NQK1/NtMEK1 is a MAPKK that acts in the NPK1 MAPKKK-mediated MAPK cascade and is required for plant cytokinesis

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The tobacco protein kinase NPK1 is a MAPKKK that regulates formation of the cell plate during cytokinesis. In the present study, we have identified tobacco NQK1/NtMEK1 and NRK1 as a MAPKK and a MAPK, respectively, downstream of NPK1. NQK1/NtMEK1 complements the mutation in the PBS2 MAPKK gene of yeast in a manner that depends on both NPK1 and its activator, NACK1, a kinesin-like protein. Active NPK1 and NQK1/NtMEK1 phosphorylate and activate NQK1/NtMEK1 and NRK1, respectively. Both NQK1/NtMEK1 and NRK1, as well as NPK1, are activated at the late M phase of the cell cycle in tobacco cells, and they are rapidly inactivated by depolymerization of phragmoplast microtubules. These results suggest the existence of a MAPK cascade that consists of NPK1, NQK1/NtMEK1, and NRK1 and functions in a process related to the architecture of phragmoplasts at the late M phase of the cell cycle. Overexpression of kinase-negative NQK1/NtMEK1 in tobacco cells generates multinucleate cells with incomplete cross-walls. Arabidopsis plants with a mutation in the ANQ1 gene, an ortholog of NQK1/NtMEK1, display a dwarf phenotype, with unusually large cells that contain multiple nuclei and cell-wall stubs in various organs. In addition, anq1 homozygotes set fewer flowers and produce large and malformed pollen grains with a tetrad structure. Thus, NQK1/NtMEK1 (ANQ1) MAPKK appears to be a positive regulator of plant cytokinesis during meiosis as well as mitosis.

[Keywords: NPK1 MAPKKK; NQK1 MAPKK; NRK1 MAPK; plant cytokinesis; cell plate; Arabidopsis knockout]

Received December 27, 2002; revised version accepted February 21, 2003.

Mitogen-activated protein kinase [MAPK] cascades play a central role in the transduction of various extra- and intracellular stimuli. They function in the control of developmental and physiological processes, such as the growth and differentiation of cells and adaptation to stress [Widmann et al. 1999; Chen et al. 2001]. They are conserved throughout eukaryotes, and individual organisms have multiple MAPK cascades. In general, each cascade depends on a MAPK kinase kinase [MAPKKK], which phosphorylates and activates a MAPK kinase [MAPKK], which, in turn, activates a MAPK by phosphorylation [Chen et al. 2001]. In higher plants, various homologs of components that might participate in MAPK cascades have been identified [Ichimura et al. 2002], and a wide variety of abiotic and biotic stimuli have been shown to activate MAPKs [Jonak et al. 2002]. In addition, the alfalfa SIMK MAPK has been shown to be involved in formation of root hairs [Sama et al. 2002]. MAPK cascades also influence various aspects of cell division [Pages et al. 1993; Minshull et al. 1994; Wang et al. 1997; Takenaka et al. 1997, 1998; Wright et al. 1999; Graves et al. 2000]. Recent studies on MAPKs of both animals and plants suggest that cytokinesis, in which complex processes separate a mother cell into two compartments, might be controlled by a MAPK cascade [Calderrini et al. 1998; Shapiro et al. 1998; Zecevic et al. 1998; Bögre et al. 1999; Nishihama and Machida 2001; Nishihama et al. 2001, 2002]. However, evidence for such a cascade was, until recently, circumstantial, being based on the activation of MAPKs at late M phase and the subcellular localization of MAPKs to the spindle midzone and the midbody [in animal cells] or the cell plate [the developing cross-wall of plant cells].

The NPK1 [nucleus- and phragmoplast-localized protein kinase 1] gene from tobacco encodes a member of the MAPKKK family, and its kinase domain can replace the functions of several yeast MAPKKKs [Banno et al. 1993; Machida et al. 1998]. NPK1 and its orthologs in Arabidopsis, ANP1, ANP2, and ANP3, are transcribed in proliferating and division-competent cells [Banno et al. 1993; Nishihama et al. 1997; Nakashima et al. 1998]. Recent studies have provided several lines of evidence for a requirement of the NPK1 group of MAPKKs in

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Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.1071103.
cytokinesis [Nishihama et al. 2001; Jin et al. 2002; Krysan et al. 2002]. The activity of NPK1 increases during late M phase. Overexpression of a kinase-negative mutant of NPK1 in tobacco cells results in the generation of multinucleate cells with incomplete cell plates. In these cells, phragmoplasts are formed but fail to expand all the way to the cell cortex [Nishihama et al. 2001]. Such overexpression or silencing of the NPK1 gene in tobacco plants results in the generation of multinucleate guard cells [Nishihama et al. 2001; Jin et al. 2002]. Moreover, anp2 anp3 double mutants of Arabidopsis exhibit defects in cytokinesis, forming unusually large multinucleate cells with cell-wall stubs [Krysan et al. 2002].

We have identified NACK1 and NACK2, novel members of the kinesin-like protein [KLP] family, as factors that increase the activity of NPK1 via formation of a complex [Nishihama et al. 2002]. NACK1 accumulates at late M phase and is consistently colocalized with NPK1 at the equatorial zone of the phragmoplast during the phragmoplast expansion from anaphase to late telophase. Overexpression of motor domain-truncated NACK1 eliminates the accumulation of endogenous NPK1 at the equatorial zone of the phragmoplast and also interrupts lateral expansion of the cell plate [Nishihama et al. 2002]. These results suggest that both the activity and the proper positioning of NPK1 are critical to cytokinesis. Mutations in the ANACK1 (HINEL) gene of Arabidopsis, which is the ortholog of NACK1, also result in the generation of multinucleate cells with incomplete cell walls [Nishihama et al. 2002; Strompen et al. 2002]. Thus, it appears that formation of a protein complex that consists of NACK1 and NPK1 controls the formation of the cell plate at cytokinesis.

In the present study, we isolated a cDNA for a tobacco MAPKK, designated NQK1, that acts downstream of NPK1 and a cDNA for a tobacco MAPK, designated NRK1, that acts downstream of NQK1. These protein kinases were activated at late M phase during the cell cycle. Overexpression of kinase-negative NQK1 in tobacco cells resulted in the generation of multinucleate cells with aberrant cell plates, and this phenotype was similar to the phenotype produced by overexpression of kinase-negative NPK1 [Nishihama et al. 2001]. Moreover, observations of Arabidopsis plants with mutations in ANQ1, an ortholog of NQK1, revealed that ANQ1 is essential for cytokinesis. A discussion is presented of the role of the NACK1/NPK1/NQK1/NRK1 signaling pathway in plant cells.

Results

Molecular cloning of a cDNA for a MAPKK that acts downstream of NPK1

To isolate a cDNA(s) for a MAPKK homolog(s) that acts downstream of NPK1, we took advantage of Saccharomyces cerevisiae cells [pbs2Δ] in which there is a mutation in the gene for the Pbs2p MAPK, which functions in the osmosensing MAPK cascade [Brewster et al. 1993; Maeda et al. 1994, 1995]. Yeast pbs2Δ cells cannot grow under high osmolality [Fig. 1A, Brewster et al. 1993]. The osmosensitivity of pbs2Δ cells was not suppressed by expression of NACK1 and NPK1 cDNAs [data not shown]. If a tobacco MAPKK that might be activated by NPK1 could activate Hog1p, then pbs2Δ cells that contain NACK1, NPK1, and such a MAPKK would be expected to grow. To isolate such a MAPKK, we made a cDNA library using mRNA prepared from tobacco BY-2 cells. The cDNAs were under the control of a galactose-inducible promoter, and they were introduced into pbs2Δ cells in which NACK1 and NPK1 cDNAs were expressed. We screened resultant transformants for cells that could form colonies under high-osmolality conditions in the presence of galactose. We obtained five positive clones from 2.3 x 10⁷ independent transformants. Plasmids containing the cDNAs of interest, derived from two clones (designated 6A and 17A), suppressed the osmosensitivity of pbs2Δ cells in a manner that depended on the expression of both NACK1 cDNA and NPK1 cDNA together [Fig. 1B, data for 17A not shown]. We characterized the two cDNAs as described below.

Sequence analysis showed that the two cDNAs included identical sequences but differed in length. They encoded a putative polypeptide that was strongly homologous to members of the MAPKK family. We named the putative protein NQK1 [Nicotiana kinase next to NPK1; GenBank accession no. BAB32405]. The predicted kinase domain of NQK1 was 58% identical in terms of amino acid sequence to that of Pbs2p [Boguslawski and Polazzi 1987]. The entire amino acid sequence of NQK1 was almost identical to that of NtMEK1, a recently identified tobacco MAPKK [the proteins differ at two positions in the kinase domain; Calderini et al. 2001]. Although NQK1 and NtMEK1 seem to be encoded by the same gene, for convenience we refer to it as NQK1 in the present paper.

We prepared a DNA construct for expression of a kinase-negative mutant of NQK1 [Fig. 4A, below], in which the lysine codon at the predicted ATP-binding site was replaced by a tryptophan codon [NQK1:KW]. Expression of NQK1:KW did not complement the pbs2Δ mutation [Fig. 4B, below], even when NPK1 and NACK1 were coexpressed. Expression of NQK1 cDNA did not suppress the osmosensitive phenotype caused by a mutation in the HOG1 gene [data not shown].

As shown in Figure 1C, histidine- and T7-epitope-tagged NQK1:KW [His-T7-NQK1:KW] was phosphorylated by the immunocomplex prepared with NPK1-specific antibodies from a lysate of yeast cells that expressed both NACK1 and NPK1 [Fig. 1C, lane 1]. A very low level of phosphorylation was detected when His-T7-NQK1:KW was incubated with the immunocomplex isolated from yeast cells that expressed either NACK1 or NPK1 [Fig. 1C, lanes 2,3].

Molecular cloning of a cDNA for a MAPK that acts downstream of NQK1

We attempted to use yeast two-hybrid screening to isolate a MAPK that might act downstream of NQK1. Using...
NQK1 as "bait," we cloned several cDNAs for proteins that were able to interact with NQK1. Two of the isolated cDNAs encoded a putative homolog of MAPK, which was designated NRK1 (Nicotiana kinase next to NQK1). An interaction between recombinant NQK1 and NRK1 proteins was also detected by examination of binding in vitro (data not shown). The predicted amino acid sequence of NRK1 was almost identical to that of NTF6 (there were different amino acids at three positions, one in the noncatalytic region at the N terminus and two in the kinase domain, Wilson et al. 1995). It has been reported that NTF6 might be a MAPK that functions downstream of NtMEK1 (Calderini et al. 2001). However, the nucleotide sequence of NRK1 cDNA (GenBank accession no. BAB32406) was significantly different from that of the NTF6 cDNA: the coding, 5'-untranslated and 3'-untranslated regions were 98%, 98%, and 80%, respectively, identical to each other. Therefore, NRK1 cDNA was apparently derived from a gene different from the NTF6 gene.

We examined two-hybrid interactions among NACK1, NPK1, NQK1, and NRK1; our results are summarized in Table 1. When NQK1 was used as "bait," it interacted with NPK1 in addition to NRK1, but not with NACK1:ST, a truncated form of NACK1 that lacked a motor domain but included the NPK1-binding region (Nishihama et al. 2002). Interaction of NPK1 with NQK1 required the kinase domain of NPK1 (NPK1:KD), whereas it did not interact with the regulatory domain at the C-terminal half of NPK1 (NPK1:RD). When NRK1 was used as the "bait," we detected interactions with NQK1 and with NRK1 itself.

NQK1 and NRK1 are activated in a parallel fashion at late M phase during the cell cycle.

Rabbit polyclonal NQK1-specific and NRK1-specific antibodies were raised against synthetic oligopeptides that corresponded to amino acid residues 337–354 of NQK1 and 1–18 of NRK1, respectively. As shown in Figure 2A, the antibodies recognized proteins of 38 kD and 45 kD, which corresponded to the estimated molecular masses of NQK1 and NRK1, respectively, in protein extracts.

Table 1. Summary of the two-hybrid interactions among NACK1, NPK1, NQK1, and NRK1

| Interaction | LexA-NQK1 | LexA-NRK1 | LexA |
|------------|-----------|-----------|------|
| VP16-NACK1:ST | - | - | - |
| VP16-NPK1 | + | - | - |
| VP16-NPK1:KD | + | ND | - |
| VP16-NPK1:RD | - | ND | - |
| VP16-NQK1 | - | ++ | - |
| VP16-NRK1 | ++ | + | - |
| VP16 | - | - | - |

Interactions (+, strong; +, weak; -, none; ND, not done) were estimated by monitoring the growth of yeast cells on histidine-free medium, which was supplemented with 20 mM and 40 mM 3-aminotriazol, respectively, when NQK1 and NRK1 were used as "bait."
prepared from tobacco BY-2 cells (Fig. 2A, lanes 1,3). When these antibodies were preincubated with the respective antigenic peptides, no signals indicative of an immunoreaction were detected (Fig. 2A, lanes 2,4). Therefore, we concluded that the respective antibodies recognized NQK1 and NRK1 specifically.

Figure 2. Activation of NQK1 and NRK1 during the cell cycle in tobacco BY-2 cells. (A) Specificity of NQK1-specific antibodies and NRK1-specific antibodies. An extract of BY-2 cells at logarithmic phase was analyzed by immunoblotting with affinity-purified NQK1-specific (lanes 1,2) and NRK1-specific (lanes 3,4) antibodies, which had been preincubated in the presence (+, lanes 2,4) or absence (−, lanes 1,3) of the respective peptide antigens. (B) Activation of NQK1 and NRK1 during the cell cycle. (Panel a) Relative proportions of mitotic cells at different times (in hours) after the removal of aphidicolin. (Panel b) Activities of NQK1, NRK1, and CDKs during the cell cycle. Proteins were extracted from cells that had been harvested at the indicated times (in hours). Immunocomplex kinase assays were performed with NQK1- (top) and NRK1-specific (middle) antibodies using His-T7-NRK1:KW and MBP, respectively, as substrate. NQK1- and NRK1-specific antibodies that had been incubated with the respective peptide antigens (+peptide) were used to confirm that phosphorylation of substrates, as detected here, was due to the activities of NQK1 and NRK1. CDKs were precipitated with p13SUC1 beads, and then CDK-bead complexes were examined for their ability to phosphorylate histone H1 (bottom). (Panel c) Extracts used for the immunocomplex kinase assay were immunoblotted with the indicated antibodies. Twenty micrograms of proteins were loaded in each lane. (C) Activities of NQK1 and NRK1 during the M-to-G1 transition. (Panel a) Mitotic indices (bars) and relative kinase activities of NQK1 (open circles) and NRK1 (filled circles), as determined in panel b, at the indicated times (in hours) after removal of propyzamide. The patterns in bars indicate populations of BY-2 cells at different phases of the M phase, as indicated. (Panel b) Activities of NQK1 (top), NRK1 (middle), and CDKs (bottom) analyzed by the immunocomplex kinase assay or the p13SUC1 bead-complex kinase assay. (Panel c) Immunoblotting of extracts with the indicated antibodies.
We measured the activities of NQK1 and NRK1 in synchronized populations of BY-2 cells by immunocomplex kinase assays. His-T7-tagged kinase-negative NRK1 [His-T7-NRK1:KW], in which the lysine residue at the predicted ATP-binding site had been replaced by a tryptophan residue (Fig. 4A, below), was used as the substrate for NQK1, and myelin basic protein (MBP) was used as the substrate for NRK1 in these assays. The cell cycle of BY-2 cells was arrested at the G1/S boundary by treatment of cells with aphidicolin, and cells were released from the blockage by washing out the drug. Analysis of mitotic indices showed that cells that were in M phase appeared from 6 to 10 h after the removal of aphidicolin and that the highest proportion of M-phase cells was found at 8 h [Fig. 2B, panel a]. The highest activities of both NQK1 and NRK1 were detected at 8 and 10 h [Fig. 2B, panel b]. Although changes in the activities of NQK1 and NRK1 occurred almost in parallel to changes in mitotic indices, high levels of activities of both protein kinases were still detected even when the mitotic index was reduced at 10 h. These results suggested that NQK1 and NRK1 were activated relatively late in M phase. Immunoblot analysis showed that both NQK1 and NRK1 themselves were present consistently throughout the cell cycle [Fig. 2B, panel c]. We also measured the activities of cyclin-dependent protein kinases (CDKs), which were activated prior to activation of NQK1 and NRK1 [2 h], although the maximum activities of these three types of protein kinase were found roughly at the same time [8 h].

To examine more precisely the timing of activation of NQK1 and NRK1 during the M phase, we blocked the cell cycle of BY-2 cells that had been synchronized with aphidicolin at prometaphase using propyzamide, a microtubule-depolymerizing drug, and then we released cells from prometaphase by washing out the drug [Fig. 2C, panel a]. No activity of either NQK1 or NRK1 was detected at prometaphase. Both activities increased transiently 0.5–2.0 h after removal of propyzamide: the peak of NRK1 activity was detected at 1 and 1.5 h, but the peak of NQK1 activity seemed to be broader than that of NRK1 activity [Fig. 2C, panel b], consistent with the observations in Figure 2B. It is noteworthy that the proportions of cells in anaphase and telophase were the highest at this time [Fig. 2C, panel a]. These patterns of activation of NQK1 and NRK1 were essentially the same as that of activation of NPK1 [Nishihama et al. 2001] and that of the accumulation of NACK1 itself [Nishihama et al. 2002].

Simultaneous decreases in the activities of NPK1, NQK1, and NRK1 upon depolymerization of microtubules

We examined whether the activities of NPK1, NQK1, and NRK1 might be affected by the depolymerization of phragmoplast microtubules [MTs]. Accordingly, 60 min after release from prometaphase arrest, when the majority of cells were in anaphase or telophase, a culture of the BY-2 cells was divided into two aliquots, one of which was again treated with propyzamide and one that was not. Treatment with the drug resulted in depolymeriza-

Figure 3. Rapid decreases in the activities of NPK1, NQK1, and NRK1 upon depolymerization of microtubules. Cultures of BY-2 cells were divided into two aliquots 60 min after the release from prometaphase-arrest [arrows], incubated with (+) or without (−) 6 µM propyzamide, and harvested at the indicated times [in minutes] after the release. (A) Activities of NPK1, NQK1, NRK1, and CDKs. (B) Immunoblotting with the indicated specific antibodies. Twenty micrograms of protein was loaded in each lane.
subjected to in gel kinase assay with contained unlabeled ATP and then NRK1 proteins were incubated in the presence of ATP to activate NRK1, we incubated the reagents to NRK1 to determine its activation, we incubated the recombinant MAPKKs. To examine whether phosphorylation of MAPKs is the motif for phosphorylation by their respective MAPKKs. To examine whether phosphorylation of NRK1 might lead to its activation, we incubated the recombinant MAPKKs with unlabeled ATP and then NRK1 proteins were subjected to in gel kinase assay with 

**NPK1 activates NQK1 through phosphorylation**

In yeast and animal cells, MAPKKs are activated upon phosphorylation of serine and threonine residues in the S/TXXXS/T motif that is located between kinase subdomains VII and VIII. However, all the plant MAPKKs isolated to date contain five residues between putative sites of phosphorylation (S/TXXXS/T). We predicted that serine (S219) and threonine (T225) residues in the SMGQRDT sequence of NQK1 would be sites of phosphorylation by NPK1. To examine this possibility, we replaced S219 and T225 with alanine residues to generate NQK1:SATA [Fig. 4A]. NQK1:SATA did not complement the pbs2A mutation even when NPK1 and NACK1 were coexpressed [Fig. 4B, panel a, sector 4].

We examined whether S219 and T225 could be phosphorylated by NPK1 by fusing His and T7 epitopes to a constitutively active form of NPK1 [His-T7-NPK1Δ374], in which the C-terminal regulatory domain of NPK1 had been removed [Banno et al. 1993]. We prepared protein extracts from yeast cells that expressed this construct and performed immunocomplex kinase assays [Fig. 4C]. As shown in Figure 4C, panel b, His-T7-NQK1:KW was phosphorylated by His-T7-NPK1Δ374 [lane 1]. No phosphorylation was detected in the absence of His-T7-NPK1Δ374 [Fig. 4C]. Recombinant His-T7-NQK1:KW/SATA protein was not phosphorylated by His-T7-NPK1Δ374 [lane 3].

**NQK1 phosphorylates and activates NRK1 in vitro and in vivo**

We examined whether NQK1 could phosphorylate and activate NRK1 in vitro using recombinant proteins. First, we examined the phosphorylation of His-T7-NRK1:KW by a chimeric recombinant NQK1 protein in which glutathione-S-transferase (GST) had been fused to the N terminus [GST-NQK1]. The activity of this recombinant protein was 60-fold higher than that of His-T7-tagged NQK1 [data not shown]. As shown in Figure 4D, GST-NQK1 efficiently phosphorylated His-T7-NRK1:KW [Fig. 4D, lane 1] but the kinase-negative mutant [GST-NQK1:KW] did not (Fig. 4D, lane 3). GST-NQK1 failed to phosphorylate His-T7-NRK1:KW/TAYA in which amino acids T196 and Y198 had been replaced by alanine residues [Fig. 4A,D]. T196 and Y198 are amino acid residues in the TEY-sequence that are conserved in MAPKs as the motif for phosphorylation by their respective MAPKKs. To examine whether phosphorylation of NRK1 might lead to its activation, we incubated the recombinant NRK1 protein and mutant forms of this protein with GST-NQK1 or GST-NQK1:KW in buffer that contained unlabeled ATP and then NRK1 proteins were subjected to in gel kinase assay with [γ-^32P]ATP and MBP as the substrate. His-T7-NRK1 that had been incubated with GST-NQK1, but not with GST-NQK1:KW, phosphorylated MBP within the gel [Fig. 4E, +MBP, lanes 1,2]. His-T7-NRK1:KW and His-T7-NRK1:TAYA [Fig. 4A] did not phosphorylate MBP, even when incubated with GST-NQK1 [Fig. 4E, +MBP, lanes 3,4].

To examine whether NQK1 could activate NRK1 in vitro, we generated BY-2 cells that had been transformed with the GST::NQK1 gene, whose expression was driven by a dexamethasone-inducible [DEX-inducible] promoter (Aoyama and Chua 1997), and with the empty binary vector, which had been used for construction of the DEX-inducible GST::NQK1 gene, as the control. The former cell line is abbreviated as GST-Q and the latter as Vec. Upon treatment of GST-Q cells with DEX, GST::NQK1 proteins were produced and accumulated to about three times the level of endogenous NQK1 proteins [Fig. 4F, panel b]. The GST-NQK1 proteins that were immunoprecipitated with the GST-specific antibodies phosphorylated His-T7-NRK1:KW in vitro [Fig. 4F, panel a, top]. An immunocomplex kinase assay with NRK1-specific antibodies revealed that NRK1 proteins were activated in GST-Q cells that had been treated with DEX [Fig. 4F, panel a, bottom]. In contrast, immunocomplexes prepared with NRK1-specific antibodies from DEX-untransformed GST-Q cells and Vec cells phosphorylated MBP only slightly [Fig. 4F, panel a, bottom]. Levels of endogenous NQK1 and NRK1 did not differ significantly among the various cell extracts [Fig. 4F, panel b]. These results showed that expression of GST-NQK1 induced the activation of endogenous NRK1 in BY-2 cells.

**Overexpression of kinase-negative NQK1 in tobacco BY-2 cells results in generation of multinucleate cells**

We examined the effects of overexpression of NQK1:KW on cytokinesis of tobacco BY-2 cells. We transformed BY-2 cells with a DEX-inducible GST::NQK1:KW construct. We selected two cell lines (GST-Q:KW cell lines) that grew normally without DEX but synthesized GST-NQK1:KW in the presence of DEX (maximum levels were obtained 18–24 h after the addition of 0.1–1.0 µM DEX) but not in the absence of DEX. Vec cell lines were used as controls. As shown in Figure 5A, when GST-Q:KW cells were incubated in the presence of 1 µM DEX, a significant proportion of cells (5%–10%) was binucleate [Fig. 5A, panel d] or multinucleate [Fig. 5A, panel e, eight nuclei] and usually large [Fig. 5A, panel e]. Cells with other than two, four, or eight nuclei were rarely seen. Without DEX, all the cells had single nuclei [Fig. 5A, panel c]. The Vec lines produced no multinucleate cells [Fig. 5A, panels a,b].

We also examined the formation of cell plates in multinucleate cells by staining with Calcofluor, which detects β-glucans in cell walls. As shown in Figure 5B, almost all the multinucleate cells had incomplete cell plates, which seemed to have been terminated prematurely [Fig. 5B, panels b,c]. In DEX-treated Vec cells and DEX-untransformed cells, there were no such abnormal structures [Fig. 5B, panel a, data not shown].
We also generated transgenic tobacco plants that harbored the DEX-inducible GST-Q:KW gene (GST-Q:KW plant lines). When seeds of the transgenic plants were allowed to germinate on DEX-containing medium,
cotyledons with rough surfaces were formed and they were poorly developed (Fig. 5C, panel b). Analysis of these cotyledons revealed that 5% of guard cells and pavement cells were binucleate and cell plates in some cells were incomplete or undetectable (Fig. 5D, panels b–d). These features can be explained by the occurrence of nuclear division with arrested or delayed cytokinesis during the development of guard cells and pavement cells. We found similar abnormalities in the epidermal cells of cotyledons in which kinase-negative NPK1 was overexpressed [Nishihama et al. 2001].

**Disruption of an Arabidopsis ortholog of NQK1 causes cytokinetic defects**

A search of sequences in the *Arabidopsis* genome revealed that the *NQK1* gene was closely related to a hypothetical gene designated At5g56580 [AtMKK6; Ichimura et al. 2002], which encoded a deduced amino acid sequence that was 84% identical to that of *NQK1*. We named this gene *ANQ1* [*Arabidopsis NQK1*]. Using a PCR-based screening system [Krysan et al. 1996], we identified a mutant allele of the *ANQ1* gene with a T-DNA insert in the first intron (*anq1*, Fig. 6A). In addition, we found that the mutant had additional copies of part of the sequence of the *ANQ1* gene, which might have been duplicated during insertion of the T-DNA. Transcripts that were synthesized from these mutated *ANQ1* sequences were detected. Analysis of nucleotide sequences of cDNAs corresponding to the transcripts showed that the 5′-noncoding region and the first exon that includes the putative initiator methionine codon were deleted in all the cDNAs [data not shown], suggesting that no active *ANQ1* protein would be synthesized from either transcript in the *anq1* mutant even if translation takes place from the first available in-frame ATG codon, which corresponds to the 98th methionine in the kinase subdomain II of wild-type *ANQ1*

The *anq1* homozygous mutant of *Arabidopsis* exhibited dwarfism, producing small leaves and short roots during vegetative growth (Fig. 6B). The cotyledons had rough surfaces (Fig. 6C) and outgrowths of cells were evident on the root epidermis (Fig. 6D). These features were observed in 26% (n = 339) of progeny of self-pollinated *anq1* heterozygotes. A normal phenotype was restored by a transgene that contained the *ANQ1* cDNA [data not shown]. The phenotype of the *anq1* mutant was similar to that of the *atnack1* mutant. However, the effects of the *anq1* mutation on plant growth and plant morphology seemed to be milder than those of the *atnack1* mutation. For example, in contrast to *atnack1* plants, whose development ceased at the vegetative stage, nine out of 24 *anq1* plants generated short inflorescence stems with two or three flowers [data not shown]. Whereas *anq1* mutants produced small leaves with smooth surfaces, *atnack1* mutants developed small leaves with rough surfaces. As shown in Figure 6E, we found large cells with incomplete cell walls and multiple nuclei in leaves and roots of *anq1* mutant plants, as well as in *atnack1* mutant plants [Nishihama et al. 2002].

In addition to the growth defect, we found that *anq1* homozygotes produced pollen grains that were abnormal in size and shape [62.7%, n = 67, Fig. 6E]. We observed unusually large and malformed pollen grains (53.7%), and pollen grains with a tetrad structure (9.0%) were observed. No similar abnormality was found in pollen grains that were produced in *anq1* heterozygotes [data not shown]. This might have been caused by the supply of ANQ1 protein from the maternal heterozygous plants to pollen cells.

**Discussion**

NPK1, NQK1, and NRK1 constitute a protein-phosphorylation cascade, the PQR pathway, that operates at late M phase

In the present study, we identified two factors that act downstream of NPK1, namely, NQK1 MAPKK and NRK1 MAPK. Our results showed that NPK1 activated NQK1 by phosphorylation and that NQK1 activated NRK1 by phosphorylation. The activities of NPK1, NQK1, and NRK1 increased coordinately during late M phase of the cell cycle in tobacco BY-2 cells (Figs. 2, 3; Nishihama et al. 2001). The timing of these increases in
activity was almost identical to that of the accumulation of the NACK1 kinesin-like protein that has the ability to activate NPK1 MAPKK (Ito et al. 2002; Nishihama et al. 2002). Thus, the accumulation of NACK1 might be the trigger for activation of these three protein kinases. In addition to the coordinated activation of NPK1, NQK1, and NRK1, our results also indicated that maintenance of the kinase activities at the late M phase might be controlled by a common regulatory mechanism that might be related to the structural state of phragmoplast MTs, because these activities decreased rapidly and simultaneously upon depolymerization of phragmoplast MTs, even though the kinase proteins themselves remained detectable (Fig. 3). Therefore, we can conclude that NPK1 (ANP1/2/3), NQK1 [ANQ1], and NRK1 [ANR1: Arabidopsis NRK1 that might correspond to AtMPK13] form a MAP kinase cascade, which may be triggered by binding of the NACK1/2 [AtNACK1/2] kinesin-like protein to NPK1 at the late M phase of the cell cycle in plant cells (Fig. 7). We have designated this cascade the NACK-PQR pathway.

Not only NACK1 and NPK1 but also NQK1 are essential for formation of the cell plate

We investigated the functions of NQK1 using two different approaches. In one case, we used an overexpression system of a dominant-negative mutant of NQK1. Because overexpression of the kinase-negative mutant form of NPK1 resulted in abortion of expansion of both the cell plate and the phragmoplast, with the subsequent generation of multinucleate cells with aberrant cell plates [Nishihama et al. 2001], we examined whether overexpression of kinase-negative NQK1 might also result in similar defects. BY-2 cells and tobacco plants that expressed this mutant protein had phenotypes that were indistinguishable from those generated by overexpression of kinase-negative NPK1 (Fig. 5; Nishihama et al. 2001). The simplest interpretation for this phenomenon is that the mutant NQK1 blocked the activation of endogenous NQK1, with resultant inhibition of the phosphorylation of a substrate protein(s) that is involved in formation of the cell plate. Thus, NQK1 seems to be a positive regulator of the formation of the cell plate. The phenotype might also have been due to an artifact caused by overexpression of the mutant NQK1 construct. However, as described below, our results support a role for NQK1 in cytokinesis.

We analyzed the phenotype associated with a loss-of-function mutation in the ANQ1 gene. Histological observations of anq1 mutant plants revealed the presence of enlarged cells with multiple nuclei and cell-wall stubs in the roots and rosette leaves, and these features are consistent with the results of overexpression of kinase-negative NQK1. The phenotype is typical of various cytokinesis-deficient mutants, such as knolle [Lukowitz et
MAPKK, which is the alfalfa ortholog of AtMKK2, and can, in turn, activate MMK3 MAPK, which is the alfalfa ortholog of NRK1/ANR1 (Cardinale et al. 2002).

For understanding of the molecular network system including NACK1, NPK1, and NQK1, it should be critical to examine functions of an NRK1 MAPK in cytokinesis control. In this context, we examined the effects of a kinase-negative NRK1 on cytokinesis of BY-2 cells, but we detected no abnormalities in cytokinesis associated with this mutant protein (data not shown). Further experiments with NRK1 and its homologs are required to clarify the roles of these MAPKs in cytokinesis.

**NQK1 and NRK1 are not localized predominantly in the phragmoplast at M phase**

We showed previously that NPK1 is localized at the equatorial zone of the expanding phragmoplast but not at the cell plate. Calderini et al. [1998] and Bögre et al. [1999] reported that antibodies against NTF6 and its alfalfa ortholog, MMK3, respectively, stained developing cell plates. However, we failed to detect the accumulation of NQK1 and NRK1 in phragmoplasts or cell plates in spite of a careful examination by immunostaining, and expression of these proteins as fusions with green fluorescent protein. Subcellular fractionation of phragmoplast-associated proteins revealed that NQK1 and NRK1 were predominantly recovered from the cytoplasmic fraction (our unpublished data), although this result does not exclude the possibility that a limited amount of NQK1 and NRK1 might be recruited to the phragmoplast, where it might be activated and function in cytokinesis. It is also possible that most of the activated NQK1 that has been phosphorylated at the phragmoplast might be released from NPK1 but might function in the formation of the cell plate around the phragmoplast.

**The NACK-PQR pathway is curtailed by depolymerization of phragmoplast MTs**

Expansion of the cell plate is led by expansion of the phragmoplast, which involves the disassembly and reassembly of MTs at the inner and the outer edges of the phragmoplast, respectively [Yasuhara et al. 1993]. It has been proposed that depolymerization of MTs on the inner side of the phragmoplast is one of the processes that is regulated by the NACK1/NPK1 complex during cytokinesis [Nishihama and Machida 2001; Corbin et al. 2002]. Arabidopsis embryos with mutations in the AtNACK1 (HINKEL) gene have aberrant phragmoplasts in which the central MTs fail to disassemble [Strompen et al. 2002]. In addition, the observation that overexpression of motor domain-truncated NACK1 prevents accumulation of NPK1 at the phragmoplast and causes the generation of incomplete cell plates [Nishihama et al. 2002] indicates the importance of colocalization of NPK1 and phragmoplast MTs. In this study, we showed that the activities of all the components of the NACK-PQR pathway disappeared rapidly after the depolymerization of phragmoplast MTs even though the compo-
ponents themselves remained detectable (Fig. 3). This finding suggests not only that activation of the NACK-PQR pathway requires phragmoplast MTs but also that there exists a mechanism for the rapid interruption of the pathway in response to the depolymerization of MTs. A regulatory circuit might operate between activation of the NACK-PQR pathway by NACK1 and various structural states of phragmoplast MTs. If the NACK-PQR pathway were to stimulate depolymerization of phragmoplast MTs, it might be reasonable to postulate that, once MTs have been disassembled, the pathway would be rapidly suppressed to prevent further depolymerization of MTs, as would be necessary for the local control of MT dynamics within the phragmoplast. Such suppression of the pathway by the depolymerization of MTs would also operate after the complete expansion of the phragmoplast at the end of cytokinesis.

Materials and methods

Plant materials and transformation of cells and plants

Tobacco BY-2 cells were maintained in suspension culture, and proliferation of cells was synchronized as described previously [Banno et al. 1993; Nishihama et al. 2001]. Transgenic BY-2 cells and tobacco plants were generated by Agrobacterium-mediated transformation [Nishihama et al. 2001]. Arabidopsis thaliana plants are described elsewhere [Nishihama et al. 2001; Tanaka et al. 2001].

Construction of plasmids and site-directed mutagenesis

Mutant derivatives of NQK1 and NRK1 were produced by site-directed mutagenesis and the polymerase chain reaction (PCR). The replacement of amino acids and the sites of such replacement are described in Results. Details of the manipulations and primers used for mutagenesis are available from the authors upon request.

cDNAs for NQK1, NRK1, and their mutant derivatives were cloned between the BamHI and NotI sites of pET28a [Novagen, Darmstadt, Germany]. A 1.1-kb BamHI-XhoI-digested fragment of pET28-NQK1 and of pET28-NQK1:KW was inserted into pGEX-KG2 to produce pGEX-NQK1 and pGEX-NQK1:KW, respectively. For genetic analysis using yeast cells, Salt–NotI-digested PCR-amplified DNA fragments that included the cDNA for NQK1:KW or the cDNA for NQK1:SATA were cloned into a yeast expression vector, pN7V. For expression of His-T7-tagged recombinant proteins and proteins fused to GST in Escherichia coli BL21(DE3) and DH10B, respectively, and we purified recombinant proteins basically as described by Kitakura et al. (2002) using HiTrap Chelating HP (Amersham Pharmacia Biotech) and glutathione-Sepharose (Amersham Pharmacia Biotech).

Kinase assays in vitro and in gels

Recombinant NRK1 [250 ng] and recombinant NQK1 [50 ng] were incubated in 20 µL of kinase buffer [Nishihama et al. 2001] containing 50 µM ATP and 10 µCi of [γ-32P]ATP [Amersham Pharmacia Biotech] at 25°C for 30 min. Reactions were terminated by addition of 20 µL of 2x sample buffer for SDS-PAGE, and reaction mixtures were fractioned by SDS-PAGE. Radioactivity was visualized with an imaging analyzer (BAS-1800, Fuji Film). For kinase assays in gels, the kinase reaction was performed as described above but without [γ-32P]ATP. The reaction products were then separated by SDS-PAGE on polyacrylamide gels prepared with and without 1 mg/mL MBP [Sigma-Aldrich]. Renaturation of proteins and kinase reactions in the gel were then performed as described by Usami et al. [1995].

Production of antibodies and immunodetection of proteins

NQK1-specific antibodies [pQC-L antibodies] and NRK1-specific antibodies [pRN-R antibodies] were raised against the oligopeptides IDFGILVSSLEPPVNFPR [pQC] and MENETNEK-MRESG [pRN], respectively, which were synthesized by Sawady Technology. Rabbits were immunized and antibodies were purified as described previously [Nishihama et al. 2001]. Immunohotting was performed as described by Nishihama et al. [2001]. For detection of NACK1, NPK1, NQK1, and NRK1, we used specific affinity-purified antibodies (0.5 µg/mL) as described by Nishihama et al. [2001, 2002]. Monoclonal T7-specific antibodies [10,000-fold dilution] were purchased from Novagen.

Preparation for lysates of yeast cells and protein extracts of BY-2 cells

Lysates of yeast cells and protein extracts of BY-2 cells were prepared with TG150 buffer (25 mM Tris-HCl at pH 7.5, 10 mM
EDTA, 10 mM EGTA, 150 mM NaCl, 10% glycerol, 1 mM DTT, 20 mM β-glycerophosphate, 1 mM sodium-o-ovanadate, 1 mM NaF, and 1 mM PMSF, plus 5 μg/mL each leupeptin, pepstatin A, mostatin, pepstatin A, and antipain), as described previously [Nishihama et al. 2001]. Extracts of BY-2 cells for immunoprecipitation of NPK1 and NRK1 were prepared with TG150 buffer that had been supplemented with 1% Triton-X100 and 0.5% NP-40 and with PG150 buffer (TG150 buffer that contained 20 mM PIPES at pH 6.2 instead of Tris-HCl), respectively. Proteins in lysates of yeast cells and extracts of BY-2 cells were quantified with a protein assay kit (Bio-Rad) with BSA as the standard.

**Immunocomplex kinase assay**

Lysates of yeast cells and extracts of BY-2 cells (0.2 mg of total proteins) were incubated with T7-specific [1 µg], NPK1-specific [1 µg], NQK1-specific [1 µg], or GST-specific antibodies [1 µL, Sigma-Aldrich] at 4°C for 2 h. For immunoprecipitation of NRK1, extracts were incubated with NRK1-specific antibodies [1 µg] at 30°C for 2 h. Then mixtures were incubated with 20 µL of rProteinA-Sepharose beads (50% slurry, Amersham Pharmacia Biotech) at 4°C for 1 h. For precipitation of plant CDKs, extracts of BY-2 cells were incubated with 10 µL of p13SUC1-agarose beads (50% slurry, Upstate Biotechnology, Lake Placid, NY, USA) for 1 h at 4°C for 1 h. Beads were washed four times with 1 mL of the buffer used for extraction and twice with 1 mL of kinase buffer, and then they were incubated in 10 µL of kinase buffer that contained 50 µM ATP, 10 µCi of [γ-32P]ATP, and substrate proteins for 30 min at 25°C for 30 min. His-T7-NQK1:KW [1 µg], His-T7-NRK1:KW [1 µg], MBP [1 µg], and histone (2 µg, Sigma-Aldrich) were used as substrates for NPK1, NQK1, NRK1, and CDKs, respectively. Reactions were terminated by addition of 30 µL of 2x sample buffer for SDS-PAGE. Radioactivity was visualized with an imaging analyzer.

**Isolation of a T-DNA-tagged anq1 mutant**

We used PCR to screen 133,440 T-DNA-tagged Arabidopsis plants that had been generated at the Arabidopsis Knockout Facility at the University of Wisconsin in a search for an anq1 mutant, following the instructions at http://www.arabidopsis.biology.wisc.edu/Arabidopsis and using primers specific for the T-DNA left-border (JL-202), ATQS2 (5'-GGATTGAGAGAAAGCGT/H11032), and ATQSR2 (5'-TACTACTACGTCTTGAGATCTTGAAATG/H11032), respectively.

**Fluorescence microscopy and light microscopy**

All techniques for microscopic analysis were described previously [Nishihama et al. 2001; Tanaka et al. 2001]

**Acknowledgments**

We thank Professor K. Matsumoto (Nagoya University, Nagoya, Aichi, Japan) for providing yeast strains and Dr. S. Usami (Hiroskima University) for providing the BY-2 cDNA library. This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas (nos. 10182102 and 14036216) from the Japanese Ministry of Education, Science, Culture and Sports, by a grant from the Research for the Future Program of the Japan Society for the Promotion of Science, and by a grant from Program for Promotion of Basic Research Activities for Innovative Biosciences. R.N. was supported by Research Fellowships from the Japan Society for the Promotion of Science for Young Scientists.

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NQK1/NtMEK1 is a MAPKK that acts in the NPK1 MAPKKK-mediated MAPK cascade and is required for plant cytokinesis

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Genes Dev. 2003, 17:
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