Supporting Information

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SI Materials and Methods

Synchronized Cultures of BY-2 Cells. For synchronization of the cell cycle of BY-2 cells from prometaphase (1, 2), 20 mL of a 7-d-old cell culture were transferred to 80 mL of fresh medium that contained 5 mg/L aphidicolin (Wako Pure Chemicals). After incubation for 24 h, cells were washed and resuspended in 100 mL of fresh medium. Propyzamide (Sumitomo Chemical) was added at a final concentration of 6 μM to the cells that had been cultured for an additional 4 h after the removal of aphidicolin. After incubation with propyzamide for a further 6 h, cells were washed and transferred to 100 mL of fresh medium. At appropriate times after the removal of propyzamide, cells were harvested, frozen in liquid nitrogen, and stored at −80 °C until use. Upon removal of propyzamide, the interrupted cell cycle recommenced, and mitosis in most cells was completed within 2 h. In such cultures, 70–75% of the cells were undergoing mitosis: More than 80% were between prometaphase and metaphase in early M phase at 0–0.5 h after removal of propyzamide, and ∼50–60% were between anaphase and cytokinesis in late M phase at 1–2 h after removal of propyzamide) (3).

DNA Constructs and Site-Directed Mutagenesis. To generate recombinant proteins, a cDNA fragment encoding the regulatory domain of *nucleus- and phragmoplast-localized protein kinase 1* (*NPK1*) was amplified by PCR with specific primers (Table S2, primers 1 and 2) and cloned into the His–T7 fusion vector pET28b (Novagen). Two cDNA fragments of *NPK1-activating kinesin-like protein 1* (*NACK1*) encoding the motor domain and the stalk region of NACK1, were amplified by PCR with specific primers (Table S2, primers 3 and 4 and primers 5 and 6, respectively) and cloned into the GST fusion vector pGEX4T-3 (GE Healthcare) and the His–Nus fusion vector pET50 (Novagen), respectively. Point mutations in each cDNA at putative sites of phosphorylation by cyclin-dependent kinases (CDKs) were introduced with a QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene), in various combinations, by use of primers 7–20 in Table S2. To express GFP fusions in cells, we introduced the GFP-fused Gateway cassette from the pGWB6 vector (4) into the pTA7001 vector (5) to yield pTAGW6. Full-length *NPK1* and *NACK1* cDNA were subcloned, separately, in the pENTR vector (Invitrogen), and point mutations were introduced at sites of CDK phosphorylation with the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) with the above-mentioned mutagenic primers. To express GFP-tagged GFP-NPK1^{3D} and GFP-NACK1^{3D}, three CDK-phosphorylation sites in the regulatory domain of NPK1 (NPK1RD) and three CDK-phosphorylation sites in the stalk region of NACK1 (NACK1ST) were replaced by aspartic acid residues. The various fragments of *NPK1* and *NACK1* cDNAs in pENTR were subcloned into the pTAGW6 vector using Gateway LR Clonase II (Invitrogen).

Immunocomplex and p13^{suc1} Bead-Complex Kinase Assays in Vitro. For precipitation of plant CDKs, we homogenized BY-2 cells that had been synchronized at prometaphase in TG150 buffer [25 mM Tris-HCl (pH 7.5), 10 mM EDTA, 10 mM EGTA, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM DTT, 20 mM β-glycerophosphate, 1 mM sodium o-vanadate, 1 mM sodium fluoride, 1 mM PMSF, and 5 μg/L each of leupeptin, chymostatin, pepstatin A, and antipain]. Extracts of cells containing 0.2 mg of protein were incubated with 10 μL of p13^{SUC1} agarose beads (Millipore) at 4 °C for 1 h on a rotator. For precipitation of plant-specific CDKB, we used specific antibodies against CDKB (αCDKB) (6). Extracts of cells containing 0.3 mg of protein were incubated for 1 h at 4 °C on a rotator with 25% (vol/vol) Protein A-Sepharose (GE Healthcare). After centrifugation, the supernatant was incubated with 250-fold diluted αCDKB for 2 h at 4 °C and subsequently for 1 h at 4 °C with 25% (vol/vol) Protein A-Sepharose beads. p13^{SUC1} and Protein A-Sepharose beads were washed four times with 1 mL of TG150 buffer and twice with 1 mL of kinase buffer [50 mM Hepes-KOH (pH 7.5), 20 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 20 mM β-glycerophosphate, and 1 mM sodium o-vanadate]. The beads then were incubated in 20 μL of kinase buffer that contained 1 μg of substrate protein, 50 μM ATP, and 5 μCi of [γ-^{32}P] ATP for 30 min at 25 °C. Histone H1 was used as a model substrate for CDKs. Reactions were terminated by addition of sample buffer for SDS/PAGE and boiling; then reaction mixtures were subjected to SDS/PAGE. Radioactivity was visualized with an imaging analyzer (BAS-1800; Fuji Film). 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.

Production of Antibodies. Antibodies against NPK1 (αNPK1) and Ser687-phosphorylated NPK1 (αNPKpS687) were raised against two synthetic peptides, CSRFASPGR and CSRFAS(PO_3H_2)PGR (residues 683–690 of NPK1), respectively, as antigens. Antibodies against NACK1 (αNACK1) were raised against a synthetic peptide, CTPDPANEKDWKIQQMEME (residues 385–402 of NACK1), as described previously (7). Antibodies against Thr675-phosphorylated NACK1 (αNACKpT675) and Thr690-phosphorylated NACK1 (αNACKpT690) were raised against two synthetic peptides, CQPDET(PO_3H_2)PTKSD (residues 671–680 of NACK1) and SSKEGT(PO_3H_2)PYRRC (residues 685–694 of NACK1) as antigens. Specific antibodies were affinity-purified from sera of immunized rabbits. Monoclonal T7-specific and Nus-specific antibodies (αT7 and αNus, respectively) were purchased from Novagen, and monoclonal GFP-specific antibodies (αGFP) were purchased from Clontech.

Immunoprecipitation Assay in Vivo. Proteins were extracted from synchronized BY-2 cells that expressed GFP-NPK1 or GFP-NACK1 with PG150 buffer (modified TG150 buffer, prepared with 20 mM Pipes and adjusted to pH 6.2 instead of 25 mM Tris-HCl adjusted to pH 7.5). Extracts containing 0.3 mg of protein were diluted to 0.5 mL with PG150 buffer and incubated with 10 μL of agarose-conjugated αGFP (MBL) at 4 °C for 2 h on a rotator. The agarose beads then were washed five times with 1 mL of PG150 buffer and suspended in 20 μL of sample buffer for SDS/PAGE. After boiling, samples were subjected to SDS/PAGE (10% polyacrylamide), and coprecipitates were analyzed by immunoblotting analysis.

Pull-Down Assays in Vitro. Purified His-T7–tagged and His–Nus fusion proteins were incubated in TBS buffer that contained 0.1% Triton X-100 at 25 °C for 1 h in various combinations. αT7-tag–conjugated agarose beads (Novagen) were added to these samples, which then were incubated on a rotator at 4 °C for 1 h. Agarose beads were washed five times with TBS buffer that contained 0.1% Triton X-100 and then were suspended in 20 μL of sample buffer for SDS/PAGE. After boiling, samples were sub-

jected to SDS/PAGE (10% polyacrylamide), and coprecipitates were analyzed by immunoblotting analysis.

Genetic Analysis. *Arabidopsis* mutant line *atnack1-1* has been described previously (7). For complementation tests, the 6.1-kb region of DNA covering the *Arabidopsis NACK1* (*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 was amplified from *Arabidopsis thaliana* (ecotype Wassilewskija; Ws) genomic DNA with specific primers (Table S2, primers 21 and 22). The fragment generated by PCR was cloned into the BamHI and NotI sites of the pGreen0029 vector and sequenced. Point mutations in *AtNACK1* were introduced at putative sites of phosphorylation by CDKs with a QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) with mutagenic primers (Table S2, primers 23–28), and products were sequenced. The genomic fragments were introduced into heterozygous *atnack1-1* plants. Genotyping was performed with the following primers: for the *atnack1-1* mutation, specific for the T-DNA left border (LB1a), AtNACK1-g1 and AtNACK1-g2; and for the transformed *AtNACK1* genomic fragment, AtNACK1-g3 and M13 reverse primer (Table S2, primers 29–33 in the z column).

RT-PCR. Total RNA and genomic DNA were isolated from 14-d-old seedlings of WT, *nack1-1* mutant, and *atnack1-1* plants with fragments that encoded G-NACK1, G-NACK1^{3A}, and G-NACK1^{3D}, separately, with TRIZOL Reagent (Invitrogen). Genotyping was performed with the above-mentioned primers and isolated genomic DNAs. First-strand cDNA was synthesized with Ready-To-Go You-Prime First-Strand Beads (GE Healthcare). Equal aliquots from each first-strand reaction were used as templates to amplify *AtNACK1* cDNA. RT-PCR was performed with primers specific for the *AtNACK1* gene (5′-GAGGAGTAACGGAGAAAGCTGTCAATG and 5′-TTTAAGCATTGCAGATGGTTTAACAG). As an internal control for RT-PCR, we amplified cDNA using primers for α-tubulin gene (5′-GGACAAGCTGGGATCCAGG and 5′-CGTCTCCACCTTCAGCACC).

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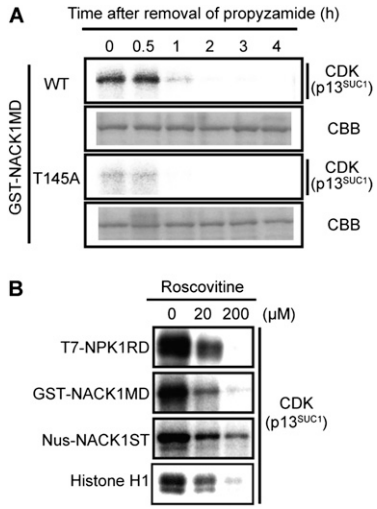

Fig. S1. Phosphorylation of NPK1 and NACK1 by CDKs in vitro. (*A*) Phosphorylation in vitro by CDKs from BY-2 cells during M phase. CDKs were purified with p13^{SUC1} beads from proteins that had been extracted from BY-2 cells, which were harvested at the indicated time after removal of propyzamide. Recombinant proteins of the GST-tagged putative motor domain of NAC1 (GST-NACK1MD) and the mutant form, in which a threonine residue at position 154 in NACK1MD had been replaced by alanine (T154A), were used as substrates in kinase assays with CDKs. Phosphorylated GST-NACK1MD was detected by autoradiography (CDK). The same gel was stained with Coomassie brilliant blue (CBB). (*B*) The effects of roscovitine on phosphorylation of NPK1 and NACK1. CDKs were purified with p13^{SUC1} beads from the proteins that were extracted from BY-2 cells at prometaphase. Kinase assays were performed with and without roscovitine, a CDK-specific inhibitor, at the indicated concentrations.

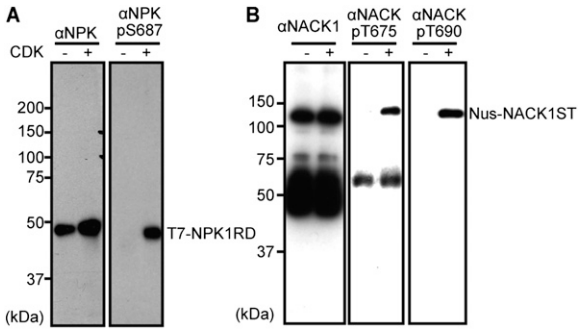

Fig. S2. Specificity of antibodies against NPK1, phospho-NPK1, NACK1, and phospho-NACK1. (*A*) Specificity of αNPK1 and αNPK1pS687. Ten nanograms each of His-T7–NPK1RD (T7-NPK1RD) and CDK-phosphorylated T7-NPK1RD were subjected to immunoblotting analysis with affinity-purified antibodies against NPK1 (αNPK1) and phospho-NPK1 (αNPKpS687). (*B*) Specificity of antibodies against NACK1 and phospho-NACK1. Ten nanograms each of His-Nus–NACK1ST (Nus-NACK1ST) and CDK-phosphorylated Nus-NACK1ST were subjected to immunoblotting analysis with affinity-purified antibodies against NACK1 (αNACK1) and two forms of phospho-NACK1 (αNACKpT675 and αNACKpT690).

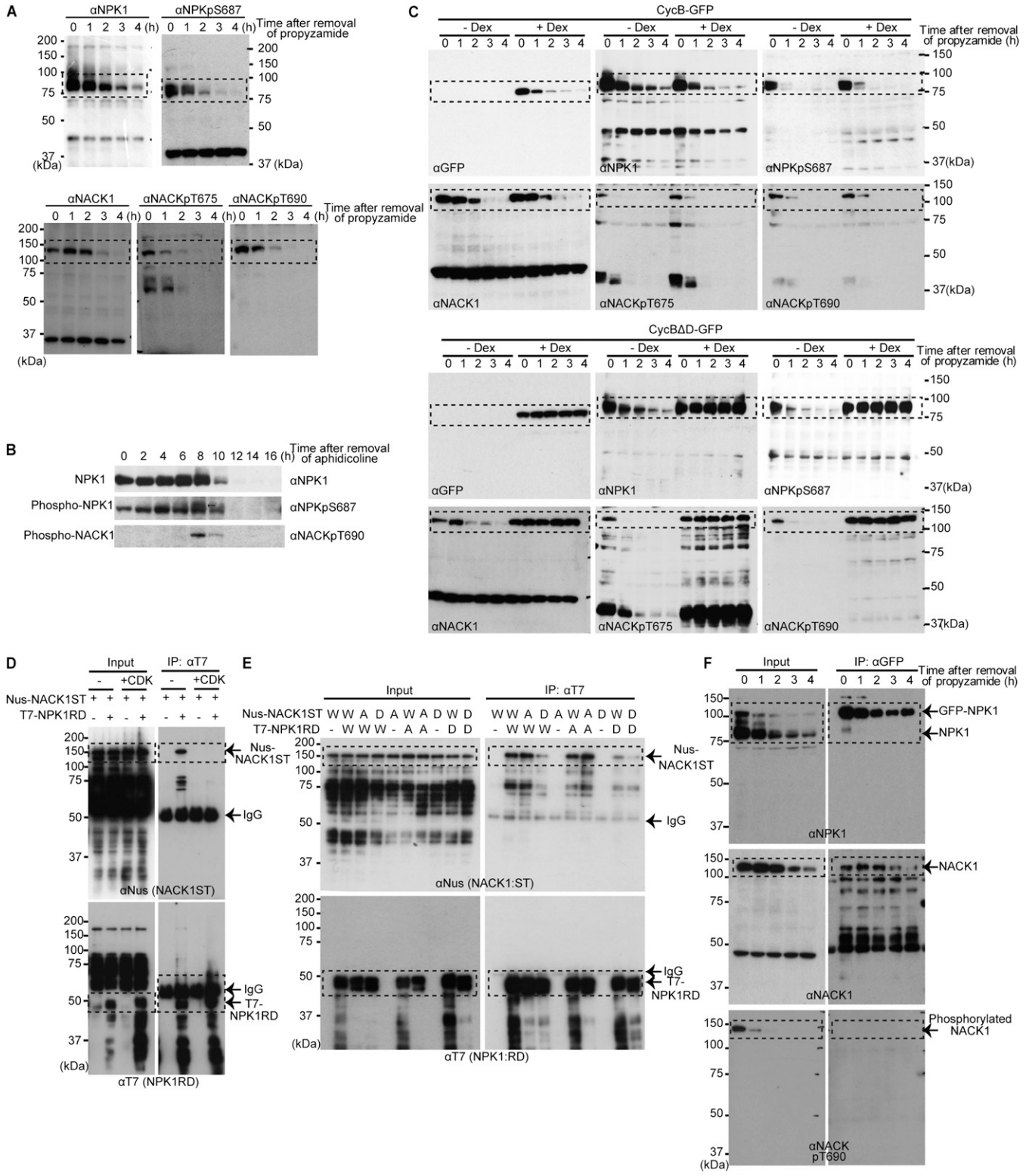

Fig. S3. Phosphorylation of NPK1 and NACK1 at the CDK-phosphorylation sites during the cell cycle and full scans of the Western blots. (*A*) Full scans of the Western blots presented in Fig. 2. (*B*) Accumulations of phopho-NPK1 and phospho-NACK1 during the cell cycle. Proteins were extracted from aphidicolin-synchronized BY-2 cells. Immunoblotting analyses were performed with antibodies against NPK1 (αNPK1), phospho-NPK1 (αNPKpS687), and phospho-NACK1 (αNACKpT690). (*C*) Full scans of the Western blots presented in Fig. 3. (*D*) Full scans of the Western blots presented in Fig. 4*A*. (*E*) Full scans of the Western blots presented in Fig. 4*B*. (*F*) Full scans of the Western blots presented in Fig. 4C.

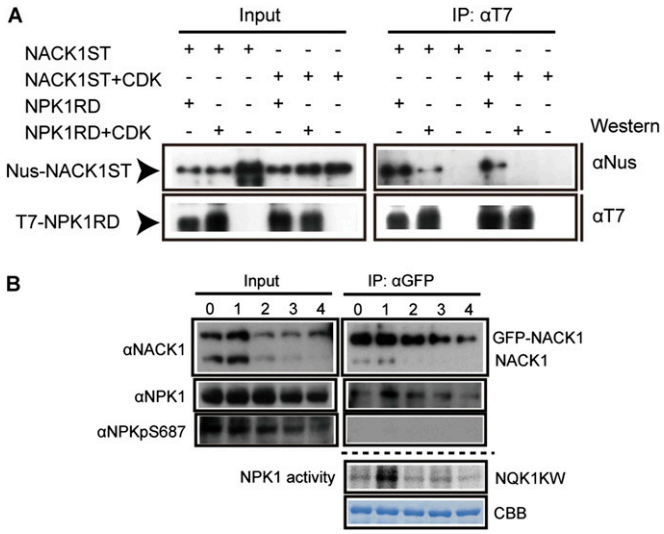

Fig. S4. The effects of phosphorylation by CDKs on the interaction between NPK1 and NACK1. (*A*) Inhibition in vitro of the interaction between NPK1 and NACK1 by CDKs. His-T7–NPK1RD (T7-NPK1RD) and His-NusA–NACK1ST (Nus-NACK1ST) recombinant proteins were used in pull-down assays with T7-specific antibodies (αT7). The target proteins were phosphorylated by CDKs that had been purified from BY-2 cells with $p13^{SUC1}$ beads and used as phosphorylated proteins in assays after the removal of $p13^{SUC1}$ beads (+CDK). Pull-down assays were performed with proteins in various combinations, as indicated. The coprecipitates were analyzed by Western blotting with αT7 and Nus-specific (αNus) antibodies. (*B*) Inhibition in vivo of the physical interaction between NPK1 and NACK1 by CDKs. Cells were two-step synchronized with aphidicolin and propyzamide in the presence of 1 μM Dex to induce the expression of GFP-NPK1. After release from propyzamide, cells were subcultured in fresh medium with 1 μM Dex. Proteins were extracted from BY-2 cells that expressed GFP-NACK1 and had been harvested at the indicated times after removal of propyzamide. Immunoprecipitation (IP) was performed with GFP-specific antibodies. Input proteins (*Left*) and precipitates (*Right*) were analyzed by Western blotting with αNACK1, αNPK1, and phospho-NPK1–specific (αNPKpS687) antibodies. NPK1 activity was analyzed in an αNPK1-immunocomplex kinase assay with kinase-negative NQK1 MAPKK (NQK1:KW) as substrate.

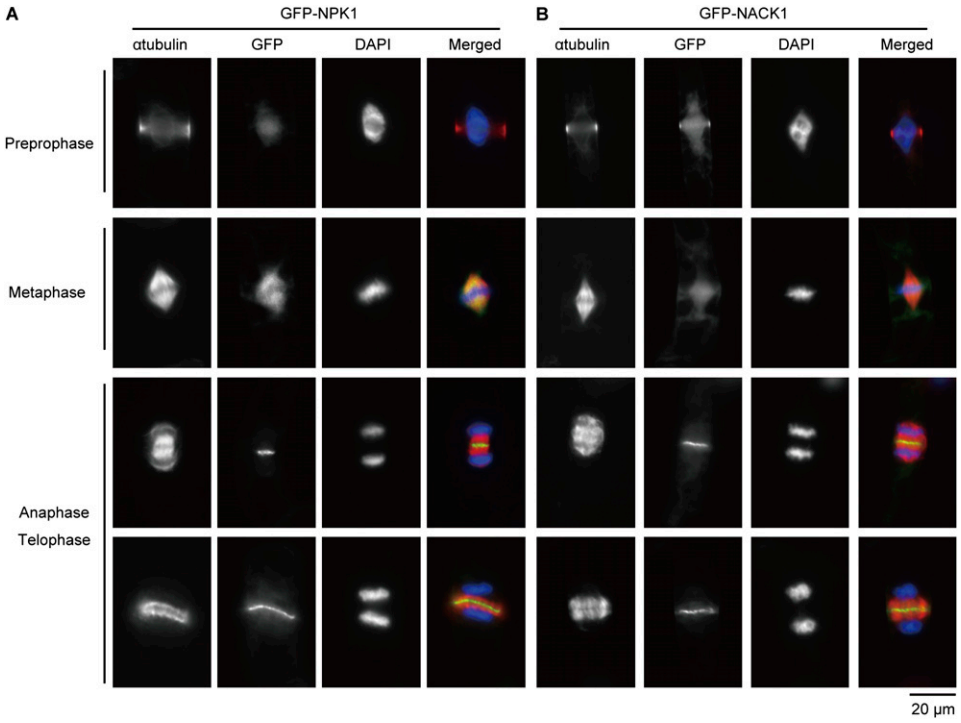

Fig. S5. Subcellular localization of GFP-NPK1 and GFP-NACK1 during mitosis in BY-2 cells. BY-2 cells expressing GFP-NPK1 (*A*) and GFP-NACK1 (*B*), separately, were incubated for 12 h in the presence of 0.5 μM dexamethasone (Dex). Cells were fixed and stained with tubulin-specific antibodies (α-tubulin) and DAPI, which is specific for nuclei. Merged images are shown on the right (α-tubulin, red; DAPI, blue; GFP, green). (Scale bar: 20 μm.)

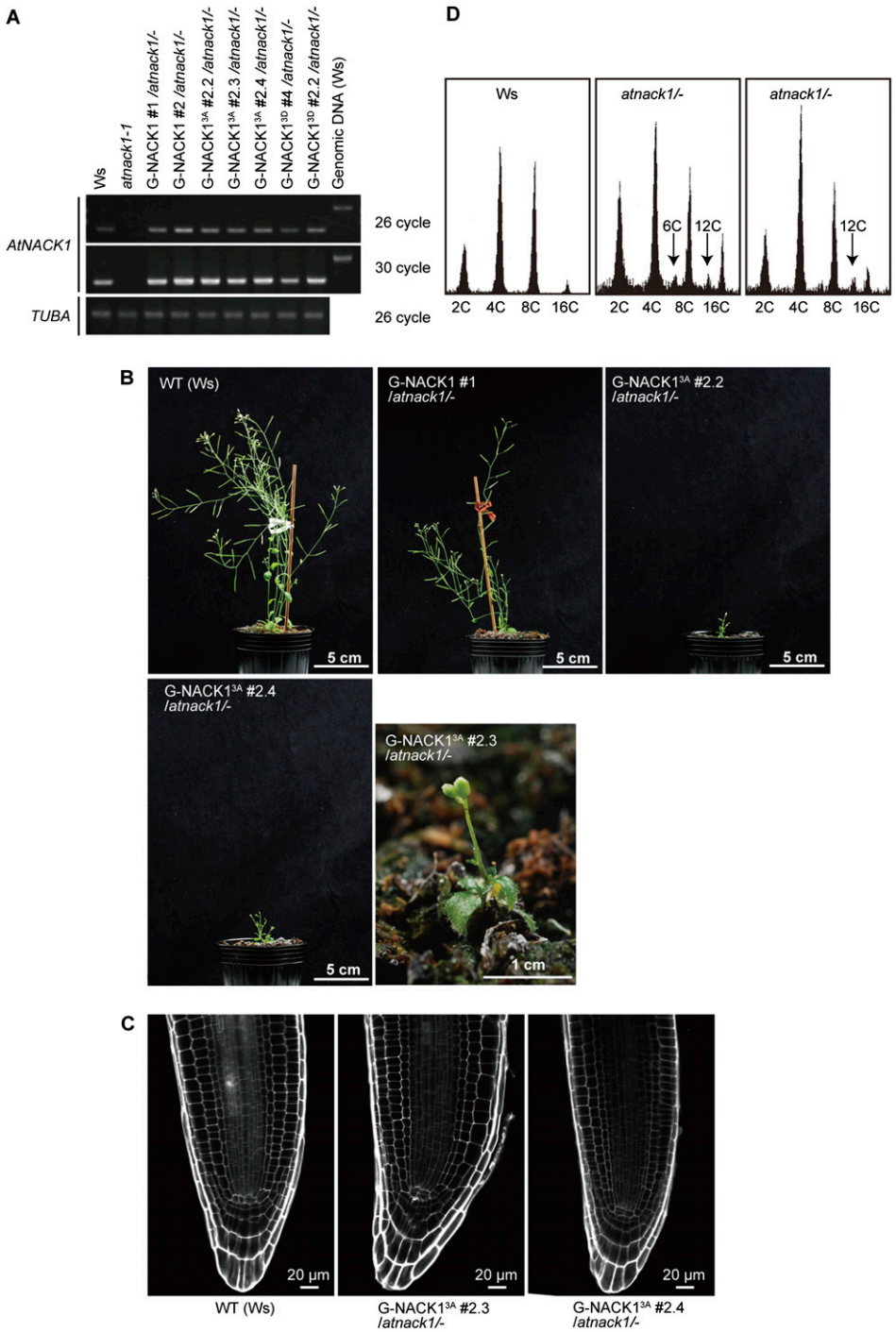

Fig. S6. Levels of *NACK1* transcripts derived from transformed G-NACK1 and mutant G-NACK1 sequences and gross morphology of transgenic *Arabidopsis* plants with G-NACK1 or G-NACK^{3A}. (*A*) Accumulation of *AtNACK1* RNAs in *atnack1-1* plants transformed with fragments of G-NACK1, G-NACK^{3A}, and G-NACK^{3D}. RT-PCR was performed using mRNA from WT plants (ecotype Wassilewskija; Ws), *atnack1-1* plants, and *atnack1-1* plants with G-NACK1, G-NACK^{3A}, and G-NACK^{3D} mutations. AtNACK1-specific primers were used (*AtNACK1*; Table S2). Primers specific to the gene for α-tubulin (*TUBA*) were used as controls. The number of PCR cycles is indicated. (*B*) The phenotype of *atnack1-1* plants transformed with fragments of AtNACK1 genomic DNA. Gross morphology of the WT plant (Ws) and of *atnack1-1* plants that harbored G-NACK1 and G-NACK1^{3A} constructs, respectively, 40 d after germination. (*C*) Median longitudinal sections of roots from 5-d-old seedlings of WT plants and *atnack1-1* plants harboring G-NACK^{3A}. (*D*) Ploidy analysis in *atnack1-1* plants. The tissue of the *atnack1-1* mutant of *Arabidopsis* occasionally includes aneuploid cells (6C and 12C, indicated by arrows). First and second true leaves in 14-d-old WT (Ws) and *atnack1-1* plants were used for the ploidy analysis with flow cytometry (Partec).

Table S1. Complementation of the *atnack1* mutation

	Genotype of T3	Transgene	Number of normal plants	Number of dwarf plants (mild)	Percentage of dwarf plants (mild)	*P* value (χ^2 test)
Atnack1/+	*atnack1*/+	–/–				
Observed			116	34	22.67	
Expected			112.5	37.5	25	0.51
G-NACK #1*	*atnack1*/+	+/–				
Observed			126	11	8.03	
Expected 1			128.44	8.56	6.25	0.39
Expected 2			102.75	34.25	25	4.49E-06†
G-NACK #2	*atnack1*/+	+/–				
Observed			106	9	7.83	
Expected 1			107.81	7.19	6.25	0.49
Expected 2			86.25	28.75	25	2.11E-05†
G-NACK^{3A} #2.2	*atnack1*/+	+/–				
Observed			149	49 (22)	24.75 (11.11)	
Expected 1			185.63	12.37	6.25	5.75E-27†
Expected 2			148.5	49.5	25	0.93
G-NACK^{3A} #2.3	*atnack1*/+	+/–				
Observed			131	49 (43)	27.22 (23.89)	
Expected 1			168.75	11.25	6.25	3.11E-31†
Expected 2			135	45	25	0.49
G-NACK^{3A} #2.4	*atnack1*/+	+/–				
Observed			104	33 (17)	24.09 (12.41)	
Expected 1			128.44	8.56	6.25	6.28E-18†
Expected 2			102.75	34.25	25	0.81
G-NACK^{3D} #4	*atnack1*/+	+/–				
Observed			146	49 (1)	25.13 (0.51)	
Expected 1			182.81	12.19	6.25	1.28E-27†
Expected 2			146.25	48.75	25	0.97
G-NACK^{3D} #2.2	*atnack1*/+	+/–				
Observed			165	45 (3)	21.43 (1.43)	
Expected 1			195.88	13.12	6.25	1.18E-19†
Expected 2			157.5	52.5	25	0.23

The actual number of plants exhibiting a mutant phenotype is shown as Observed. Expected 1 indicates the number of plants expected if the transgene had fully rescued the phenotype associated with the *atnack1* mutation. Expected 2 indicates the number of plants expected if the transgene had not rescued the phenotype associated with the *atnack1* mutation. Mild indicates the number of plants showing a milder dwarf phenotype than the dwarf phenotype of *atnack1-1* plants.

*The 6.1-kb fragment of genomic DNA and the same fragments in which alanine or aspartate was substituted for predicted sites of phosphorylation by CDKs, as shown in Fig. 5, were introduced into *atnack1* heterozygotes. Transformants were designated G-NACK, G-NACK^{3A}, and G-NACK^{3D}, respectively.

†*P* values indicating significant differences.

Table S2. Primers used for molecular cloning, site-directed mutagenesis, and genotyping of *atnack1-1* mutation in *Arabidopsis* plants

z	Primer	Primer sequence
1	NPK1RD_F	SalI site-5′-GCAGAACATCAGGAAGCTCGCCC
2	NPK1RD_R	NotI site-5′-TTATCTTCCAGGAGATGCAAACCTTGAT
3	NACK1MD_F	BamHI site-5′-ATGACTGTTAGAACTCCTGGTACTCC
4	NACK1MD_R	NotI site-5′-CACTTGGGCGTTATTCGTGACTTCC
5	NACK1ST_F	BamHI site-5′-AACATGGTTGTATCCGACAAACAGCTT
6	NACK1ST_R	NotI site-5′-TTAGATATGAAGGAGGTCAGAGATTTG
7	NPK_S575A	5′-CCACCAAAAAGCAGGGCACCAAAACGTATGCTTAGC
8	NPK_S669A	5′-CGTCAGGCAGTCAACTTAgCACCACCAAAGGATC
9	NPK_S687A	5′-AGTAAATCAAGGTTTGCAgCTCCTGGAAGATAAGCGGC
10	NPK_S575D	5′-CCACCAAAAAGCAGGGATCCAAAACGTATGCTTAGC
11	NPK_S669D	5′-CGTCAGGCAGTCAACTTAgatCCACCAAAGGATCC
12	NPK_S687D	5′-AGTAAATCAAGGTTTGCAgaTCCTGGAAGATAAGCGGC
13	NACK_T145A	5′-CTGTAAACGACATATATGCACATATAATGAGTGCCCCAG
14	NACK_T675A	5′-CAGCCGGATGAGGCTCCTACAAAAAGTGATGGAGGAG
15	NACK_T690A	5′-CAAAGGAAGGCGCTCCATATCGCCGTTCCAGTTCTGTG
16	NACK_T836A	5′-GAGCTTGGAAATGCAGCCCCGGCTCGTGTTGGAAATG
17	NACK_T145D	5′-CTGTAAACGACATATATGCACATATAATGAGTGACCCAGAAAGAG
18	NACK_T675D	5′-CAGCCGGATGAGGATCCTACAAAAAGTGATGGAGGAG
19	NACK_T690D	5′-CAAAGGAAGGCGATCCATATCGCCGTTCCAGTTCTGTG
20	NACK_T836D	5′-GAGCTTGGAAATGCAGACCCGGCTCGTGTTGGAAATG
21	gAtNACK1_F	BamHI site-5′-AATCAAAAACACACCATTGAATAAATAGC
22	gAtNACK1_R	NotI site-5′-CTAATGGCTACTTCCTCATCTACCACCGTCGTC
23	AtNACK1_T145A	5′-CACTGTTGTTTCCAGGCCCCTGAAAGAGATTTTACTATCAAGA
24	AtNACK1_T687A	5′-CAGGGGTCTGAGAAAGAGGCTCCACAGAAAGGAGAAGAAAGCGGA
25	AtNACK1_T857A	5′-GCAGAAGTAGGAAACGCAGCTCCAGCTCGAAACTGCGATGAATCT
26	AtNACK1_T145D	5′-CACTGTTGTTTCCAGGACCCTGAAAGAGATTTTACTATCAAGA
27	AtNACK1_T687D	5′-CAGGGGTCTGAGAAAGAGGATCCACAGAAAGGAGAAGAAAGCGGA
28	AtNACK1_T857D	5′-GCAGAAGTAGGAAACGCAGATCCAGCTCGAAACTGCGATGAATCT
29	LB1a	5′-CATTTTATAATAACGCTGCGGACATCTAC
30	AtNACK1-g1	5′- CGACGGGAGGTCAGCGACGGAGAAGACG
31	AtNACK1-g2	5′-CACACGCACTAAGTCCACAAAGTTCTAG
32	AtNACK1-g3	5′-TGAAGAAAATGCAGCAAATG
33	M13R	5′-CAGGAAACAGCTATGAC