokinesis is achieved by formation of cell plates in the phragmoplast, a plant-specific cytokinetic apparatus, which consists of microtubules (MTs) and microfilaments. During cytokinesis, the cell plate is expanded centrifugally outward from the inside of cells in a process that is supported by dynamic turnover of MTs. M-phase-specific kinesin NACK1, which comprises the motor domain at the amino-terminal half to move on MT bundles and the stalk region in the carboxyl-terminal half, is a key player in the process of MT turnover. That

is, the specific region in the stalk binds the MAP kinase kinase to activate the whole MAP kinase cascade, which stimulates depolymerization of MTs for the MT turnover. The stalk is also responsible for recruiting the activated kinase cascade to the mid-zone of the phragmoplast, which corresponds to the cell-plate formation site. It should be crucial to uncover roles of the NACK1 kinesin stalk as well as the motor domain in the formation of cell plates in order to understand the mechanisms of cell plate formation. Using dissected Arabidopsis NACK1 (AtNACK1/HINKEL) molecules and AtNACK1-fused GFP, we showed that the C-terminal tail of the stalk in addition to the motor domain is critical for its proper localization to the site of cell plate formation in the phragmoplast, probably by affecting its motility activity.

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Introduction

Cytokinesis is the process whereby one cell physically separates into two at the end of the cell cycle, which is essential for the growth and development of multicellular organisms. If cytokinesis fails to occur in animals, multinucleate cells, heteroploid cells, and aberrantly amplified centrosomes are formed, which are often related to tumorigenicity and apoptosis of cells (Normand and King 2010). In plants, defects in cytokinesis cause the generation of multinucleate cells and accumulation of stubs containing premature cell plates in aberrantly enlarged cells, which results in dwarfism of plant bodies and organs (Nishihama and Machida 2001; Nishihama et al. 2002; Sazuka et al. 2005; Strompen et al. 2002).

Plant cytokinesis is achieved by the formation of a cell plate in the characteristic cytokinetic machinery of plant cells called the phragmoplast, which is mainly composed of aligned microtubules (MTs) and actin filaments (Müller et al. 2009; Nishihama and Machida 2001; Otegui et al. 2005). Formation of the phragmoplast starts at a certain time in anaphase of the late M phase between two separating daughter nuclei in the internal region of the cytoplasm (Nishihama and Machida 2001; Otegui et al. 2005). As cytokinesis proceeds in a cell, bundles of phragmoplast MTs expand centrifugally from the interior to the periphery of the cell, driving the centrifugal development of a cell plate behind the expanding phragmoplast MTs. In the newly synthesized cell plate, a new cell wall is constructed within the lumens of membranous tubules that are formed by the fusion of membrane vesicles transported by membrane trafficking systems with MTs (Jürgens 2005; McMichael and Bednarek 2013; Van Damme and Geelen 2008; Woollard and Moore 2008). Although some vesicle trafficking-related components such as KNOLLE, Rab-A GTPases, and PATL1 have been identified (Chow et al. 2008; Lauber et al. 1997; Peterman et al. 2004; Waizenegger et al. 2000), the molecular mechanisms for the trafficking involving these factors in the formation of cell plates have yet to be determined.

The centrifugal expansion of the phragmoplast is supported by a dynamic turnover of MTs involving the depolymerization of MTs inside of the bundle, while MTs are newly generated (elongation) outside of the bundle (Murata et al. 2013; Nakaoka et al. 2012; Smertenko et al. 2011; Yasuhara et al. 1993). Several MT-associated proteins (MAPs) including MAP65, MOR1/GEM1 (XMAP215), and kinesin-like proteins stimulate the turnover of MTs (Müller et al. 2004; Nishihama et al. 2002; Sasabe et al. 2006; Strompen et al. 2002; Twell et al. 2002).

Expansion of the phragmoplast in tobacco BY-2 cells is positively regulated by the mitogen-activated protein (MAP) kinase cascade, all the components of which localize to the equatorial zone of the phragmoplast corresponding to the site of cell plate formation (Ishikawa et al. 2002; Nishihama et al. 2001; Soyano et al. 2003). Activity of the cascade is controlled by cytokinesis-specific kinesins, which are designated NACK1 and NACK2 in tobacco cells. NACK1 protein is located in the fraction that is purified biochemically from phragmoplasts by following the microtubule-based motility activity, suggesting that NACK1 may have a phragmoplast-associated motility activity (Nishihama et al. 2002). Both kinesins localize to the equatorial zone of the phragmoplast during cytokinesis and directly bind to the NPK1 MAP kinase kinase kinase (MAPKKK) to increase NPK1 activity at late M phase during cytokinesis (Nishihama et al. 2002), and this process is inhibited by phosphorylation of NACK1 and NPK1 by cyclin-dependent kinase

(CDKs) before metaphase (Sasabe et al. 2011a). These kinesins have the MT-based motor domains at the amino-termini and the stalk regions in the carboxyl-terminal halves that are rich in several predicted coiled-coils structures, one of which provide binding sites for NPK1 MAPKKK, and they also consistently localize to the equatorial region of the phragmoplast during its expansion. Thus, NACK1 kinesin recruits NPK1 MAPKKK, which binds NQK1 MAP kinase kinase (MAPKK) and NRK1 MAP kinase (MAPK) to the phragmoplast equator, in addition it activates NPK1 and the whole kinase cascade (Nishihama et al. 2002). On the basis of the above results, the NACK-PQR MAPK pathway including these components is proposed to positively facilitate expansion of the phragmoplast (Soyano et al. 2003). Activated NRK1 MAPK phosphorylates MAP65 to reduce its activity of bundling MTs, which increases the rate of the MT turnover to allow phragmoplast MT arrays to expand centrifugally (Sasabe et al. 2006).

Genetic analyses have shown that a similar pathway is also conserved in *Arabidopsis thaliana* (Kosetsu et al. 2010; Sasabe et al. 2011b; Takahashi et al. 2010). AtNACK1/HINKEL (HIK), an *Arabidopsis* homolog of NACK1, appears to be a key regulator of the progression of cytokinesis, because knockout and knock-down mutants of *AtNACK1/HIK* and/or *AtNACK2/STUD (STD)/TETRASPORE (TES)* show defects in cell plate structures and gametophytic lethality in *Arabidopsis* plants (Tanaka et al. 2004). The myc-tagged AtNACK1/HIK protein is also localized to the phragmoplast equator (Krupnova et al. 2013). In addition to *Arabidopsis* and rice (Sazuka et al. 2005), the genomes of all multicellular plants so far examined encode for proteins that are homologous to NACK1 (Miki et al. 2014), suggesting a ubiquitous role of these homologues in cytokinesis. The cellular biological information of AtNACK1/HIK in *Arabidopsis*, however, is still limited. In the present study, we took advantage of the tobacco cell line BY-2, which is considered to be highly proliferative, to investigate the molecular and cellular bases for the characteristics of AtNACK1/HIK. We have investigated which regions of AtNACK1 are required for its localization to the equatorial zone of the phragmoplast corresponding to the site of cell plate formation. The results in the present study show that the C-terminal region of the stalk, as well as the motor domain, is necessary for the proper localization of AtNACK1/HIK to the phragmoplast equatorial zone.

Materials and methods

DNA constructs

The deletion constructs were made by PCR and overlap PCR. Each fragment of *AtNACK1* cDNA was amplified

by PCR with specific primers and/or overlap primers (sequences of these primers will be sent on request) and cloned into the pENTR1A vector (Invitrogen, Carlsbad, CA). To express GFP fusions in BY-2 cells, the fragments of *AtNACK1* cDNAs in pENTR were subcloned into the binary vector pGWB452 (Nakagawa et al. 2007) by using Gateway[®] LR Clonase II (Invitrogen). All of these constructs were N-terminally GFP-fused. To express N-terminal GFP fusion in cells by estradiol, we introduced the sGFP-fused Gateway[®] cassette from the pGWB6 vector (Nakagawa et al. 2007) into the pER8 vector that contains the estradiol-inducible promoter sequence (*XVE* sequence) (Zuo et al. 2000), which yielded the pER8GW6 vector. The full length cDNA fragment of *AtNACK1* was also subcloned into the binary vector pER8GW6 by the Gateway system. To express the C-terminal GFP fusion in cells with estradiol, the *AtNACK1-GFP* fragment was amplified by PCR with the C-terminally GFP-fused *AtNACK1* sequence cloned in pGWB451 (Nakagawa et al. 2007) as a template, and cloned into pER8 vector. The 6.1-kb DNA fragment covering the *AtNACK1* locus from a position 1.3 kb 5'-upstream of the initiation codon to a position 0.75 kb 3'-downstream of the termination codon (Sasabe et al. 2011a) was used as a template to make a construct which expressed a GFP-fusion protein under the original promoter of *AtNACK1*. The 5.4-kb DNA fragments, excluding the 3' UTR of *AtNACK1*, were amplified by specific primers and cloned into the pCR8 vector (Invitrogen). This fragment was sequentially subcloned into the binary vector pGWB504 (Nakagawa et al. 2007) to fuse sGFP to the C-terminal of *AtNACK1*. The sequences of all constructs were confirmed by sequencing analysis.

Plant materials, transformations, and selection of transgenic lines

Tobacco BY-2 cells were maintained in suspension culture at 26 °C in darkness with weekly subculture in modified Linsmaier and Skoog medium. *Arabidopsis thaliana* plants (Col-0) were grown with a 16-h photoperiod at 22 °C either in soil or on Murashige and Skoog (MS) plates that contained 0.8 or 1.5 % agar. Each construct was transformed into BY-2 cells and/or wild-type Col-0 plants by using the *Agrobacterium tumefaciens*-mediated method (An 1985; Clough and Bent 1998). More than 10 lines of transformants including each construct of BY-2 were used for observation of subcellular localization, and the offspring of seven independent T1 plants were used for subsequent analysis. The BY-2 cells transformed with transgenes under the control of an estradiol-inducible promoter were cultured in liquid medium containing 0.1 μM 17-β-estradiol (Sigma-Aldrich, St Louis, MO, USA) for 16 h to express GFP fusion.

Microscopy

BY-2 calli were suspended in liquid medium and used for observation. GFP fluorescence was detected with an AXIO Imager (Carl Zeiss, Oberkochen, Germany) and a confocal microscope, FV1000 (Olympus, Tokyo, Japan). Transgenic plants for microscopic observation were grown on MS plates containing 1.5 % agar. Plates were placed vertically in growth chambers for 7–14 days. To observe the dividing cells of root-tips in the 7-day-old transgenic plants, a 10 μM solution of FM4-64 (Invitrogen) was applied dropwise onto the root-tips, which were then incubated for 10 min. Fluorescence due to GFP and FM4-64 was sequentially recorded by confocal microscopy (LSM780; Carl Zeiss).

Phenotypic analysis

Leaves from 14-day-old plants grown on the MS plates were cleared, basically, as described by Ishikawa et al. (2003). Leaves were fixed overnight at room temperature in a mixture of ethanol and acetic acid (9:1, v/v). After rehydration in a graded ethanol series (90, 70, 50, and 30 % for 20 min each), they were cleared in a solution of chloral hydrate (trichloroacetaldehyde monohydrate, 80 g; glycerol, 10 ml; distilled water, 20 ml). Cleared leaves were observed with by Nomarski differential interference microscopy (AxioPlan2; Carl Zeiss).

Results

GFP-AtNACK1 localizes to the site of cell plate formation, expands to the parental cell walls, and disappears in living tobacco BY-2 cells

As is the case for tobacco NACK1 (Nishihama et al. 2002), in silico analysis by means of the InterPro (<http://www.ebi.ac.uk/interpro/>) and COILS programs (Lupas et al. 1991) predicts with high probability the presence of a putative motor domain (amino acids 1–360) and several coiled-coil structures in the carboxyl-terminal (C-terminal) half, which is called the stalk region in NACK1, of AtNACK1/HIK (Fig. 1a, residues 361–974). In addition to the highly probable coiled-coil structures, a single coiled-coil region is predicted with lower probability in the C-terminal tail (Fig. 1a, residues 764–974).

We made the *XVE::GFP-AtNACK1* construct, in which the coding sequence for green fluorescent protein (GFP) was fused in frame to the amino-terminal (N-terminal) coding sequence of *AtNACK1/HIK* cDNA (Fig. 1b). This fusion gene was driven by the estrogen-inducible promoter *XVE* (Zuo et al. 2000) and introduced into cells of the tobacco cultured-cell line BY-2. We cultured the transformed BY-2

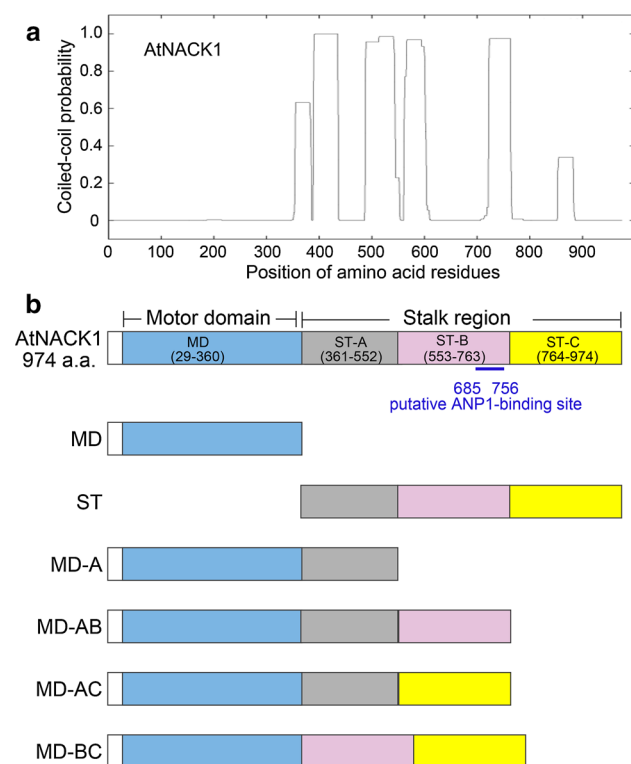


Fig. 1 Domain and motif organizations of AtNACK1. **a** The probability of coiled-coil formation plotted against the amino acid number for AtNACK1 by using the COIL program and a 28-amino acid prediction window (Lupas et al. 1991). **b** Schematic representation of wild-type and deletion mutant constructs of AtNACK1/HIK. Predicted domain organization and relevant amino-acid positions for the AtNACK1 protein sequence are indicated in the wild-type schematic diagram. The coding region of the stalk (ST) is divided into three parts (ST-A, ST-B, and ST-C), which were fused to the region corresponding to the predicted motor domain (MD) and designated as AtNACK1:MD-A, MD-AB, MD-AC, and MD-BC. The coding sequence corresponding to each construct was fused to the C-terminus of the sequence of G3GFP or sGFP

cells in liquid medium containing 0.1 μM 17- β -estradiol for 16 h to induce expression of *GFP-AtNACK1*. We observed subcellular localization of GFP-AtNACK1 in the transformed cells (Fig. 2), where it appeared in the central region corresponding to the expanding cell plate. The GFP signals disappeared as the formation of cell plates ended, suggesting that AtNACK1 disappears upon the maturation of cell plates. These results are consistent with the pattern of a sharp accumulation of tobacco NACK1 protein seen at the M phase of the cell cycle (Nishihama et al. 2002).

The amino-terminal motor and the carboxyl-terminal region of the stalk of AtNACK1/HIK are required for its localization to the site of cell plate formation

To examine which sequences of AtNACK1/HIK are involved in the localization to the site of cell plate

formation, we made a series of deletion constructs of *AtNACK1* cDNA (Fig. 1b), which contained the DNA sequence corresponding to the full length cDNA (full), the motor domain (MD), the stalk (ST) sequence, the MD-A sequence covering the MD and A regions in ST, the MD-AB sequence covering MD, the A and B regions in ST, the MD-AC sequence covering MD, the A and C regions in ST, and the MD-BC sequence covering the MD and BC regions in ST. Note that the B region contained the predicted binding sites of ANP1 MAPKKKs and the putative CDK phosphorylation sites (Ishikawa et al. 2002; Takahashi et al. 2010). These DNA constructs were fused to the C-terminus sequence of the *GFP* gene, whose expression was under the control of the constitutive active Cauliflower mosaic virus (CaMV) 35S promoter, and the chimeric DNA constructs were introduced into cells of the cultured tobacco cell line BY-2.

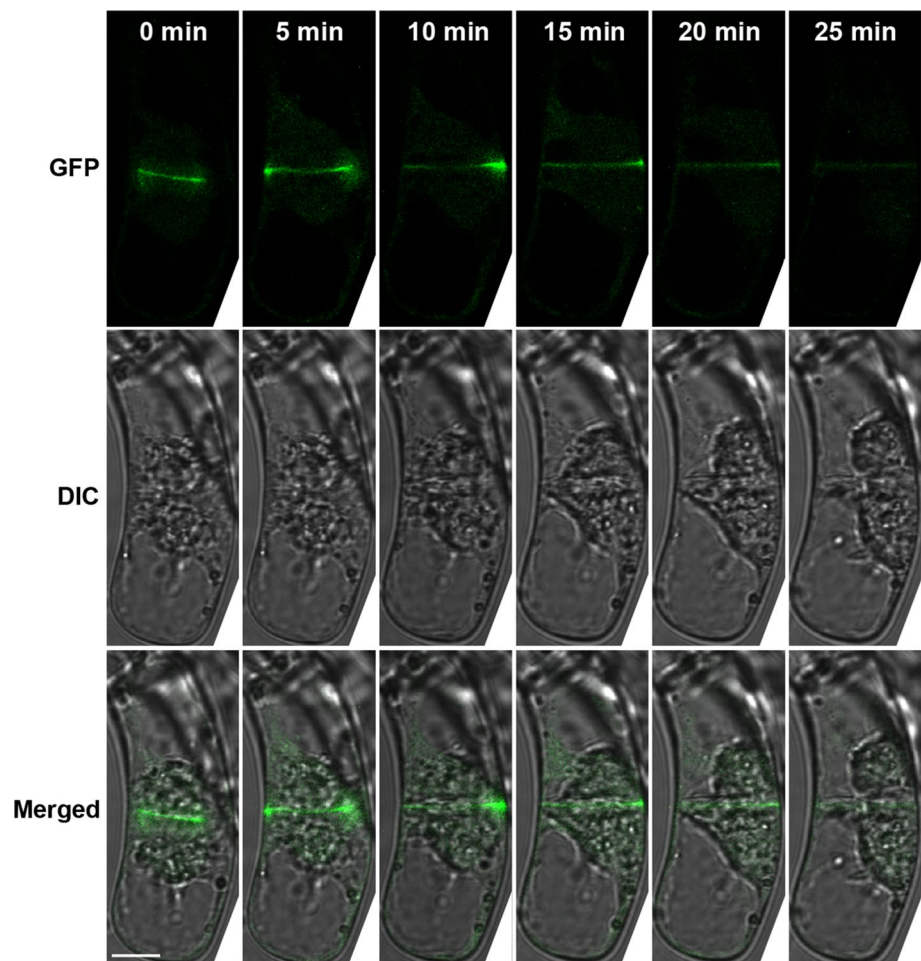
We observed GFP signals during cytokinesis in transformed BY-2 cells (Fig. 3). In cells expressing *GFP-AtNACK1:MD*, no GFP signals were observed in either the phragmoplast region or the site of cell plate formation (Fig. 3b). Although we also transformed BY-2 cells with the *GFP-AtNACK1:ST* and obtained some calli, these calli were unable to grow further (data not shown), which might be due to the mislocalization of the motor-less NACK1:ST, because this observation is consistent with the result that the overexpression of the stalk region of tobacco NACK1 prevents NPK1 MAPKKK from locating to the phragmoplast equator and has a dominant-negative effect (Nishihama et al. 2002). Taken together, the motor domain seems to play a critical role in the AtNACK1 localization to the site of cell plate formation, but only the motor domain is not sufficient for its proper localization.

GFP signals were not detected at the site of cell plate formation in the transformed cells with *GFP-AtNACK1:MD-A*, *GFP-AtNACK1:MD-AB*, and *GFP-AtNACK1:MD-AC* (Fig. 3c–e). Obvious GFP signals were observed, however, at the cell plate formation site in the transformed BY-2 cells expressing GFP-AtNACK1:MD-BC (Fig. 3f). These results indicate that the BC region in ST, in addition to the motor domain of AtNACK1/HIK, is sufficient for the localization of AtNACK1 to the site of cell plate formation, but that only the AB region and the C region when separated from the B region are not sufficient for the appropriate localization. These results also indicate that the A region is not required for its localization to the site of cell plate formation.

AtNACK1-GFP fusion does not localize to the site of cell plate formation in the living cells of Arabidopsis roots and causes dominant-negative effects on cytokinesis in Arabidopsis plants

We fused the C-terminal coding sequence of the entire *AtNACK1/HIK* genomic sequence, which covered the

Fig. 2 Subcellular localization patterns of GFP-AtNACK1 during the formation of cell plates in BY-2 cells. BY-2 cells transformed with estradiol-inducible *GFP-AtNACK1* were incubated for 16 h in the presence of 0.1 μ M 17- β -estradiol. Living cells expressing GFP-AtNACK1 were observed by confocal fluorescence microscopy to detect fluorescence of sGFP (*upper*) and recorded as time-lapse micrographs at the indicated times during expansion of the cell plate. Normarski (*DIC*) and merged images (*merged*) are shown in the *center* and *lower* panels, respectively. Bar 10 μ m

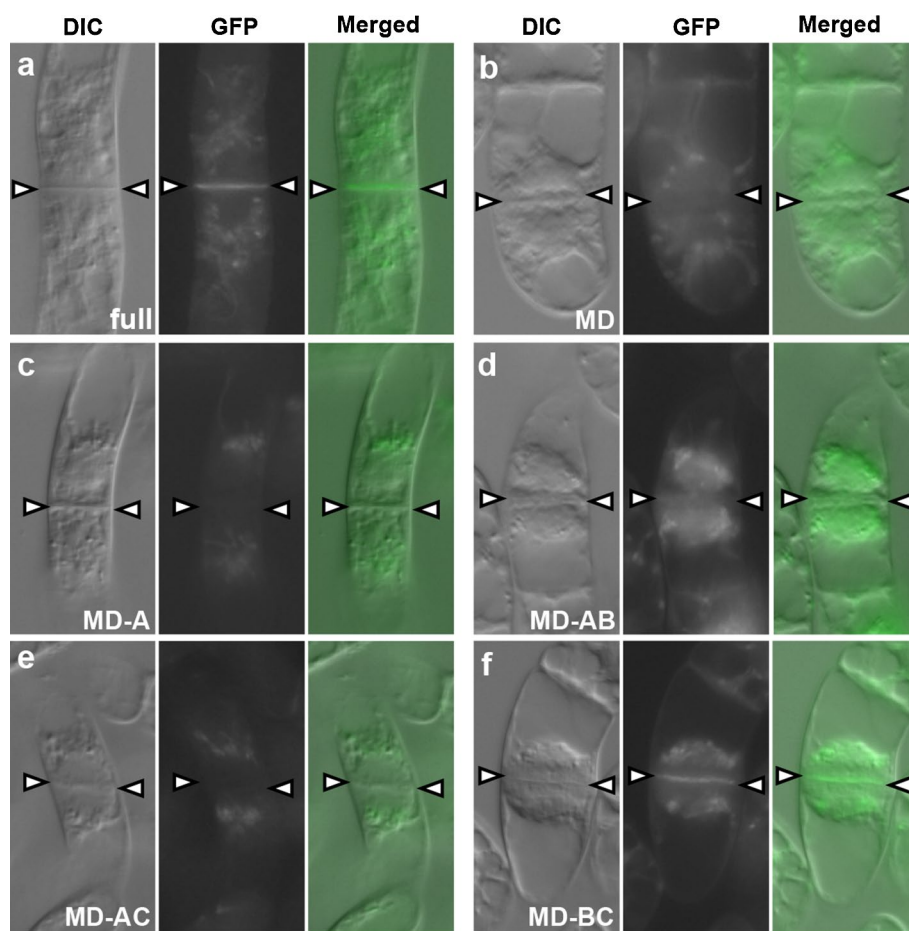


5'-upstream promoter and coding regions, to the N-terminus of the *GFP* sequence. The genomic sequence we used has the ability to complement the phenotypes of *atnack1* mutants (*pAtNACK1::genome AtANCK1*; Sasabe et al. 2011a). We used this construct to transform the wild-type Arabidopsis plants (Col-0). We observed the GFP signals by means of confocal laser scanning microscopy. By analyzing time-lapse images of living cells in the transgenic plants (Fig. 4a), we observed that the signals derived from *pAtNACK1::genome AtNACK1-GFP* did not localize at the site of cell plate formation, as visualized by staining with FM4-64, but were detected within wide areas corresponding to the whole phragmoplast in transformed root cells. The cell plate stained by FM4-64 in the transgenic Arabidopsis expressing AtNACK1-GFP appeared to expand more slowly than that in non-transgenic Arabidopsis (Fendrych et al. 2010; Kosetsu et al. 2010). These results indicate that the C-terminal GFP fusion of AtNACK1 appears to interfere with its localization to the site of cell

plate formation, suggesting that the C-terminal region of AtNACK1/HIK plays an important role in its localization to the site of cell plate formation as well as in the cell plate formation itself.

We observed the phenotypes of Arabidopsis plants transformed with *pAtNACK1::genome AtNACK1-GFP* (Fig. 4b–d). In T2 plants, 6 out of 7 transgenic lines displayed a dwarf phenotype (Fig. 4b; 6–72 %), although the extent of the phenotype depended on each transgenic line. Epidermal cells in the #1 transgenic line, which displayed the highest proportion (72 %) of the most severe dwarf phenotype, exhibited cytokinesis defects with incomplete cell walls (Fig. 4c). Although the #8 transgenic plant line did not show dwarfism in T2 plants, the T3 plants homozygously expressing AtNACK1-GFP showed 8 % of dwarf phenotypes (Fig. 4d, nested image; 4 of 50 seedlings). The dwarf phenotypes might be due to a growth defect of the transgenic plants owing to the mislocalization of AtNACK1-GFP as described above.

Fig. 3 Subcellular localization of GFP-AtNACK1 and its derivatives during formation of cell plates in BY-2 cells. Expression of indicated constructs of AtNACK1 was controlled under the 35S promoter. Transformed BY-2 cells expressing GFP-AtNACK1 (a), GFP-AtNACK1:MD (b), GFP-AtNACK1:MD-A (c), GFP-AtNACK1:MD-AB (d), GFP-AtNACK1:MD-AC (e), and GFP-AtNACK1:MD-BC (f) were observed by fluorescence microscopy to detect fluorescence of G3GFP (*center*) during cell plate formation. Nomarski images (*DIC*) and merged images (*merged*) are shown in the *left* and *right* panels, respectively. Arrowheads indicate the site of expanding cell plates. Bar 20 μm



AtNACK1-GFP localizes to the site of cell plate formation and the wide area outside of the site in BY-2 cells

We also examined subcellular localization of AtNACK1-GFP in BY-2 cells and compared the pattern of its localization with that of the pattern of localization of AtNACK1-GFP in Arabidopsis. We made the *XVE::AtNACK1-GFP* construct, in which the *GFP* sequence was fused to the C-terminal coding sequence of *AtNACK1* cDNA, and introduced into BY-2 cells. We cultured the transformed BY-2 cells in liquid medium containing 0.1 μM estradiol for 16 h to induce expression of *AtNACK1-GFP*. As compared with the GFP signal derived from *XVE::GFP-AtNACK1* (Fig. 4e), those derived from *XVE::AtNACK1-GFP* were detected in the wide regions around the site of cell plate formation (Fig. 4f, g), which might have corresponded to the phragmoplast area. These results indicate that the C-terminal GFP fusion of AtNACK1 appears to disturb its localization to the site of cell plate formation in BY-2 cells as well as in Arabidopsis plants. We have also made the *AtNACK1-GFP* construct with a constitutive active CaMV 35S promoter and have attempted to analyze the

subcellular localization of AtNACK1-GFP in tobacco BY-2 cells. Although we obtained transformed BY-2 cells, we failed to detect GFP signals.

Discussion

AtNACK1/HIK is localized to the cell plate formation site in the phragmoplast of tobacco BY-2 cells and degraded after cell plate formation

The results of the present study with the *GFP-AtNACK1/HIK* construct have shown that the GFP-AtNACK1 protein is localized to the site of cell plate formation during cytokinesis in tobacco BY-2 cells (Fig. 2). The GFP signals disappeared when the signals reached the parental cell walls (Fig. 2), suggesting that the fusion protein degrades after cell plate formation, which is consistent with the previous results obtained by western blot analysis: tobacco NACK1 protein is accumulated specifically during M phase and disappears at the end of the M phase in synchronized BY-2 cells (Nishihama et al. 2002). The M-phase-specific accumulation partially depends on

the specific transcription at the M phase by the Myb transcription factors (Ito et al. 2001; Araki et al. 2004). The specific accumulation should also be due to the disappearance of NACK1 protein around the end of M phase, which is inhibited by the persistent CDK activity (Sasabe et al. 2011a) and the treatment with MG-132, a proteasome inhibitor (our unpublished data), suggesting that NACK1 is degraded by the action of the ubiquitin–proteasome pathway. AtNACK1 of Arabidopsis might be degraded by a similar mechanism at the end of cytokinesis in tobacco BY-2 cells (Fig. 2). The mechanism involved in this phase-specific degradation of NACK proteins should be further investigated experimentally.

We also transformed Arabidopsis plants with the same *GFP-AtNACK1* construct, and obtained sufficient numbers of transgenic plant lines. We, however, failed to find plants that were GFP-positive after induction with estrogen.

The BC region in the stalk of AtNACK1/HIK is active in its binding to phragmoplast microtubules and localizing to the site of cell plate formation

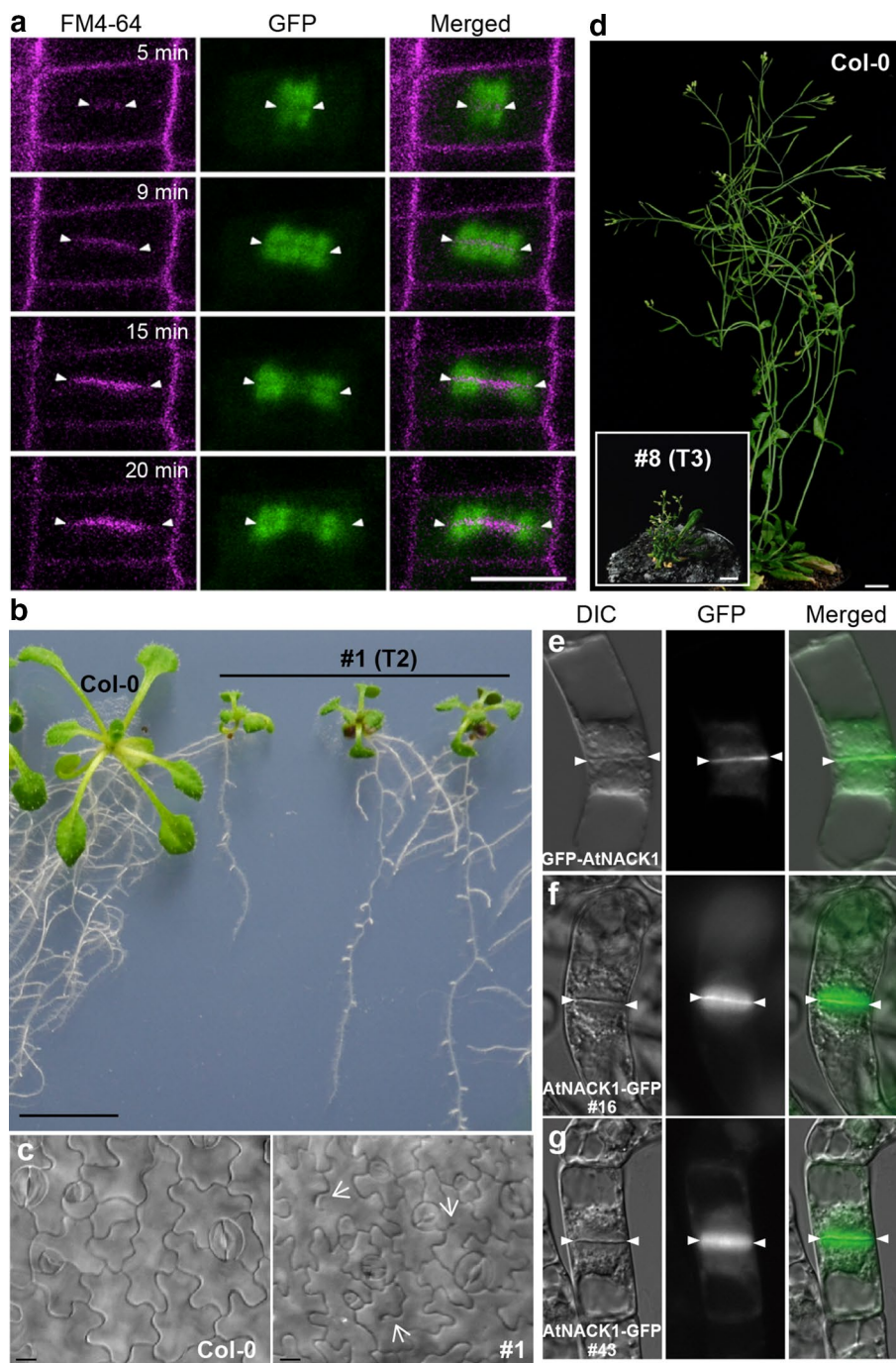
The sequences covering the motor domain and the BC region of the stalk of AtNACK1 (Fig. 1) are sufficient for its localization to the site of cell plate formation in the phragmoplast, but the A region is not required (Fig. 3). Characteristic features of the BC region in AtNACK1 are the presence of three predicted coiled-coil structures (4th, 5th, and 6th positions of coiled-coils in Fig. 1a), one of which overlaps with the site of interaction with ANP1 MAPKKK (corresponding to NPK1 in tobacco), a component of the PQR MAPK pathway that positively controls the formation of cell plates (Ishikawa et al. 2002; Kosetsu et al. 2010; Nishihama et al. 2002; Sasabe et al. 2011a; Takahashi et al. 2010). Considering the general concepts of configurations and dynamics of kinesin motors (Seeger and Rice 2010; Sablin 2000; Verhey and Hammond 2009), these coiled-coil segments together with the motor domain of AtNACK1 possibly play at least two critical roles in their specific localization. First, these coiled-coil structures might be involved in oligomerization of AtNACK1 kinesin, which might be required for their binding to microtubules and motility along the microtubules to move to the site for cell plate formation. The motility property is generated by coordinate stepping of at least two motor domains in the kinesin oligomers.

Second, the coiled-coil structures of the BC region might provide binding sites for cargos to be transported along the microtubules, and the binding of the AtNACK1 kinesin motor to microtubules and its motility activity on the microtubule might be stimulated by the interaction with the PQR MAPK pathway similarly as by interactions between motors and cargos of other known kinesins. Kinesins, in

general, bind to proper cargos at their non-motor regions, such as stalks and C-terminal tail regions, and transport the cargos along microtubule filaments to genetically determined destinations in specific subcellular compartments where they exert their precise functions (Verhey and Hammond 2009; Verhey et al. 2011). In the absence of cargos, kinesins are inactive, in terms of binding to microtubules and motility activities, due to their conformational repressive states, which have been recently defined as autoinhibition mechanisms (Hammond et al. 2010; Kaan et al. 2011; Verhey and Hammond 2009; Verhey et al. 2011). Since the complex of components in the PQR MAPK pathway binds to the B region of the AtNACK1 stalk (Ishikawa et al. 2002; Takahashi et al. 2010), the protein complex might release the autoinhibition of AtNACK1 to bind microtubules and activate its motility function. A well-characterized example is the cargo protein of the mammalian Kinesin-1 motor, which is the JIP scaffold protein that assembles the JNK MAPK pathway consisting of MAPKKK, MAPKK, and JNK MAPK and activates the Kinesin-1 motor (Blasius et al. 2007; Verhey et al. 2001). Activation of the JNK pathway seems to negatively regulate the interaction of kinesin motors with their cargos (Horiuchi et al. 2007), suggesting the presence of crosstalk between the MAPK signaling and trafficking pathways. It must be worth testing whether similar crosstalk between PQR MAPK and the vesicle trafficking systems modulated by AtNACK1 kinesin might be involved in the formation of cell plates.

The C-terminus of AtNACK1/HIK is involved in the processive motility of AtNACK1/HIK on microtubules

It has been reported that Myc-tagged AtNACK1/HINKEL proteins expressed under the original promoter are localized at the midzone of dividing cells in Arabidopsis plants (Krupnova et al. 2013). AtNACK1-GFP proteins that were synthesized in the Arabidopsis plant from the *pAtNACK1::genome AtNACK1-GFP* construct and in BY-2 cells from the *XVE::AtNACK1-GFP* construct were not clearly localized to the site of cell plate formation, however, but rather distributed over wide areas, which appear to correspond to phragmoplast microtubules, around the newly formed midzone (Fig. 4a, f, g). Considering the potential microtubule-binding activity and the motor activity of NACK proteins (Nishihama et al. 2002), the observed diffusive localization of AtNACK1-GFP might be due to inhibitory effects of GFP, which had been joined to the C-terminus of AtNACK1, on its motility activity; however, AtNACK1-GFP might still maintain the binding activity to microtubules because the GFP signals appear to localize on the phragmoplast area (Fig. 4a, f, g). Within the population of these transgenic plants expressing *pAtNACK1::genome AtNACK1-GFP*, we



observed plants showing the dwarf phenotype and cytokinetic defects (Fig. 4b–d), suggesting that the AtNACK1-GFP protein has a dominant-inhibitory effect on wild-type AtNACK1 in the transgenic plants. The *Arabidopsis* mutation of *RUNKEL* exhibits severe cytokinetic defects and results in seedling lethal (Krupnova et al. 2009). Recently, it has been reported that the transgenic plants expressing truncated *RUNKEL* proteins cause the mislocalization of

Myc-AtNACK1/HIK, such that it is dispersed around the cell plate and between the daughter nuclei from early to late stages of cytokinesis in these plants (Krupnova et al. 2013). Again, GFP fusion to the C-terminus of AtNACK1 might affect the interaction between AtNACK1 and *RUNKEL*, or other unknown cargos such as vesicles to be required for the cell plate formation, which might cause the mislocalization of AtNACK1-GFP during cell plate

◀ **Fig. 4** Subcellular localization of AtNACK1-GFP during the formation of cell plates in Arabidopsis plants and BY-2 cells, and effects of its expression on the plant phenotypes. **a** Time-lapse micrographs of AtNACK1-GFP in the living root-tip cell of an Arabidopsis plant that was transformed with genomic *AtNACK1*-fused *GFP*. Genomic *AtNACK1* constructs were controlled under the original promoter. Confocal images were recorded at indicated times after the detection of cell plate formation. Fluorescence images show the signals due to FM4-64 (*left, magenta*) and GFP (*center, green*). The merged images (*merged*) are shown in the *right panels*. *Arrowheads* indicate the site of expanding cell plates. *Bar* 10 μ m. **b** Comparison of gross morphology of one 14-day-old wild type Col-0 plant and three T2 plants transformed with genomic *AtNACK1*-fused *GFP*. T2 plant line 1 (#1) showed the most severe dwarf phenotype. *Bar* 10 mm. **c** Cytokinetic defects in epidermal cells of leaves in transgenic plants. Fourteen-day-old Col-0 plants and T2 plants, line 1, were cleared and the epidermal cells in their leaves were observed. *Arrows* indicate the incomplete cell walls. *Bars* 10 μ m. **d** Comparison of gross morphology of 46-day-old wild type Col-0 plant and T3 plant transformed with genomic *AtNACK1*-fused *GFP*. These plants were grown on soil under the long-day condition. T3 plant line 8 (#8) that homozygously expressed AtNACK1-GFP showed dwarf phenotypes at an efficiency of 8 %. *Bars* 10 mm. **e–g** Comparison of the patterns of subcellular localization of AtNACK1-GFP and GFP-AtNACK1 in BY-2 cells. Expression of *GFP-AtNACK1* (**e**) and *AtNACK1-GFP* (**f, g**) were induced by 0.1 μ M estradiol and fluorescence due to GFP was observed by fluorescence microscopy during cell plate formation. Fluorescence images (*GFP*), Nomarski images (*DIC*) and merged images (*merged*) in the cell exhibiting the representative patterns are shown. Images observed in transformed lines 16 (**f**, #16) and 43 (**g**, #43) are shown. *Arrowheads* indicate the sites of expanding cell plates. *Bar* 20 μ m

formation. Functions of NACK kinesins and their regulatory mechanisms in the formation of cell plates should be further investigated.

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