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A MAP Kinase Cascade That Controls Plant Cytokinesis

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Several components of mitogen-activated protein kinase (MAPK) cascades have been identified in higher plants and have been implicated in cellular responses to a wide variety of abiotic and biotic stimuli. Our recent work has demonstrated that a MAP kinase cascade is involved in the regulation of cytokinesis in plant cells. The MAP kinase cascade in tobacco includes NPK1 MAPK kinase kinase, NQK1 MAPK kinase, and NRK1 MAPK, and its activation is triggered by the binding of NACK1/2 kinesin-like protein to the NPK1 MAPK kinase kinase at the late M-phase of the cell cycle. We refer to this cascade as the NACK-PQR pathway. In this review, we introduce a mechanism for the regulation of plant cytokinesis, focusing on the role of the NACK-PQR pathway.

Key words: plant cytokinesis, NACK1, NPK1 MAPKKK, NQK1 MAPKK, NRK1 MAPK.

Abbreviations: BFA, brefeldin A; DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein; KLP, kinesin-like protein; MAPs, MT-associated proteins; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MT, microtubule; NACK1, NPK1-activating kinesin-like protein; NPK1, nucleus- and phragmoplast-localized protein kinase.

Mitogen-activated protein kinase (MAPK) cascades are conserved as units of cellular signal transmission in all eukaryotes that have been examined to date. Each species has multiple MAPK cascades that link various extra- and intracellular stimuli to a wide range of cellular responses. In general, each cascade consists of at least three protein kinases, which mediate sequential phosphorylation reactions, as follows: a MAPK kinase kinase (MAPKKK) phosphorylates and activates a MAPK kinase (MAPKK), which, in turn, activates a MAPK by phosphorylation (*1*).

The availability of the complete sequence of the genome of the model dicot plant *Arabidopsis* allowed the identification of the entire set of components of MAPK cascades in this plant (*2*). *Arabidopsis* has close to twenty genes that might encode MAPKs (known as MPKs in this plant) that have TEY or TDY phosphorylation motifs in the region between kinase subdomains VII and VIII, which are structurally similar to typical motifs in animal and yeast MAPKs. *Arabidopsis* also has ten genes that appear to encode MAPKKs (known as MKKs in this plant). Nine of the putative MAPKKs have the consensus sequence S/T-X_5-S/T at their putative sites of phosphorylation. This consensus sequence is distinct from that in animal and yeast. Computational analysis predicts that more than 60 genes should encode MAPKKK homologs in the *Arabidopsis* genome (*2*, *3*). Since members of the plant MAPKKK family have diverged considerably in terms of the primary structures of their kinase domains, it is necessary to examine the biochemical and genetic characteristics of each of them in order to assign their positions in the various cascades. *Arabidopsis* is not the only higher plant that is known to have a number of putative homologs of components of MAPK cascades (*2*). Such components have been found in tobacco, alfalfa and maize, for example.

Plant MAPK cascades are associated with defense responses to pathogens, cellular responses to hormones, regulation of the cell cycle, and responses to abiotic stresses (for review, *4*). The subcellular localization and patterns of activation of Ntf6 MAPK in tobacco (*5*) and of MMK3 MAPK in alfalfa (*6*) suggest that these MAPKs might participate in cytokinesis. Some MAPK cascades also appear to be involved in the growth and differentiation of plant organs. For example, SIMK MAPK is required for the formation of root hairs in alfalfa (*7*) and YODA MAPKKK regulates the first cell fate decision in embryogenesis in *Arabidopsis* (*8*). However, identification of components of the predicted cascades remains incomplete, and modes of regulation and/or activation of these protein kinases have yet to be characterized.

We review here our recent studies of a MAPK cascade that is involved in the regulation of cytokinesis in plant cells. This MAPK cascade includes NPK1 MAPKKK, NQK1 MAPKK and NRK1 MAPK (Fig. 1A). We have also found a kinesin-like protein (KLP) that can activate the cascade through protein-protein interaction with NPK1.

Plant cytokinesis

Cytokinesis of plant cells occurs in the phragmoplast, a cytokinetic apparatus that consists mainly of microtubules (MTs) (for review, *9*). The phragmoplast is generated between the two daughter nuclei at a certain stage of anaphase and, in its equatorial zone, antiparallel bundles of MTs interdigitate at their plus ends (Fig. 1B) (*10*). Immediately after it has formed, the phragmoplast assumes a barrel-like shape that is loaded with MTs. Then it develops into a ring that expands centrifugally

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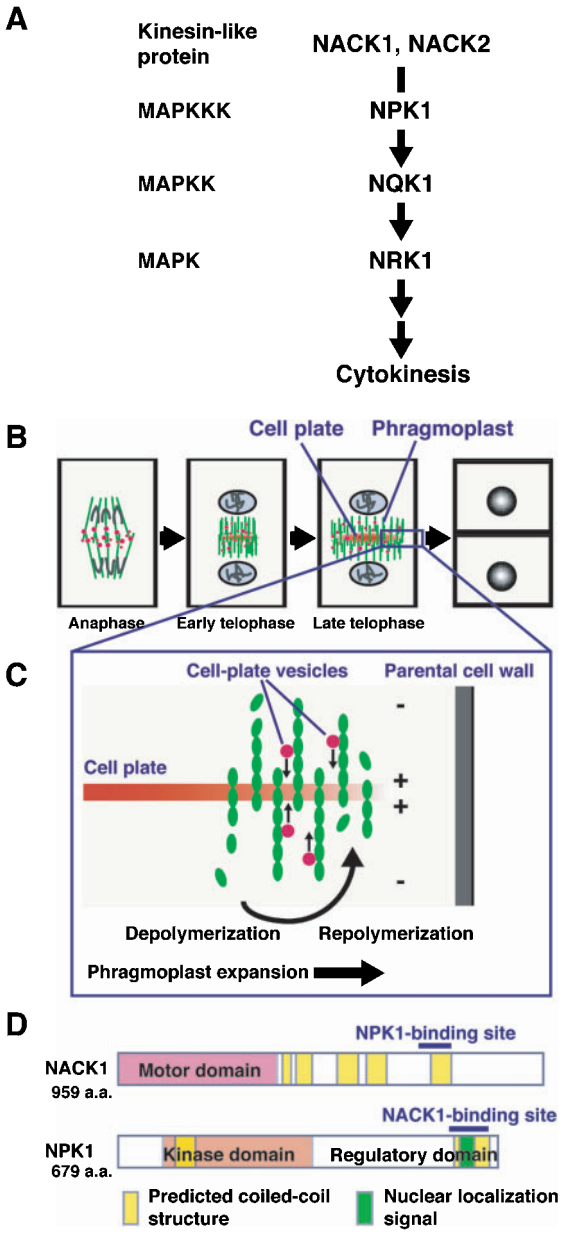

Fig. 1. **Plant cytokinesis.** (A) The NACK-PQR pathway. (B) A schematic diagram of plant cytokinesis. Spindle and phragmoplast MTs are drawn in green. Cell-plate vesicles are shown in red. (C) A schematic diagram of the expansion of the phragmoplast and cell plate. Disassembly of MTs at the inner edge of the phragmoplast and reassembly of tubulin at the outer edge allow the phragmoplast to expand centrifugally. MTs migrate towards their minus ends concomitantly with the polymerization of tubulin at their plus ends (*31*). (D) Schematic illustration of the domains and motifs in NACK1 and NPK1. The sites of coiled-coil structures (yellow) were predicted by the COILS program (*32*) and the nuclear localization signal (green) was identified by Ishikawa *et al.* (*24*).

until it reaches the cell wall of the parent cell. The expansion of the phragmoplast appears to involve disassembly of the bundles of MTs on the inside and polymerization of tubulin at the periphery. Treatment with taxol, a stabilizer of MTs, inhibits disassembly of the MTs and blocks centrifugal expansion of the ring (*11*). The blockage is thought to be a result of a reduction in the availability of tubulin molecules that is due to inhibition of the disassembly of MTs (*11*).

As the phragmoplast ring expands, the cell plate forms inside the ring (Fig. 1B), as a result of the fusion of Golgi body-derived vesicles (cell-plate vesicles) that contain components of the plate and migrate to the equatorial zone of the phragmoplast along the MTs (Fig. 1C) (for review, *12*). Brefeldin A (BFA; an inhibitor of the formation and movement of Golgi vesicles) inhibits the accumulation of the vesicles and the formation of the cell plate (*13*).

Treatment with BFA inhibits not only the accumulation of cell-plate vesicles, but also the expansion of the phragmoplast. Thus it appears that some mechanism might cooperatively control the formation of the cell plate, which involves the movement and fusion of cell-plate vesicles, and the centrifugal expansion of the phragmoplast, which involves the disassembly and reassembly of MTs (*14*). Caffeine prevents the phragmoplast from changing its initial barrel-like structure, and cells are generated with an incomplete cell plate (*15*). In the equatorial zone of the phragmoplast, fused vesicles become organized as a mesh via several intermediate stages, and caffeine inhibits passage from one of these stages to the next (*15*). The results of the caffeine treatment support the putative existence of a cooperative control mechanism.

The analysis of *Arabidopsis* mutants has clarified the involvement of multiple genes in formation of the cell plate. In mutants of these genes, cells with common features of incomplete cell plate and multiple nuclei can be observed. For example, the *KNOLE* and *KEULE* genes encode a syntaxin-like protein and Sec1-like protein, respectively, and mutations in these genes demonstrate that fusion and transportation of cell-plate vesicles are indispensable for cell-plate formation (*16*, *17*).

Nucleus- and phragmoplast-localized protein kinase (NPK1) MAPKKK controls formation of the cell plate

We identified NPK1 MAPKKK as a protein kinase that might play a role in the division of tobacco cells when we found transcripts of the *NPK1* gene in cultured cells at the logarithmic phase of growth specifically (*18*). The amino acid sequence of the amino- terminal half of NPK1 is 47% homologous to that of the kinase domain of STE11 MAPKKK from budding yeast. By contrast, the carboxy-terminal half has a novel amino acid sequence (Fig. 1D) (*18*). Deletion of the carboxy-terminal half increased the activity of the NPK1 protein kinase, indicating that this region contains an element that negatively regulates the activity of NPK1 (*18*, *19*)

The *NPK1* gene and its orthologs in *Arabidopsis*, namely, *ANP1*, *ANP2* and *ANP3*, are strongly expressed in organs that are rich in dividing cells (*20*, *21*). In tobacco BY-2 cultured cells, the transcript and the product of translation of the *NPK1* gene accumulate from the S-phase to the end of the M-phase of the cell cycle. The kinase activity of NPK1 increases from late anaphase to telophase (Fig. 2) (*22*). The mode of expression and the mode of activation of NPK1 are clearly compatible with a role for NPK1 in cell division.

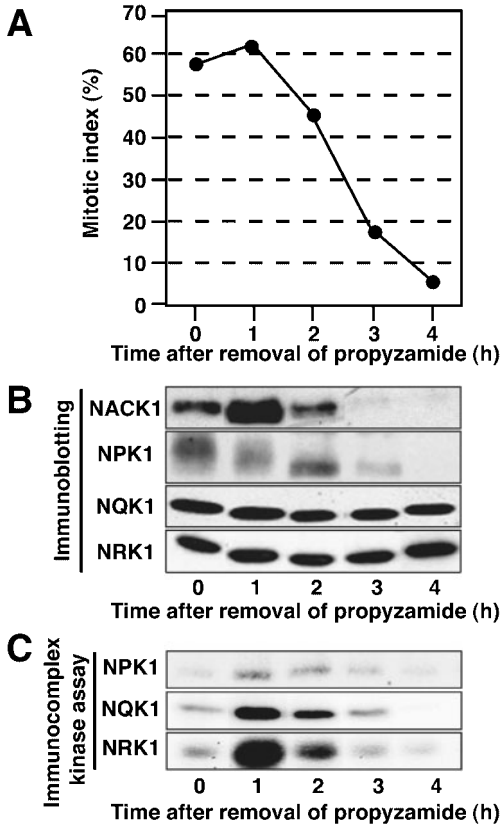

Fig. 2. **Patterns of activation of NPK1 MAPKKK, NQK1 MAPKK, and NRK1 MAPK at late M-phase.** (A) The graph shows proportions of mitotic cells at the indicated times after removal of propyzamide. The cell cycle of tobacco BY-2 cells was arrested at prometaphase by treatment with aphidicolin and propyzamide. The subsequent removal of propyzamide allowed the cell cycle to progress (*22*, *33*). (B) Patterns of accumulation of NPK1, NQK1, and NRK1, as determined by immunoblotting with antibodies specific for the respective proteins. (C) Activities of the various protein kinases, as analyzed by an immunocomplex kinase assay (for details, *22*, *27*). Note that the kinase activities of NPK1, NQK1, and NRK1 and the accumulation of NACK1 increased almost synchronously. Although it is clear that both NQK1 and NRK1 were present consistently throughout the cell cycle, NACK1 and NPK1 disappeared after the M-phase.

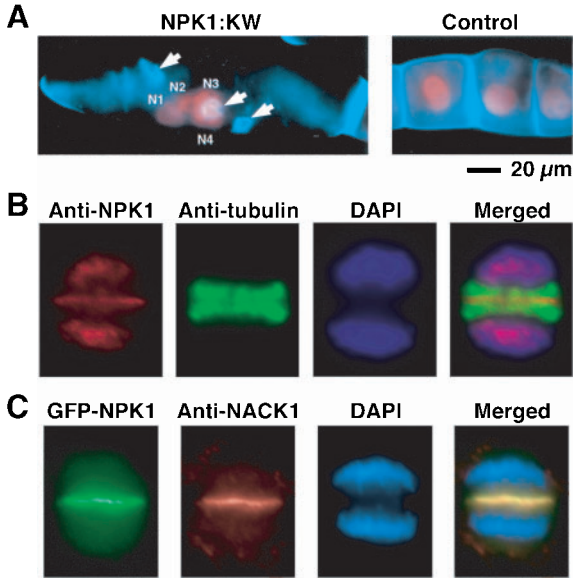

Fig. 3. **Microscopic observations of multinucleate BY-2 cells and the colocalization of NPK1 MAPKKK and NACK1 KLP.** (A) Generation of multinucleate cells with incomplete cell plates upon overexpression of NPK1:KW. Cell walls and nuclei are stained with calcofluor (blue) and propidium iodide (red), respectively. Arrows indicate incomplete cell plates. N1 through N4 indicate the nuclei found in a single cell. (B) Subcellular localization of NPK1 at telophase. BY-2 cells were triple-stained with NPK1-specific antibodies, tubulin-specific antibodies, and 4′,6-diamidino-2-phenylindole (DAPI). (C) Colocalization of NACK1 and NPK1 at telophase. Cells expressing GFP-NPK1 were stained with NACK1-specific antibodies and DAPI.

The function of NPK1 in cytokinesis was first revealed upon overexpression of a kinase-negative form of NPK1 (NPK1:KW). In this kinase-negative form, the lysine residue in the ATP-binding site of the kinase domain was changed to a tryptophan residue, and the protein was expected to have a dominant-negative effect. Overexpression of NPK1:KW in BY-2 cells suppressed the expansion of the cell plate and resulted in the formation of multinucleate cells with imperfect septa (Fig. 3A) (*22*). This phenotype resembles that of typical cytokinesis-defect mutants of *Arabidopsis*. Similar multinucleation has also been observed in *anp2 anp3* double-mutants (*23*). These observations suggest that NPK1 might positively control some step(s) in cytokinesis, in particular, some step(s) in the formation of the cell plate.

The subcellular localization of NPK1 also supports its involvement in cytokinesis. NPK1 includes a nuclear localization signal (Fig. 1D), and it is found in the nuclei of interphase cells (*24*). After breakdown of the nuclear membrane at the entry into the M-phase, the localization of NPK1 is much less clear. When the phragmoplast begins to form at the late anaphase, NPK1 is localized in its equatorial plane (Fig. 3B) (*22*). Analysis of fluorescence signals in BY-2 cells that expressed green fluorescent protein-fused NPK1 (GFP-NPK1) indicated that ring-like fluorescence grew towards the wall of the parental cell, reflecting the expansion of the phragmoplast as a doughnut-like structure. When a similar experiment was performed with GFP-NPK1:KW, the signal failed to expand all the way to the parental wall (*22*). These results showed that NPK1 was consistently localized in the equatorial zone of the phragmoplast during its lateral expansion and that this expansion required the kinase activity of NPK1.

A kinesin-like protein that controls the activity and localization of NPK1

To clone cDNAs for factors that might activate NPK1 MAPKKK, we took advantage of a yeast system that had been used to isolate the cDNA for a factor that stimulated the activity of Raf MAPKKK (*25*). Using this system, we isolated cDNAs for two proteins that increased the activity of NPK1. We named the genes that corresponded to these cDNAs *NACK1* (NPK1-activating kinesin-like protein 1) and *NACK2*, because they appeared to encode novel members of the kinesin superfamily (*26*). The amino acid sequences of the amino-terminal halves of

these proteins were strongly homologous to those of the motor domains of KLPs. However, the primary structures of the stalk and tail regions in the carboxy-terminal half were unique to NACK1 and NACK2. Computer-based analysis of the carboxy-terminal halves predicted that these regions should form several coiled-coil structures at similar positions (Fig. 1D). Such analysis also predicted that NPK1 MAPKKK should form a coiled-coil structure at its carboxy-terminal end (Fig. 1D). Biochemical experiments demonstrated that NPK1 was activated as a result of interaction with NACK1 and that this interaction was mediated by association between the coiled-coil structures of NACK1 and of NPK1 (*24*, *26*).

The NACK1 protein accumulates in the M-phase of the cell cycle, with the highest levels occurring at anaphase and telophase, when NPK1 is activated (*26*), and immunoprecipitation experiments confirmed that NPK1 binds NACK1 in tobacco cells. In addition, NACK1 is colocalized with NPK1 in the equatorial zone of the phragmoplast (Fig. 3B) (*26*). A mutant form of NPK1 that lacks the NACK1-binding site does not accumulate at the phragmoplast (*26*). In BY-2 cells, overexpression of truncated NACK1 that lacks the motor domain (NACK1:ST) interferes with the localization at the phragmoplast of endogenous NPK1. This observation can be explained if we assume that most of the endogenous NPK1 is bound to large amounts of NACK1:ST, which cannot move to the phragmoplast. It seems likely that NACK1 regulates both the activity and the subcellular localization of NPK1 during the M-phase. In populations of BY-2 cells that overexpress NACK1:ST, there are many multinucleate cells with incomplete cell plates (*26*). In these cells, time-lapse observations by Nomarski microscopy revealed that expansion of cell plates was markedly suppressed. This phenotype is identical to that of cells that overexpress NPK1:KW, indicating that localization of NPK1 in the phragmoplast is important for regulation of cell-plate formation by this kinase.

Factors that act downstream of NPK1

We isolated cDNAs for a MAPKK and a MAPK that might act downstream of NPK1 and named the gene products NQK1 and NRK1, respectively (Fig. 1A) (*27*). Biochemical analysis *in vitro* showed that NPK1 phosphorylates and activates NQK1 and that activated NQK1 then activates NRK1 by phosphorylation. Moreover, NQK1, NRK1 and NPK1 are all activated from anaphase to telophase during the cell cycle in tobacco (Fig. 2). In addition, NQK1 is required for the formation of cell plates, as demonstrated in BY-2 cells that expressed a kinase-negative mutant form of NQK1 and in mutants of the *Arabidopsis* ortholog of the *NQK1* gene (*ANQ1*) (*27*). Moreover, GFP-fused NQK1 is localized at the phragmoplast equator (Y.T. and Y.M., unpublished observation), and preliminary observations in our laboratory also indicate that a mutation in the gene for a MAPK (*ANR1*) that operates downstream of ANQ1 MAPKK is associated with defects in cytokinesis (T.S. and Y.M., unpublished observation). All these results together indicate that a MAP kinase cascade (designated the NACK-PQR pathway), which includes NPK1, NQK1 and NRK1, is activated by the interaction between NACK1 and NPK1 and might regulate formation of the cell plate.

The molecular role(s) of the NACK-PQR pathway, which is initiated by the formation of the NACK1/NPK1 complex, during cytokinesis

Formation of the cell plate can be divided into four stages: (*1*) the initiation of formation of the phragmoplast; (*2*) the transport of cell-plate vesicles to the division plane and fusion of these vesicles; (*3*) the expansion of the phragmoplast to the parental cell wall, which involves the turnover of microtubules (MT dynamics); and (*4*) synthesis of the cell wall inside the phragmoplast. When we consider the process(es) during cytokinesis that the NACK-PQR pathway might control directly, it seems unlikely that this pathway is related to the initial step in formation of the phragmoplast, since a phragmoplast is consistently observed in BY-2 cells that overexpress dominant inhibitory forms of NACK1 KLP, NPK1 MAPKKK, and NQK1 MAPKK, even though the phragmoplast fails to expand (*22*, *26*, *27*). In addition, such cells have immature cell walls, which can be stained with calcofluor (a stain specific for cellulose) and aniline blue (a stain specific for callose, which is present only in newly synthesized cell plates). Thus, it appears that synthesis of cell-wall components is not seriously affected by overexpression of these dominant inhibitory proteins.

A clue to the molecular role of the pathway might be found in the sites of subcellular localization of NACK1, NPK1, and NQK1. As noted above, these proteins are localized at the periphery of the phragmoplast equator, at sites at and/or around the sites at which the turnover of MTs occurs and cell-plate vesicles are fused. Therefore, this pathway might be involved in either process. The phenotypes induced by the overexpression of the dominant inhibitory proteins resemble those of cells treated with a taxol, which blocks the depolymerization of MTs (*11*), suggesting that the NACK-PQR pathway might be involved in expansion of the cell plate through control of MT dynamics. Strompen *et al.* reported that, in mutants of an *Arabidopsis* ortholog of NACK1 (*atnack1/hinkel*), MTs that normally disassemble inside the phragmoplast remain intact (*28*). This observation is consistent with the hypothesis that the NACK-PQR pathway might regulate MTs dynamics inside the phragmoplast. However, it remains to be determined whether vesicle fusion is controlled by this pathway.

Perspectives

Results from our laboratory and another have shown that NACK1 KLP and NPK1 MAPKKK are indispensable for cytokinesis in plant cells: double-mutants of *Arabidopsis* homologs of *NACK1* and *NACK2* and triple-mutants of *Arabidopsis* homologs of *NPK1* are gametophyte lethal (H. Tanaka and Y.M., unpublished observation; *23*). However, requirements for other components of this pathway in cytokinesis remain to be examined, since single mutants of ANQ1 MAPKK and ANR1 MAPK are not lethal in spite of the presence of severe defects in cytokinesis. Since the *Arabidopsis* genome appears to encode 10 MAPKKs and 20 MAPKs, including ANQ1 and ANR1, respectively, other members of the corresponding families might function redundantly with respect to ANQ1 and ANR1 in *Arabidopsis* plants. It will be interesting to determine how many MAPK cascades are involved in the progression of plant cytokinesis.

In this review, we have proposed that a MAPK cascade, designated the NACK-PQR pathway, plays a role in regulating the MT dynamics of the phragmoplast. We do not yet know how this pathway regulates MT dynamics. Preliminary results in our laboratory suggest the involvement of this cascade in the depolymerization of MTs in plant cells. Some MT-associated proteins (MAPs) might be reasonable target(s) of the NACK-PQR pathway. Recently, the AtMAP65–3/PLE of *Arabidopsis* was shown to be involved in the regulation of cytokinesis (*29*). In addition, the MAP215 family (XMAP215 in frog, ch-TOGp in mammals, Dis1 in fission yeast, and MOR1/GEM1 in *Arabidopsis*) and Kin I KLPs (XKCM1 in frog and MCAK in mammals) have been shown to control the stabilization and destabilization of MTs, respectively (for review, *30*). We look forward to investigations of the relationship between the role(s) of the NACK-PQR pathway and these MAPs, since the *Arabidopsis* genome encodes homologs of such proteins. We are also interested in the biological relevance of the involvement of the MAPK cascade in the turnover of MTs. Studies of these kinase cascades will shed much light on the processes that control the division of plant cells.

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