Phosphorylation of a mitotic kinesin-like protein and a MAPKKK by cyclin-dependent kinases (CDKs) is involved in the transition to cytokinesis in plants

Michiko Sasabe[a], Véronique Boudolf[b,c], Lieven De Veylder[b,c], Dirk Inzé[b,c], Pascal Genschik[d], and Yasunori Machida[a,1]

[a]Division of Biological Science, Graduate School of Science, Nagoya University, Nagoya 464-8602, Japan; [b]Department of Plant Systems Biology, Flanders Institute for Biotechnology, 9052 Ghent, Belgium; [c]Department of Plant Biotechnology and Genetics, Ghent University, B-9052 Ghent, Belgium; and [d]Institut de Biologie Moléculaire des Plantes, Centre National de la Recherche Scientifique, Unité Propre de Recherche 2357, 67084 Strasbourg, France

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Cytokinesis in eukaryotes involves specific arrays of microtubules (MTs), which are known as the "central spindle" in animals, the "anaphase spindle" in yeasts, and the "phragmoplast" in plants. Control of these arrays, which are composed mainly of bundled nonkinetochore MTs, is critically important during cytokinesis. In plants, an MAPK cascade stimulates the turnover of phragmoplast MTs, and a crucial aspect of the activation of this cascade is the interaction between the MAPKKK, nucleus- and phragmoplast-localized protein kinase 1 (NPK1) and the NPK1-activating kinesin-like protein 1 (NACK1), a key regulator of plant cytokinesis. However, little is known about the control of this interaction at the molecular level during progression through the M phase. We demonstrated that cyclin-dependent kinases (CDKs) phosphorylate both NPK1 and NACK1 before metaphase in tobacco cells, thereby inhibiting the interaction between these proteins, suggesting that such phosphorylation prevents the transition to cytokinesis. Failure to inactivate CDKs after metaphase prevents dephosphorylation of these two proteins, causing incomplete mitosis. Experiments with *Arabidopsis* NACK1 (AtNACK1/HINKEL) revealed that phosphorylated NACK1 fails to mediate cytokinesis. Thus, timely and coordinated phosphorylation by CDKs and dephosphorylation of cytokinetic regulators from prophase to anaphase appear to be critical for the appropriate onset and/or progression of cytokinesis.

cell cycle | cell plate | phospho-mimic | MAP65

Cytokinesis mediates the precise distribution of cytoplasm and duplicated nuclear genomes to daughter cells. In eukaryotes, cytokinesis involves specific arrays of microtubules (MTs), and the dynamic control of these arrays, which are composed mainly of bundled nonkinetochore MTs, is critically important during cytokinesis (1–3). Plant cytokinesis is achieved by the formation of cross walls; this formation is promoted by the expansion of the cytokinetic MT-containing machinery, known as the "phragmoplast," from the interior to the periphery of the dividing cell (4, 5). Unlike the stable spindles observed during cytokinesis in animal and yeast cells, the phragmoplast in plants expands dynamically via the turnover of MT; this turnover involves the depolymerization of MTs and the polymerization of tubulins (6–8). Thus, controlling systems of cytokinesis must be diverse among species.

Cyclin-dependent kinases (CDKs) play an essential role in the progression of the cell cycle in eukaryotes, and they drive early mitosis as far as metaphase. Furthermore, delay in the inactivation of CDKs inhibits not only chromosome decondensation and formation of the nuclear envelope but also formation of the cytokinetic MT-containing machinery of yeasts, animals, and plants (9–13). In budding yeast, Ask1 (associated with spindles and kinetochores protein 1) (12), Fin1 (filaments in between nuclei 1), a spindle-stabilizing protein (14, 15), and Ase1 (anaphase spindle elongation 1), a homolog of MT-associated protein 65 (MAP65) which is an MT-bundling protein (14, 16, 17), have been identified as substrates of CDKs; these proteins are involved in the events after anaphase. Iqg1 (IQ motif containing GTPase-activating protein 1), a component of the actomyosin ring, also is a target of CDK for cytokinesis in *Candida albicans* (18). In animals, the phosphorylation of protein regulator of cytokinesis 1 (PRC1) and mitotic kinesin-like protein 1 (MKLP1) by CDK1 reduces the affinity of these proteins for MTs and/or their specific binding partners before metaphase (19–23). It seems plausible that CDKs might be involved in control of the stability and organization of MTs from anaphase to cytokinesis. However, only limited information is available about substrates for CDKs that might be relevant to cytokinesis.

Two key regulators of plant cytokinesis have been identified: a MAPKKK, nucleus- and phragmoplast-localized protein kinase 1 (NPK1); and a kinesin-like protein, NPK1-activating kinesin-like protein 1 (NACK1). Both are concentrated at the midzone of the phragmoplast in plant cells during cytokinesis (24–30). Turnover of MTs in the phragmoplast is stimulated by NACK1 (HINKEL in *Arabidopsis*) and a MAPK cascade, which consists of NPK1 MAPKKK, NQK1 MAPKK, and NRK1/NTF6 MAPK in tobacco and the respective orthologs in *Arabidopsis*, via the phosphorylation of MAP65 by tobacco NRK1 and *Arabidopsis* MPK4 MAPK (31–35). The kinase cascade is inactive before metaphase, even though NACK1 and all components of the MAPK cascade are present throughout M phase (31), and is activated via the proper interaction of NACK1 with NPK1 at late mitosis (25, 26). However, a control mechanism of the interaction between NACK1 and NPK1 during M phase is unknown.

In the present report, we show that CDKs phosphorylate NACK1 and NPK1 at early M phase and that phosphorylation inhibits the interaction between these proteins. Our results further suggest that this phosphorylation prevents the transition to cytokinesis, presumably via the inhibition of the interaction between NACK1 and NPK1. Thus, CDKs repress the activation of the NACK1-controlled MAPK cascade that is a major regulatory system in plant cytokinesis.

Results

CDKs Phosphorylate the Cytokinetic Kinesin NACK1 and MAPKKK NPK1 in Vitro. We purified recombinant proteins that contained the carboxyl-terminal regulatory region of NPK1 (NPK1RD; amino acids 344–690), the putative motor domain of NACK1 (NACK1MD; amino acids 1–359), and the putative stalk region of NACK1 (NACK1ST; amino acids 360–959) (Fig. 1*A*) and used

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[1]To whom correspondence should be addressed. E-mail: yas@bio.nagoya-u.ac.jp.

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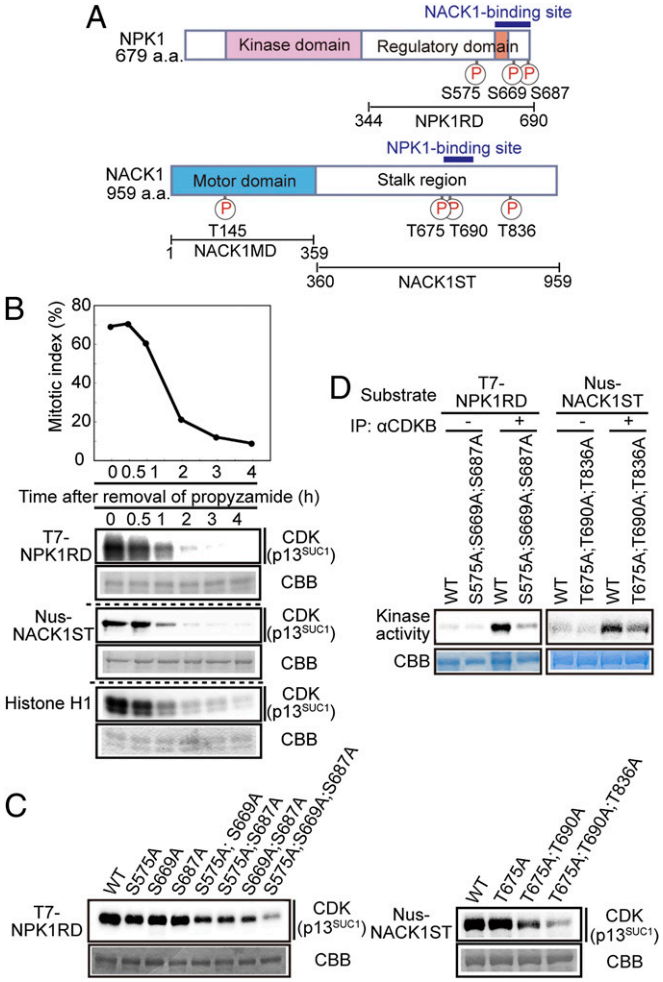

Fig. 1. Phosphorylation of NPK1 and NACK1 by CDKs in vitro. (*A*) Schematic representations of NPK1 and NACK1. Conserved sites of phosphorylation by CDKs are indicated as "P." (*B*) Phosphorylation of NPK1 and NACK1 in vitro by CDKs from BY-2 cells during M phase. CDKs were prepared from synchronized BY-2 cells as described in *Materials and Methods*. Recombinant proteins listed on the left were used as substrates of CDKs. Phosphorylated proteins were detected by autoradiography (CDK). The same gels were stained with Coomassie brilliant blue (CBB). (*C*) Identification of sites of phosphorylation in NPK1 and NACK1 by CDKs. Kinase assays in vitro were performed with indicated mutant proteins of T7-NPK1RD and Nus-NACKST and CDKs prepared as described in *B*. Weak signals in triple mutants might be caused by phosphorylation at minimum phosphorylation consensus sites (S/T-P) by CDKs. (*D*) Phosphorylation of NPK1 and NACK1 by CDKB purified by immunoprecipitation (IP) from prometaphase BY-2 cells with CDKB-specific antibodies (αCDKB).

them as substrates in kinase assays of CDKs in vitro. Proteins were generated as His- and T7-tagged NPK1RD (T7-NPK1RD), GST-tagged NACK1MD (GST-NACK1MD), and His- and NusA-tagged NACK1ST (Nus-NACK1ST). We synchronized tobacco BY-2 cultured cells at prometaphase by treating them with aphidicolin and propyzamide and purified CDK complexes from the synchronized cells with p13SUC1 beads at the indicated times during M phase (Fig. 1*B*). Within 1 h CDK complexes from cells efficiently phosphorylated T7-NPK1RD, GST-NACK1MD, and Nus-NACK1ST, as well as histone H1, a known substrate for CDKs (Fig. 1*B* and Fig. S1*A*). Phosphorylation was inhibited dose-dependently by the CDK-specific inhibitor roscovitine (Fig. S1*B*).

NPK1 and NACK1 possess three and four potential sites of phosphorylation by CDKs, respectively (Fig. 1*A*: serine residues at positions 575, 669, and 687 in NPK1 and threonine residues at positions 145, 675, 690, and 836 in NACK1; these sites are designated S575, S669, S687, T145, T687, T690, and T836, respectively). We replaced these resitues by alanine residues in various combinations, generating mutant derivatives of T7-NPK1RD, GST-NACK1MD, and Nus-NACK1ST. All the above-mentioned residues were phosphorylated in vitro by CDK complexes (Fig. 1*C*). The extent of phosphorylation of mutant NPK1RD with a single mutation (S575A, S669A, or S687A) was slightly lower than that of WT NPK1RD, and phosphorylation of NPK1RD with two mutations (S575A/S669A, S575A/S687A, or S669A/S687A) demonstrated additive decreases (Fig. 1*C*). The triple-mutant derivative of NPK1RD (S575A/S669A/S687A) was barely phosphorylated by CDKs (Fig. 1*C*). NACK1ST with three mutations (T675A/T690A/T836A) also was barely phosphorylated by CDKs (Fig. 1*C*), and NACK1MD mutated at T145 was not phosphorylated by CDKs (Fig. S1*A*).

In plants, two types of CDK are responsible for cell-cycle control: (*i*) CDKA, an ortholog of mammalian CDK1, and fission yeast Cdc2, which has a conserved PSTAIRE cyclin-binding motif; and (*ii*) plant-specific CDKBs, which cannot be replaced by Cdc2 of yeast (36). Although the functions of CDKB remain to be demonstrated, the relatively strong activity of CDKB kinases during mitosis (37) suggests a role for CDKBs during M phase. Although $p13^{SUC1}$ beads can bind to both CDKAs and CDKBs, CDKAs have a higher affinity for $p13^{SUC1}$ beads than CDKBs (38). Therefore, we performed immunocomplex kinase assays in vitro using CDKB-specific antibodies (αCDKB) (37). The CDKB complex efficiently phosphorylated both WT NPK1RD and NACK1ST but phosphorylated mutant proteins with substitution of three alanine residues at much lower efficiency (Fig. 1*D*).

NPK1 and NACK1 Are Phosphorylated During Early Mitosis. We raised antibodies against NPK1 (αNPK1) and against NPK1 that had been phosphorylated at S687 (αNPKpS687). We also raised antibodies against NACK1 that had been phosphorylated at T675 and T690 (αNACKpT675 and αNACKpT690, respectively). These residues are located at sites of binding of NACK1 to NPK1 (Fig. 1*A*) (39). Anti–phospho-NPK1 (αNPKpS687) and anti–phospho-NACK1 (αNACKpT675 and αNACKpT690) specifically recognized CDK-phosphorylated T7-NPK1RD and Nus-NACK1ST, respectively, but not nonphosphorylated proteins (Fig. S2). By contrast, αNPK1 recognized both CDK-phosphorylated and nonphosphorylated T7-NPK1RD, and αNACK1 recognized both CDK-phosphorylated and nonphosphorylated Nus-NACK1ST (Fig. S2).

We extracted proteins from synchronized-BY-2 cells and subjected them to Western blotting analysis with αNPK1 and αNACK1 (Fig. 2 and Fig. S3*A*). In aphidicolin-synchronized cells, NPK1 phosphorylated at S687 (phospho-NPK1) consistently accumulated from S phase immediately after the release of

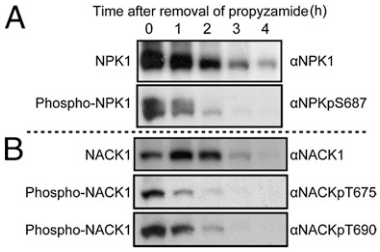

Fig. 2. Phosphorylation of NPK1 and NACK1 at the CDK-phosphorylation sites during M phase in vivo. Proteins were extracted from synchronized BY-2 cells. Immunoblotting analyses were performed with antibodies against NPK1 (αNPK1) and against phospho-NPK1 (αNPKpS687) (*A*) and with antibodies against NACK1 (αNACK1) and against phospho-NACK1 (αNACKpT675 and αNACKpT690) (*B*).

aphidicolin, and the signals of phospho-NPK1 were shifted up 8 h after the removal of aphidicolin, suggesting that NPK1 is fully phosphorylated by CDKs at M phase (Fig. S3*B*). The accumulation pattern of the shifted phospho-NPK1 protein was similar to that of phospho-NACK1 (Fig. S3*B*). In propyzamide-synchronized cells, we detected the highest level of shifted phospho-NPK1 immediately after the removal of propyzamide (0 h), but the level of phospho-NPK1 fell rapidly within 2 h of removal of propyzamide, when maximal activity of NPK1 is observed (Fig. 2*A*, *Lower*) (25), even though the total level of NPK1 gradually decreased during progression through the M phase. The greatest accumulation of NACK1 was observed 1–2 h after the removal of propyzamide. By contrast, the highest level of NACK1 that had been phosphorylated at T675 and T690 (phospho-NACK1) was observed at 0 h, resembling the pattern of accumulation of phospho-NPK1 (Fig. 2*B*). The patterns of phosphorylation of S687 in NPK1 and of T675 and T690 in NACK1 corresponded closely to the pattern of activity of CDKs during earlier M phase (Fig. 1*B*). Taken together with the results of the phosphorylation of NPK1 and NACK1 by CDKs in vitro, these results suggest that CDKs phosphorylate NPK1 and NACK1 in vivo at predicted sites of phosphorylation before metaphase, when CDK activities are high and NPK1 activity is low (25). Phosphorylated NPK1 and NACK1 appeared to undergo dephosphorylation after metaphase.

Expression of Nondegradable Cyclin B Maintained the Phosphorylated Status of NPK1 and NACK1 During Mitosis. Using nondegradable *Nicotiana* cyclin B (NtCycB), we examined whether phosphorylation of NPK1 and NACK1 actually was dependent on the activity of CDKs. We introduced constructs that encoded NtCycB1;1-GFP (CycB-GFP) and nondegradable NtCycB1;1-GFP (CycBΔD-GFP) (40), both under the control of a dexamethasone (Dex)-inducible promoter, into BY-2 cells. In synchronized BY-2 cells, in the absence of Dex (−Dex), normal progression of M phase was observed in cell lines that harbored the CycB-GFP and CycBΔD-GFP constructs (Fig. 3*A*, blue lines). There were no obvious differences between CycB-GFP cells treated with and without Dex in terms of M-phase progression, patterns of accumulation of NPK1, phospho-NPK1, NACK1, and phospho-NACK1, or patterns of CDK activity (Fig. 3 *A*, *Left*, and *B* and Fig. S3*C*). By contrast, metaphase arrest occurred in CycBΔD-GFP cells exposed to Dex (Fig. 3*A*, *Right*, red line), as reported previously (11). CycBΔD-GFP remained undegraded more than 4 h after the release from propyzamide (Fig. 3*C*, lane 1 and Fig. S3*C*), and kinase activities of CDKs were maintained in the presence of Dex (Fig. 3*C*, lane 7). Moreover, NPK1, phospho-NPK1, NACK1, and phospho-NACK1 accumulated for more than 4 h under these conditions (Fig. 3*C*, lanes 2–6). Parallel relationships between the phosphorylation status of NPK1 and NACK1 and the kinase activity of CDKs suggest that NPK1 and NACK1 are phosphorylated by CDKs in vivo.

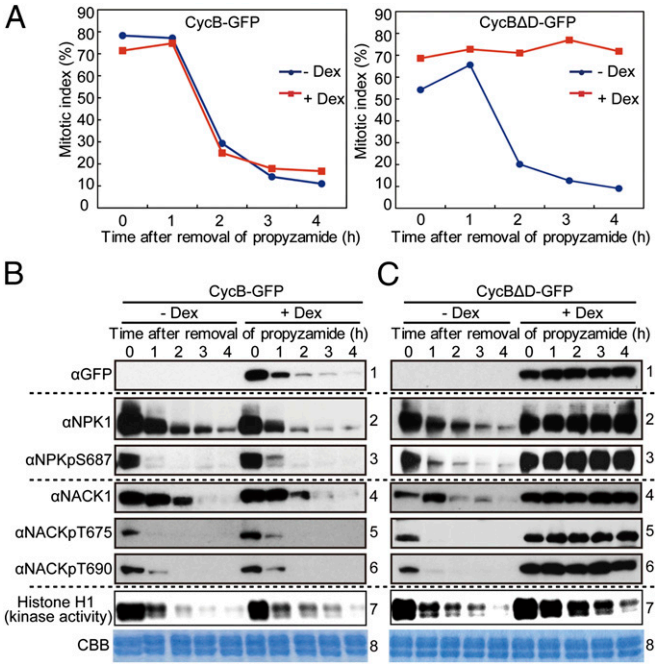

Fig. 3. CDK-dependent phosphorylation of NPK1 and NACK1 in vivo. (*A*) Mitotic indices in BY-2 cells which were synchronized at prometaphase and cultured in the absence (−Dex, blue) or presence (+Dex, red) of 1 μM Dex to induce the expression of CycB:WT-GFP (*Left*) and nondegradable CycB:ΔD-GFP (*Right*). (*B* and *C*) Accumulation of NPK1, phospho-NPK1, NACK1, and phospho-NACK1 in the absence or presence of Dex during mitosis. Lanes 1 to 6 show immunoblotting analyses using αGFP and antibodies as described in Fig. 2; lane 7 shows CDK activity; lane 8 shows CBB staining.

Phosphorylation by CDKs Interferes with Direct Binding of NACK to NPK1. Because the sites of phosphorylation by CDKs are located at the sites at which NPK1 and NACK1 bind to one another, we examined the effect of phosphorylation by CDKs on the interaction between the two proteins in vitro. We generated recombinant T7-NPK1RD and Nus-NACK1ST and performed pull-down assays in vitro. When T7-NPK1RD was immunoprecipitated with αT7 from protein extracts, Nus-NACKST was detected in the immunoprecipitate (Fig. 4*A*, −CDK and Fig. S3*D*), demonstrating binding of these proteins, in harmony with results in previous reports (26, 39). However, when both T7-NPK1RD and Nus-NACK1ST were phosphorylated by CDKs, Nus-NACK1ST was not detected in the immunoprecipitate (Fig. 4*A*, +CDK and Fig. S3*D*). When either NPK1RD or NACK1ST was phosphorylated, the efficiency of coprecipitation was markedly reduced (Fig. S4*A*). Thus, phosphorylation by CDKs of both NPK1 and NACK1 inhibited their mutual interaction.

We also prepared nonphosphorylatable mutant and phosphomimic mutant derivatives of T7-NPK1RD and Nus-NACK1ST in which all three consensus residues at CDK-phosphorylation sites (Fig. 1*A*) were replaced by alanine or aspartic acid residues (NPK1RD^{3A}, NPK1RD^{3D}, NACK1ST^{3A}, and NACK1ST^{3D}, respectively) and used them in pull-down assays in vitro. As shown in Fig. 4*B* and Fig. S3*E*, the efficiency of coprecipitation of NACK1ST^{3D}, which carried three substitutions with aspartic acid residues, with NPK1RD (lane 9) and the efficiency of coprecipitation of NACK1ST with NPK1RD^{3D} (lane 4) was markedly lower than that of NACK1ST with NPK1RD (lane 2). The efficiency of coprecipitation of NACK1ST^{3D} with NPK1RD^{3D} (lane 10) was even lower, indicating that phospho-mimic NACK1ST^{3D} was unable to bind to phospho-mimic NPK1RD^{3D}. NACK1ST^{3A}, however, was coprecipitated efficiently with both NPK1RD (lane 6) and NPK1RD^{3A} (lane 7). NACK1ST also was coprecipitated efficiently with NPK1RD^{3A} (lane 3). In summary, the substitutions with aspartic acid residue reduced the efficiency of the interaction between NPK1RD and NACK1ST.

We examined whether the phosphorylation of NPK1 and NACK1 by CDKs might affect the physical interaction between these proteins in vivo. We established lines of BY-2 cells that expressed GFP-NPK1 and GFP-NACK1 (Fig. S5) separately and used them in pull-down assays in vivo. Cells were synchronized with aphidicolin and propyzamide, and proteins were extracted at indicated times after the removal of propyzamide from cells that expressed GFP-NPK1 (Fig. 4*C* and Fig. S3*F*). When we immunoprecipitated extracted proteins with αGFP, we detected endogenous NACK1 in the immunoprecipitate (Fig. 4*C*, *Right*). However, we did not detect phospho-NACK1 in the immunoprecipitate (Fig. 4*C*, *Right*), even though phospho-NACK1 ac-

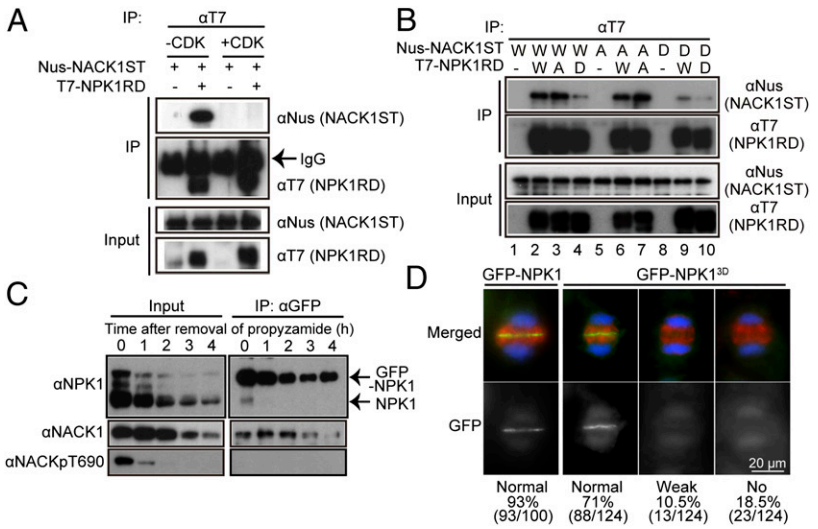

Fig. 4. Inhibition of the physical interaction between NPK1 and NACK1 upon phosphorylation of these proteins by CDKs. (*A*) The effects of phosphorylation by CDKs on the interaction between NPK1 and NACK1 in vitro. T7-NPK1RD and Nus-NACK1ST were used in pull-down assays with αT7 (−CDK). These proteins phosphorylated with CDKs also were used in assays (+CDK). The coprecipitates were detected with αT7 and αNus. (*B*) The effects of mutations at sites of phosphorylation by CDKs in NPK1 and NACK1 on the interaction between NPK1 and NACK1 in vitro. T7-NPK1RD ("W") and Nus-NACK1ST (W) were used in pull-down assays with αT7. Mutant proteins in which each of three CDK-phosphorylation sites in T7-NPK1RD and in Nus-NACK1ST had been replaced with alanine ("A") or aspartic acid ("D") residues also were used in pull-down assays. The coprecipitates (IP) and input samples were detected with αT7 and αNus. (*C*) Physical interaction between NPK1 and NACK1 was prevented by CDKs in vivo. BY-2 cells were synchronized at prometaphase and cultured in the presence of 1 μM Dex to induce the expression of GFP-NPK1. Proteins were extracted from these cells, and immunoprecipitation was performed with αGFP. Input proteins (*Left*) and precipitates (*Right*) were detected with αNPK1, αNACK1, and phospho-NACK–specific (αNACKpT690) antibodies. (*D*) Suppression of the recruitment of phospho-mimic NPK1 to the phragmoplast midzone during cytokinesis. BY-2 cells were transformed with DEX-inducible GFP-NPK1 and GFP-NPK1^{3D} and were incubated for 12 h in the presence of 0.5 μM Dex. Cells were fixed and stained with αtubulin and DAPI. (*Upper*) Merged images (α-tubulin, red; DAPI, blue; GFP, green). (*Lower*) Fluorescence caused by GFP.

cumulated in cells at early M phase (Fig. 4*C*, *Left*). Similarly, in cells that expressed GFP-NACK1, endogenous NPK1 was immunoprecipitated with GFP-NACK1, but the immunoprecipitate did not include phospho-NPK1 (Fig. S4*B*). These results suggest that phospho-NACK1 and phospho-NPK1 do not interact physically each other. Moreover, the efficiency of localization of phospho-mimic GFP-NPK1^{3D} at the midzone of phragmoplast was reduced significantly (71%) compared with the efficiency of localization of GFP-NPK1 (93%) (Fig. 4*D*). However, the efficiency of localization of phospho-mimic GFP-NACK1^{3D} at the phragmoplast midzone (95%) was the same as that of localization of GFP-NACK1 (93%). The less efficient localization of GFP-NPK1^{3D} might be caused by the low ability of this mutant protein to interact with the endogenous WT NACK1 protein.

Phospho-Mimic Mutations in the *AtNACK1* Gene Do Not Rescue the Phenotype of *Arabidopsis atnack1-1* Plants. We addressed the biological significance of the phosphorylation of NACK1 by CDKs using a loss-of-function mutant of the *Arabidopsis* homolog of *NACK1* (*AtNACK1*, also known as "*HINKEL*"). We isolated a 6.1-kb fragment of the *Arabidopsis* genome that encompassed the 5′-upstream region and the entire coding region (G-NACK1), and we introduced point mutations to replace the threonine residues at CDK-phosphorylation sites by alanine residues (G-NACK1^{3A}) and by phospho-mimic aspartic acid residues (G-NACK1^{3D}) (Fig. 5*A*). Homozygous *atnack1-1* mutants exhibit severe dwarfism, seedling lethality (Fig. 5*B*), and defects in cytokinesis (Fig. 5*D*) (26, 28). We transformed the heterozygous *atnack1-1* mutant with fragments that encoded G-NACK1, G-NACK1^{3A}, and G-NACK1^{3D}, separately, and selected homozygous mutants that harbored exogenous DNA. Levels of transcripts of exogenous WT and mutant *AtNACK1* genes were similar to each other and to that of the endogenous *AtNACK1* transcript (Fig. S6*A*). As shown in Fig. 5 *B* and *C* and Fig. S6*B*, the *atnack1-1* mutation was rescued fully by the G-NACK fragment. However, homozygous *atnack1-1* mutants harboring the G-NACK1^{3D} construct were barely distinguishable from *atnack1-1* plants (Fig. 5*B*). Results of quantitative analyses of this experiment are shown in Table S1. These results demonstrate that G-NACK1^{3D} was unable to reverse the seedling lethality of *atnack1*. In addition, it did not rescue cytokinetic defects or the disordered cell lineage in *atnack1* roots (Fig. 5 *C* and *E*). These results suggest that AtNACK1 with the phospho-mimic mutation failed to activate the MAPK cascade for cytokinesis in *Arabidopsis*. Moreover, the *atnack1-1* phenotype was rescued only incompletely by the G-NACK1^{3A} construct: Cytokinetic defects in root cells were rescued, but the dwarfism of whole plants was not rescued (Fig. 5*B*, Fig. S6 *B* and *C*, and Table S1), suggesting that G-NACK1^{3A} might be a weak mediator of cytokinesis and that its function might be insufficient for the normal growth of an entire plant.

Discussion

Here we provide the results of an initial study pointing toward a mechanism for the control of cytokinesis in plants. CDKs inhibit the interaction of two cytokinetic regulators, NACK1 and NPK1, by phosphorylating these proteins during early mitosis, and dephosphorylation of these regulators occurs at late mitosis, in parallel with activation of the NACK1-mediated MAPK cascade [the NACK–PQR pathway (31)]. We propose that CDKs might control the transition from early mitosis to cytokinesis via the repression of activation of the MAPK cascade (Fig. 5*F*).

Three possible models, which are not mutually exclusive, can explain how CDKs control the transition to cytokinesis. In the first model, phosphorylation by CDKs might inhibit premature initiation of cytokinesis at early mitosis. Premature cytokinesis before the separation of sister chromatids would result in improper nuclear division, which would be deleterious to cells. In animals, it has been reported that the localization and activity of a mitotic kinesin (ZEN-4 in *Caenorhabditis elegans* and MKLP1 in humans) that is included in the centralspindlin complex are controlled by CDK, and phosphorylation of ZEN-4/MKLP1 inhibits the assembly of the central spindle and the proper segregation of chromosomes (21). The *atnack1-1* mutant of *Arabidopsis* occasionally includes aneuploid cells (Fig. S6*D*), suggesting that there might be a close relationship between maintenance of a stable karyotype and appropriate execution of cytokinesis.

In a second possible model, CDKs might inhibit aberrant events unrelated to cytokinesis that result from the inappropriate activation of NPK1 before metaphase; such events might disturb the normal progression of M phase. PRC1 (MAP65 in plants) is one of the important MT-bundling proteins involved in assembly of the central spindle and recruitment of multiple mitotic kinesins and polo-like kinase1 (Plk1) to the central spindle (19, 20, 22, 23, 41, 42). Mutation of CDK-phosphorylation sites in PRC1 causes extensive bundling of the prometaphase spindle and

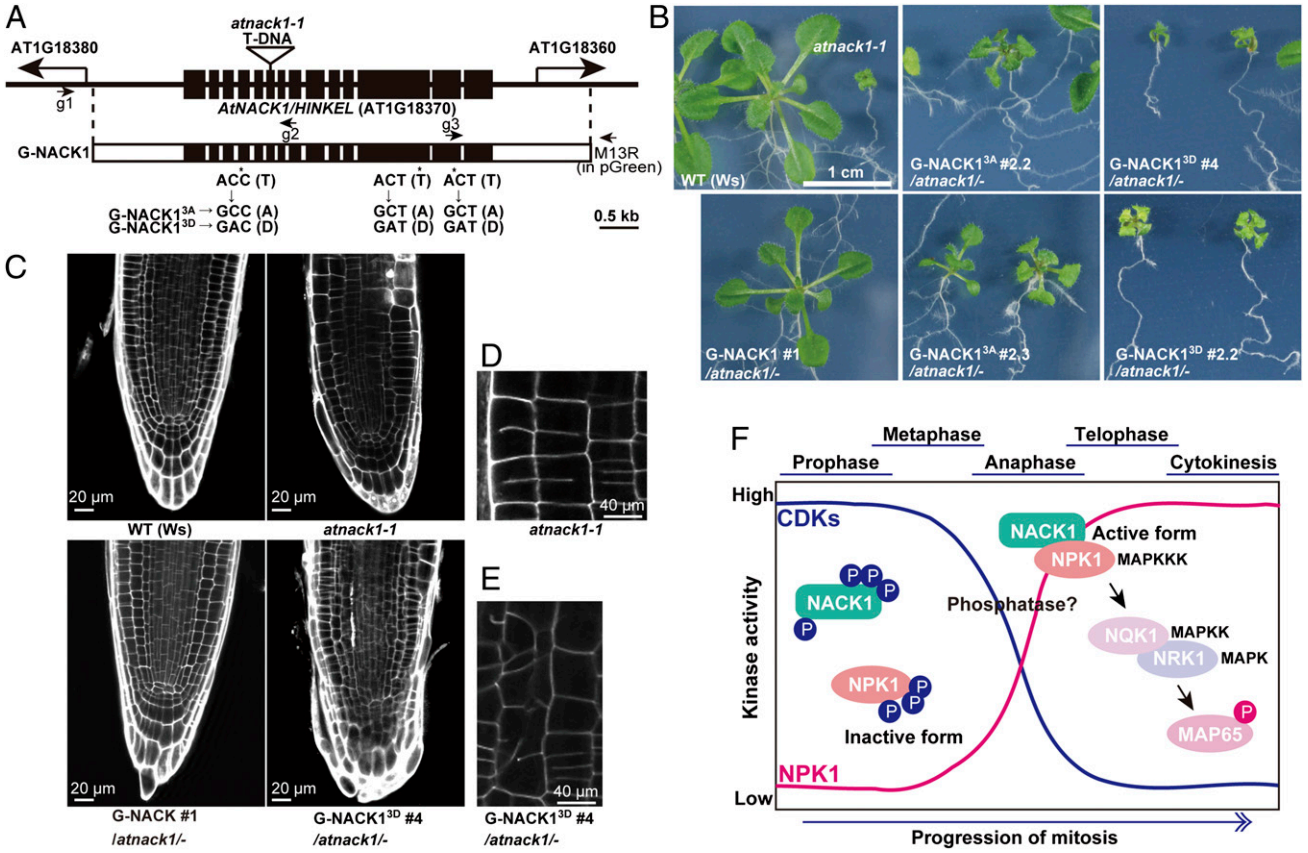

Fig. 5. CDK-phospho mimic AtNACK1/HINKEL was nonfunctional in cytokinesis in *Arabidopsis*. (*A*) Structure of the *AtNACK1* locus and the transformed *AtNACK1* genomic fragments. Black boxes represent exons. Fragments of genomic DNA (G-NACK1: ~6.1 kb) were used for complementation of the *atnack1* mutation. G-NACK1^{3A} and G-NACK1^{3D}, in which alanine or aspartic acid residues had been substituted for respective predicted sites of phosphorylation by CDKs (indicated by asterisks), were constructed. Primers used for genotyping (g1, g2, g3, and M13R) are indicated by arrows. (*B*) Gross morphology of WT (ecotype Wassilewskija; Ws) plants, *atnack1-1* plants, *atnack1-1* plants harboring G-NACK, *atnack1-1* plants harboring G-NACK^{3A} (two independent isolates), and *atnack1-1* plants harboring G-NACK^{3D} (two independent isolates). (*C*) Median longitudinal sections of roots from 5-d-old seedlings of WT plants, *atnack1-1* plants, *atnack1-1* plants harboring G-NACK, and *atnack1-1* plants harboring G-NACK^{3D}. (*D* and *E*) Examples of cytokinetic defects in sections from an *atnack1-1* plant (*D*) and an *atnack1-1* plant harboring G-NACK^{3D} (*E*). (*F*) Schematic representation of the control of cytokinesis by phosphorylation with CDKs. Before metaphase, NACK1 and NPK1 are phosphorylated by CDKs; this phosphorylation prevents their interaction. CDK is inactivated after metaphase, and NPK1 and NACK1 are dephosphorylated by as yet unidentified protein phosphatase(s), with the resultant direct interaction of NACK1 with NPK1. This interaction activates NPK1, with subsequent activation of the MAPK cascade (NACK–PQR pathway). Because the NPK1 protein is present from G1/S to the end of cytokinesis, it is possible that NPK1 might play additional roles during these phases (25).

premature recruitment of Plk1 to the spindle during prometaphase (20, 23). Although plant MAP65s also are phosphorylated in vitro by CDKs, the phosphorylated forms do not affect MT-bundling activity (32). Note that the MT-bundling activity is controlled by phosphorylation by MAPKs during cytokinesis of plant cells (32, 43).

In a third possible model, phosphorylation by CDKs might stabilize both NACK1 and NPK1 for proper progression of cytokinesis. Accumulation of stable forms of these proteins, from prophase onwards, might be necessary for prompt and strong activation of the MAPK cascade within a short period. Such high-level activation of the MAPK cascade might be required for the rapid turnover of the copious phragmoplast MTs, because the expansion of the phragmoplast is completed within a mere 12–15 min in *Arabidopsis* cells (33, 34). In animal and yeast cells, several effector proteins of MT status, such as MAPs and kinesins, have been identified as substrates for CDKs, and it appears that multiple mechanisms prevent inappropriate cytokinesis. In contrast, in plant cytokinesis, CDKs might act predominantly to inhibit the NACK–PQR pathway, which is a key regulator in the control of expansion of the phragmoplast (32, 33).

The genetic analysis that involved mutations at sites of phosphorylation by CDKs demonstrated that the phospho-mimic variant of NACK1 (G-NACK1^{3D}) was inactive in terms of cytokinesis and that nonphosphorylatable NACK1 (G-NACK1^{3A}) also caused growth defects (Fig. 5), suggesting that the transitional stage controlled by CDKs might be critical for appropriate execution of cytokinetic events. Thus, control by CDKs of the transition to late M phase might be an essential feature ensuring the progression of cytokinesis in both plant and animal cells, even though plants have no known functional Plk orthologs or a centralspindlin complex. However, we could not rule out the possibility that other kinases might contribute to phosphorylation of the sites by CDKs.

A protein phosphatase that counteracts the activity of CDKs, such as Cdc14 phosphatase in yeast and animal cells (44), must play a crucial role in the interaction between NACK1 and NPK1. However, plant genomes that have been sequenced to date do not include genes for Cdc14 orthologs. Efforts to isolate and characterize the phosphatases required for plant cytokinesis are underway in our laboratories.

Materials and Methods

Plant Materials and Growth Conditions. 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 (ecotype Wassi-

lewskija; Ws) were grown with a 16-h photoperiod at 22 °C either in soil or on Murashige and Skoog plates. Procedures for synchronization of BY-2 cells and genetic analysis are given in *SI Materials and Methods*.

DNA Constructs and Site-Directed Mutagenesis. To generate recombinant proteins, partial cDNA fragments of *NPK1*and *NACK1* were cloned into the His–T7 fusion vector pET28b (Novagen), the GST fusion vector pGEX4T-3 (GE Healthcare), and the His–Nus fusion vector pET50 (Novagen). Point mutations in each cDNA at putative sites of phosphorylation by CDKs were introduced with a QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene), in various combinations. To express GFP fusions in cells, full-length *NPK1*, *NACK1*, and mutant forms of these cDNAs were subcloned, separately, in the pTAGW6 vector. Detailed information is given in *SI Materials and Methods*. See Table S2 for a list of primers used.

Immunocomplex and p13^{suc1} Bead-Complex Kinase Assays in Vitro. For precipitation of plant CDKs, extracts of BY-2 cells were incubated with p13^{SUC1} agarose beads (Millipore). For precipitation of plant-specific CDKB, we used specific antibodies against CDKB (αCDKB) (37). To prepare phosphorylated proteins for pull-down assays in vitro, His-T7–NPK1RD and Nus-NACK1ST were incubated separately in kinase buffer that contained 500 μM ATP and p13^{SUC1} beads containing CDK complex for 1 h at 4 °C. After p13^{SUC1} beads were removed by centrifugation, phosphorylated recombinant proteins were dialyzed against TBS buffer for 12 h at 4 °C and then were used for pull-down assays in vitro. Detailed information is given in *SI Materials and Methods*.

Immunoprecipitation Assay in Vivo and Pull-Down Assays in Vitro. Proteins were extracted from BY-2 cells that expressed GFP-NPK1 or GFP-NACK1, and extracts were incubated with agarose-conjugated αGFP (MBL International) at 4 °C for 2 h on a rotator. For pull-down assays in vitro, purified His-T7–tagged and His–Nus fusion proteins were incubated at 25 °C for 1 h in various combinations and incubated with αT7-tag–conjugated agarose beads (Novagen) at 4 °C for 1 h on a rotator. The beads then were washed five times and suspended in sample buffer for SDS/PAGE. After boiling, samples were subjected to SDS/PAGE and immunoblotting. Detailed information is given in *SI Materials and Methods*.

Microscopy. Plant material was incubated for 2 min in a working solution of 10 μM propidium iodide in water. Images were recorded with a confocal microscope (LSM510; Carl Zeiss). All techniques for immunofluorescence analysis have been described previously (25). Fluorescence caused by GFP, TRITC, and DAPI was detected with an AXIO Imager (Carl Zeiss).

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