Control of plant cytokinesis by an NPK1-mediated mitogen-activated protein kinase cascade

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Cytokinesis is the last essential step in the distribution of genetic information to daughter cells and partition of the cytoplasm. In plant cells, various proteins have been found in the phragmoplast, which corresponds to the cytokinetic apparatus, and in the cell plate, which corresponds to a new cross wall, but our understanding of the functions of these proteins in cytokinesis remains incomplete. Reverse genetic analysis of NPK1 MAPKKK (nucleus- and phragmoplast-localized protein kinase 1 mitogen-activated protein kinase kinase kinase) and investigations of factors that might be functionally related to NPK1 have helped to clarify new aspects of the mechanisms of cytokinesis in plant cells. In this review, we summarize the evidence for the involvement of NPK1 in cytokinesis. We also describe the characteristics of a kinesin-like protein and the homologue of a mitogen-activated protein kinase that we identified recently, and we discuss possible relationships among these proteins in cytokinesis.

Keywords: plant cytokinesis; NPK1 MAPKKK; kinesin-like protein

1. INTRODUCTION

Cell division is essential for all living organisms. The cell division cycle includes the S phase and the M phase, which are essential because duplication of sets of chromosomes and separation of the sets of chromosomes and cell components to two daughter cells occur during these phases, respectively. Cytokinesis is a crucial step in the partition of cellular components during the M phase. Compared with earlier steps in the cell cycle, such as DNA replication and the separation of chromosomes, which have been relatively well conserved, modes of cytokinesis have diverged considerably in plants and animals (Field *et al.* 1999). In animal cells, cytokinesis is achieved by constriction, namely an 'outside-in' phenomenon. A contractile ring of actin and myosin, which is anchored to the cell cortex, is critical for constriction. By contrast, cytokinesis in plant cells occurs in the opposite direction (Gunning 1982; Staehelin & Hepler 1996; Heese *et al.* 1998; Smith 1999). At late anaphase, a plant-specific and complex array of MTs and microfilaments, known as the phragmoplast, is formed at the mid-zone of the spindle in the centre of the cell. Anti-parallel MTs interdigitate at their 'plus' ends in the equatorial zone of the phragmoplast. Fusion of Golgi-derived vesicles, which are probably transported along the MTs toward this zone, generates a membranous network, which eventually matures into a new cross wall that is known as the cell plate. The growth

of the cell plate is accompanied by the lateral expansion of the phragmoplast, which is achieved by translocation of phragmoplast MTs and which allows fusion of vesicles to occur continuously at the periphery of the cell plate, with a resultant 'inside-out' mode of cytokinesis.

MAPK cascades were originally identified as protein phosphorylation systems that control the division and growth of yeast and animal cells. Subsequently, such cascades were found to be distributed ubiquitously among all eukaryotes, including plants. These cascades consist, in general, of three types of protein kinase: MAPKs, MAPKKs and MAPKKKs. MAPKKKs activate the respective downstream MAPKKs by phosphorylation and these, in turn, activate their own downstream MAPKs. The amino acid sequences of the respective protein kinases that act at each step have been strongly conserved. In addition, multiple distinct MAPK cascades are found in a single species, and this multiplicity of cascades has led to the general hypothesis that eukaryotic cells exploit related, but functionally different, MAPK cascades that are involved in a variety of cellular processes, such as the response to osmotic stress and apoptosis (for reviews, see Herskowitz 1995; Robinson & Cobb 1997; Widmann *et al.* 1999).

Many of homologues of components of MAPK cascades have been identified in higher plants (Hamal *et al.* 1999; Jouannic *et al.* 1999; Machida *et al.* 1997; Mizoguchi *et al.* 1997; Nishihama *et al.* 1995), and a wide variety of stimuli, including wounding, cold stress, osmotic imbalance, mechanical stimulation and attack by pathogens, have been shown to activate MAPKs (Seo *et al.* 1995; Suzuki & Shinshi 1995; Usami *et al.* 1995; Jonak *et al.* 1996; Bögre *et al.* 1997; Ligterink *et al.* 1997; Ichimura *et al.*

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al. 2000; Zhang *et al.* 1998; Zhang & Klessig 1998*a,b*; Munnik *et al.* 1999; Romeis *et al.* 1999). Recent biochemical studies *in vitro* have established relationships between some MAPKs (e.g. AtMPK4, SIPK, WIPK and SIMK) and the respective MAPKKs (AtMEK1, NtMEK2 and SIMKK) (Kiegerl *et al.* 2000; Huang *et al.* 2000; Yang *et al.* 2001). In addition, genetic analysis of mutants of *Arabidopsis* has shown that AtMPK4 negatively regulates systemic acquired resistance to pathogen attack (Petersen *et al.* 2000), and that EDR1, a putative MAPKKK, is also a negative regulator of the defence response that is induced by salicylic acid (Frye *et al.* 2001). However, current evidence for a MAPK cascade that controls the proliferation of plant cells is restricted to the demonstration, by our group, of the involvement of NPK1 MAPKKK in the formation of the cell plate, as discussed below (Nishihama *et al.* 2001).

In yeast and animal cells, MAPK cascades influence various aspects of cell division, namely: entry into the cell cycle, control of nucleotide biosynthesis, transition from the G2 to the M phase, fragmentation of the Golgi apparatus at the M phase, and the so-called spindle assembly checkpoint (Pages *et al.* 1993; Minshull *et al.* 1994; Acharya *et al.* 1998; Takenaka *et al.* 1998; Wright *et al.* 1999; Graves *et al.* 2000). The localization of MAPKs to the spindle mid-zone and the mid-body of animal cells (Shapiro *et al.* 1998; Zecevic *et al.* 1998) and to the phragmoplast of plant cells (Calderini *et al.* 1998; Bögre *et al.* 1999) suggests that cytokinesis might be controlled by an MAPK cascade. However, our understanding of the role of the MAPK pathways in plant cytokinesis is very limited. To gain further insight into such pathways, both genetic analysis of mutants with altered components of these pathways and biochemical and cell biological analysis is clearly essential.

The *NPK1* gene from tobacco encodes a member of the MAPKKK family and the kinase domain of NPK1 can replace the functions of several yeast MAPKKKs (Banno *et al.* 1993; Machida *et al.* 1998). The *NPK1* gene and its homologues in *Arabidopsis* are transcribed in proliferating and division-competent cells in meristematic tissues and at the periphery of such tissues, an observation that suggests that they play a role in cell division (Banno *et al.* 1993; Nishihama *et al.* 1997; Nakashima *et al.* 1998). In a recent study, we obtained evidence for the involvement of NPK1 in cytokinesis in tobacco cells (Nishihama *et al.* 2001). During our efforts to identify factors that might increase the activity of NPK1, we isolated a tobacco cDNA for a protein (designated NACK1) that is a novel member of the family of kinesin-like proteins and accumulates only at the late M phase of the cell cycle (Nishihama *et al.* 2002). Genetic and biochemical analysis of NACK1 revealed that it is associated with NPK1 and increases the ability of NPK1 to activate MAPKKs in yeast cells. Both of NPK1 and NACK1 were consistently co-localized at the equatorial zone of the phragmoplast from anaphase to late telophase. The mutant construct of NPK1 lacking the NACK1-binding site was not co-localized with NACK1. Overexpression of the kinase-negative NPK1 resulted in generation of multinucleate cells with aberrant cell walls. Thus, these proteins appear to act together as regulators of cytokinesis in tobacco cells. In the present review, we shall summarize the results of recent investigations of

NPK1 and NACK1 and we shall consider the processes that might be controlled by two proteins. In addition, we describe the characteristic activation of a tobacco MAPK that we identified recently as a factor that acts downstream of NPK1. We shall discuss the possible involvement of this MAPK in cytokinesis and, on the basis of our observations, we shall propose a model wherein the NACK1/NPK1 system is essential for the control of cytokinesis in plant cells.

2. CYTOKINESIS OF PLANT CELLS REQUIRE NPK1

Recent studies have provided four lines of evidence for involvement of NPK1 MAPKKK in formation of the cell plate (Nishihama *et al.* 2001), as follows: (i) the activity of NPK1 increases during the late M phase; (ii) NPK1 is localized at the equatorial zone of the expanding phragmoplast; (iii) expression of a kinase-negative mutant of NPK1 resulted in the generation of multi-nucleate cells with aberrant cell plates; and (iv) phragmoplasts generated in the presence of mutant NPK1 ceased to expand toward the cell cortex.

(a) *Structural characteristics of NPK1*

Figure 1*a* is a schematic representation of the domain and motif organization of NPK1. NPK1 consists of essentially two domains, with a short kinase-unrelated region at the N-terminus. The KD, with catalytic activity, is in the N-terminal half and is structurally similar to the kinase domain of MAPKKKs, whereas the RD is in the C-terminal half, whose amino-acid sequence is found only in plant homologues of NPK1. The C-terminal region of the RD contains short amino acid residues that are similar to those of known functional motif, such as a NLS and consensus sequences for sites of phosphorylation by the Cdc2 kinase (indicated by Cdc2 in figure 1*a*; Nishihama *et al.* 1997). In addition, this region contains a putative coiled-coil structure, as predicted at high probability by the COILS program (Lupas *et al.* 1991). This region is the binding site of the NACK1 kinesin-like protein (see § 1 and below). Thus, the RD, and in particular the C-terminal region, has motifs that seem to be important for the functions of NPK1.

(b) *NPK1 activity increases in late M phase of the cell cycle*

The BY-2 line of tobacco cells that can be grown in suspension culture is useful for studies on the division of plant cells because the cell cycle can be synchronized under optimal conditions. To detect the activity of NPK1 during the M phase of the cell cycle, we performed an immunocomplex kinase assay using NPK1-specific antibodies for immunoprecipitation and a kinase-negative mutant of a MAPKK as the substrate protein (Nishihama *et al.* 2001). This MAPKK was recently identified as a factor that acts downstream of NPK1 (designated NQK1; T. Soyano, K. Morikiyo, R. Nishihama and Y. Machida, unpublished data). The cell cycle of BY-2 cells was first synchronized by treatment with aphidicolin, an inhibitor of DNA polymerase α , and the removal of this drug, and then arrested at prometaphase of the M phase by the treatment of propyzamide, an MT-depolymerizing drug, and then cells were released from arrest by removal of the

Figure 1. Evidence for a role for NPK1 in cytokinesis. (a) Domain organization and critical regions in the NPK1 protein. See text for organization and critical regions of NPK1 protein. (b) Activity and accumulation of NPK1 during the M phase of the cell division cycle of BY-2 cells. The cell cycle of BY-2 cells was arrested at prometaphase by treatment with propyzamide after release of aphidicolin block. At the indicated times after the removal of propyzamide, cells were harvested and proteins were extracted. Mitotic index (top, bars) indicates that the cell cycle progressed through anaphase (1 h), telophase (1–2 h) and the next G1 phase (after 2 h) after the removal of propyzamide. Kinase activities of NPK1 were measured by an immunocomplex kinase assay with recombinant histidine-tagged kinase negative NQK1 (His-NQK1KW) as the substrate (top, open circles; middle). Western blotting (bottom) with NPK1-specific antibodies, which were raised against a synthetic peptide (see a). (c) Subcellular localization in BY-2 cells of GFP-NPK1 during the late M phase of the cell cycle. BY-2 cells were transformed with a *GFP-NPK1* gene driven by a DEX-inducible promoter. Transformed cells were cultured with 0.1 μ M DEX for 24 h. Nuclei and MTs were double-stained with DAPI (blue) and mouse antibodies against α -tubulin, respectively. MTs were visualized with Texas red-conjugated antibodies raised against mouse IgG (red). Fluorescence due to GFP is apparent in green. Merged images are shown on the right. (d) Generation of multinucleate cells upon expression of kinase-negative derivative of NPK1. Transformed BY-2 cells in which synthesis of the kinase-negative derivative of NPK1 was driven by a DEX-inducible promoter were subcultured in the absence of 0.1 μ M DEX ((i), -DEX) or in the presence of DEX ((ii), +DEX). Three days after the onset of culture, the cells were stained with orcein. Tetra- and bi-nucleate cells were found. Scale bar, 50 μ m for (d).

drug. As the cell cycle progressed through anaphase (1 h), telophase (1–2 h), and the next G1 phase (after 2 h), the mitotic index decreased within 2 h after the removal of propyzamide. At various times after they were released from the prometaphase arrest, we prepared extracts of the synchronized cells, isolated immunocomplexes, and measured the activity of NPK1 in the immunocomplexes using the substrate MAPKK protein. Figure 1b shows that the activity of NPK1 increased transiently 1–2 h after the removal of propyzamide, at a time that corresponded with late anaphase and telophase. The amount of NPK1 protein fell to below the limit of detection with the rapid decrease in the mitotic index (3–4 h). During this period, the state of NPK1 protein changed dramatically. At prometaphase (time 0), NPK1 was strongly phosphorylated, and phosphorylation caused a decrease in mobility during electrophoresis on a polyacrylamide gel. Dephosphorylation of NPK1 proceeded as the cell cycle progressed

(figure 1b; Nishihama *et al.* 2001). Our data suggest that NPK1 was extensively phosphorylated at least at prometaphase and that its activity increased after some of the phosphate groups had been removed. It is likely that NPK1 kinase that has been phosphorylated to a certain extent might have elevated kinase activity.

(c) *NPK1 is concentrated at the equatorial zone of the phragmoplast at late M phase*

The subcellular localization of NPK1 during the M phase supports the hypothesis that NPK1 functions in cytokinesis. Immunofluorescence analysis of endogenous NPK1 with NPK1-specific antibodies and GFP-NPK1 revealed the presence of NPK1 in the nucleus at prophase before breakdown of the nuclear envelope, with distribution in the cytoplasm at metaphase (Nishihama *et al.* 2001). No distinct subcellular localization of NPK1 was detected at metaphase. However, at anaphase, NPK1 was

widely distributed around the spindle midzone between the two sets of chromosomes and around spindle poles. Moreover, no NPK1 was detected on chromosomes (figure 1c(i)). When cells at early telophase began to form a phragmoplast, NPK1 was localized on the equatorial plane of the phragmoplasts (figure 1c(ii)). During expansion of the phragmoplast, from early to late telophase, NPK1 was consistently localized in the MT-interdigitating region of the ring-shaped phragmoplast and was also redistributed to the newly forming daughter nuclei (Nishihama *et al.* 2001).

(d) Expression of kinase-negative NPK1 results in multinucleate cells with aberrant cell plates

To investigate the function of NPK1 in further detail, we adopted a dominant-inhibitory strategy using a kinase-negative mutant of NPK1 because, as in the case of kinase-negative mutants of other MAPKKKs (Posas & Saito 1998; Takekawa & Saito 1998; Sakurai *et al.* 1999), we anticipated that overexpression of the mutant NPK1 might interfere with the proper association of endogenous NPK1 with NACK1, with potential resultant inhibition of the NPK1-mediated signalling pathway. We made a mutant cDNA construct that encoded a kinase-negative derivative of NPK1, designated NPK1KW, with replacement of a lysine residue (K) by a tryptophan residue (W) in the ATP-binding region (as shown in figure 1a). We manipulated the cDNA construct such that its expression was under the control of the DEX-inducible transcription system (Aoyama & Chua 1997) and we then introduced it into BY-2 cells. We examined the effects of expression of the cDNA that encoded NPK1KW in the transformed BY-2 cells on the progression of the cell cycle. When the transformed BY-2 cells were cultured in the presence of DEX, multinucleate cells appeared at a significant level (tetranucleate cells are shown in figure 1d(ii)). No such abnormal cells were observed in the absence of DEX or in the population of control BY-2 cells that had been transformed with the empty vector, in the presence and in the absence of DEX (figure 1d(i); Nishihama *et al.* 2001).

All the multinucleate cells that were induced upon expression of NPK1KW had incomplete cell plates with disc-like or stub-like structures instead of an authentic cell plate. These malformed structures could be stained with calcofluor, which interacts with β -glucans in cell walls, or with aniline blue, which interacts with callose that accumulates exclusively in newly synthesized cell plates after fusion of Golgi-derived vesicles. These observations suggest that NPK1KW might not inhibit the initial step(s) in the formation of the cell plate, such as fusion of vesicles at the early stage, but might rather affect progression of the phragmoplasts toward the outer margins of the cell surfaces.

(e) Expression of NPK1KW inhibits expansion of the phragmoplast

We examined whether expression of NPK1KW could inhibit the expansion of the phragmoplast. In these experiments, we analysed signals due to the fluorescence of GFP on phragmoplasts in living cells in which GFP-NPK1 or GFP-fused NPK1KW was expressed. We postulated that the extent of fluorescent signals from these GFP-fused proteins might reflect the extent of the phragmoplast

because dimensions of phragmoplasts in the equatorial direction were roughly equal to those of bands of NPK1 or the GFP signals. In cells that express GFP-NPK1, fluorescent signals extended toward the peripheries of the cells during the first 30 min and reached plateau values. The fluorescent signal was finally recorded as a ring-like structure. By contrast, the signal in cells that expressed GFP-NPK1KW barely expanded at all. This time-lapse analysis of the behaviour of fluorescence due to GFP in living cells demonstrated directly that phragmoplast-localized GFP-NPK1KW inhibited the lateral expansion of the phragmoplast.

3. NACK1 KINESIN-LIKE PROTEIN IS RESPONSIBLE FOR THE SUBCELLULAR LOCALIZATION AND ACTIVATION OF NPK1 MAPKKK

We attempted to identify the activator(s) of NPK1 MAPKKK. In spite of the conservation of structure that is apparent among components of MAPK cascades, various proteins that differ in terms of amino acid sequence and biochemical function have been shown to control the activation of MAPKKKs. Some of these proteins in animal and yeast systems act via protein-protein interactions: for example, the interaction between the small G protein Ras (Farrar *et al.* 1996; Luo *et al.* 1996) or the 14-3-3 protein and Raf MAPKKK (Irie *et al.* 1994), TAB1 and TAK1 MAPKKK (Shibuya *et al.* 1996), SSK1 response regulator of the two-component regulatory system and SSK2 MAPKKK in the yeast cell (Posas & Saito 1998). STE20 and its related protein kinases also activate yeast cell STE11 MAPKKK and MEKK1 MAPKKK of animals by phosphorylation of the latter kinases, respectively (Wu *et al.* 1995). No information is, however, available about factors that activate MAPKKKs in plant systems.

To clone cDNAs for factors that might activate the NPK1 protein kinase, we took advantage of a cloning system using yeast cells that had been used to isolate cDNA for a stimulator of the activity of Raf MAPKKK (Irie *et al.* 1994). This system is based on suppression of a mutation in the *STE11* gene for MAPKKK by the combined effects of a MAPKKK and its potential activator which are introduced into yeast cells from a heterologous organism. We first transformed yeast *ste11* mutant cells with the cDNA for a yeast promoter-fused NPK1 construct and then we introduced a tobacco cDNA library, which had been generated with a yeast gene expression vector, into the yeast cells. We then looked for yeast cells in which the STE11-mediated MAPK cascade was activated upon expression of both NPK1 and introduced cDNA(s). Using this procedure, we isolated two tobacco cDNA clones in our search for factors that increased the activity of NPK1 in the yeast cells. These clones appeared to encode novel members of the kinesin superfamily of MT-based motor proteins since the amino acid sequences of the N-terminal halves of the deduced protein were homologous to those of the motor domains of KLPs. The genes that corresponded to these cDNAs were named *NACK1* and *NACK2* (for NPK1-activating kinesin-like proteins). The amino acid sequences of the predicted motor domains in the N-terminal halves of NACK1 and NACK2 were strongly homologous to each other (68%

identity) while the remaining stalk and tail regions in the C-terminal halves exhibited less extensive homology (52%). However, computer-based analysis with the COILS program (Lupas *et al.* 1991) predicted that the probability is high that the C-terminal halves of both proteins form coiled-coil structures at the same positions. We characterized the protein encoded by *NACK1* cDNA in further detail, as follows.

We first examined biochemically whether NACK1 could associate with NPK1 and increase the activity of NPK1. We generated NPK1 and NACK1 in yeast cells, prepared a cell extract and performed a co-immunoprecipitation assay with NPK1-specific antibodies. The isolated immunocomplex contained NACK1 and the activity of NPK1 in the complex was markedly higher than the activity of the NPK1 produced in yeast cells in the absence of NACK1. These results indicated that NACK1 interacts with NPK1 and that the activity of NPK1 increases as a consequence of such an interaction. We also demonstrated direct binding of NPK1 to NACK1 *in vitro* using recombinant proteins that had been produced in *Escherichia coli*. A search for binding sites that would allow interactions between the proteins revealed that the C-terminal region of NPK1 interacted with the middle region of the C-terminal half of NACK1, and this region corresponds to one of the predicted coiled-coil structures.

Patterns of accumulation of transcripts of the *NPK1* and *NACK1* genes during the cell cycle of suspension-cultured BY-2 tobacco cells were examined, together with the pattern of accumulation of transcripts of the *NRK1* gene, which was shown recently to encode a tobacco MAPK that acts downstream of NPK1 (see figure 3*b*; T. Soyano, R. Nishihama and Y. Machida, unpublished data). As shown in figure 2, transcripts of *NACK1* accumulated only at the M phase of the cell cycle while transcripts of *NPK1* and *NRK1* were detected from the S phase to the M phase, with levels decreasing at the end of the M phase. The pattern of accumulation of the *NACK1* transcript was similar to that of NACK1 protein though the peak level of the protein was seen at the late M phase (data not shown). The patterns of accumulation of *NPK1* and *NRK1* transcripts were essentially similar to those of the corresponding proteins. Thus, the patterns of accumulation of the *NACK1* transcript and of the NACK1 protein were consistent with the increase in the activity of NPK1 at the late M phase (see figure 1*b*).

The results of our immunofluorescence analysis indicated that NACK1 was localized in the equatorial zone of the phragmoplast from anaphase to telophase of the cell cycle in tobacco BY-2 cells. NPK1 was also found in the same region of the phragmoplast at the late M phase (Nishihama *et al.* 2001). In a recent study, we found that deletion of the NACK1-binding site in the C-terminal region of NPK1 prevented its localization to the equator of the phragmoplast (data not shown). This observation suggests that NACK1 might be responsible for the localization of NPK1 to this region, where cytokinesis proceeds, as well as for the activation of NPK1.

As described above, overexpression of the kinase-negative derivative of NPK1 in BY-2 cells interrupted formation of the cell plate, with generation of multi-nucleate cells with aberrant cell walls. This effect might have been due to interference by the mutant NPK1 with the proper

Figure 2. Accumulation of transcripts of the *NACK1*, *NPK1* and *NRK1* genes during tobacco cell cycle. The cell cycle of the BY-2 cells was arrested at the G1–S boundary by treatment with aphidicolin and released by washing out the drug. At the indicated times after the removal of aphidicolin, aliquots of the cell culture were harvested and total RNA was prepared. Total RNA (20 µg) was analysed by Northern blotting with 32 P-labelled probes specific for transcripts of the *NACK1*, *NPK1* and *NRK1* genes. (a) Mitotic index. (b) Northern blots.

association of endogenous wild-type NPK1 with NACK1. We are currently attempting to investigate whether overexpression of a truncated form of NACK1 that lacked the motor domain of NACK1 might have a similar phenotypic effect on cytokinesis. The results of such an examination will provide information on the functional relationship between NACK1 and NPK1 in plant cytokinesis.

4. MOLECULAR ASSOCIATION OF NACK1 WITH NPK1 LEADS TO ACTIVATION OF THE NRK1 MAPK

Using a yeast genetic system, we have shown recently that expression of a NQK1 cDNA replaced the function of the yeast MAPKK in the presence of an active form of NPK1. Recombinant NQK1 protein was phosphorylated by the active form of NPK1 (details to be published elsewhere). These results suggest that NQK1 is a member of the MAPKK family that can act downstream of NPK1 (see figure 3*b*).

As described above, *NRK1* transcripts accumulated throughout the cell cycle (figure 2). We examined the pattern of activation of NRK1 during the cell cycle. We arrested the cell cycle of BY-2 cells at prometaphase by treating them with aphidicolin and propyzamide and then allowed them to start dividing again by washing away pro-

Figure 3. Activity of NRK1 MAPK during the M phase of the tobacco cell cycle. (a) The cell cycle of BY-2 cells was arrested at prometaphase by treatment with aphidicolin and propyzamide (see figure 1b). At the indicated times after removal of propyzamide, aliquots of the culture were collected and cell extracts were prepared. Kinase activity of NRK1 was determined by the immunocomplex kinase assay with MBP as the substrate. Mitotic index is shown below activity. (b) Hypothetical representation of the NPK1-mediated pathway that regulates some aspect(s) of cytokinesis in plant cells. Kinase activity and the subcellular localization of NPK1 are regulated by direct binding to NACK1. Unknown signals (?) upstream of NACK1 might be required for the association of NACK1 with NPK1. NPK1 kinase controls formation of the cell plate. The signal pathway includes NQK1 MAPKK and NRK1 MAPK, which act downstream of NPK1, as recently demonstrated in our laboratory (T. Soyano, K. Morikiyo, R. Nishihama and Y. Machida, unpublished data; NQK1 is not described in the present article). Since the involvement of NQK1 and NRK1 in cytokinesis remains to be established, the relationships between these factors are represented by pale grey arrows.

pyzamide. We prepared cell extracts at various times after removal of the drug and performed an immunocomplex kinase assay of NRK1 kinase with MBP as the substrate. Figure 3a shows that the phosphorylation by NRK1 of MBP increased 1.5 h and decreased 3.5 h after removal of the drug. The profile of activation was similar to that of NPK1 (figure 1) and to the pattern of accumulation of *NACK1* transcripts (figure 2). These results therefore support the hypothesis that activation of NPK1 MAPKKK by NACK1 results in activation of NRK1 MAPK, probably via activation of NQK1 MAPKK (figure 3b).

Although the activation of NRK1 is tightly coupled with the accumulation of *NACK1* transcripts and the activation of NPK1, involvement of NRK1 in the formation of the cell plate remains to be confirmed. We have made various mutant constructs of *NRK1* cDNA and investigations of

the effects of expression of the mutant proteins on the cell cycle of BY-2 cells are in progress in our laboratory.

5. THE NACK1/NPK1 SYSTEM MIGHT BE UNIQUE TO PLANT CYTOKINESIS AND MIGHT BE REQUIRED FOR THE 'INSIDE-OUT' TRANSLOCATION OF PHRAGMOPLAST MICROTUBULES

Our results demonstrate that NACK1 KLP and NPK1 MAPKKK constitute a regulatory system (the NACK1–NPK1 system) that controls cytokinesis of plant cells from inside the phragmoplast. It is not clear, however, which particular cytokinetic event(s) is/are directly controlled by this system. The formation of the cell plate involves three mutually dependent processes (Heese *et al.* 1998) as follows: (i) transport of Golgi-derived vesicles along phragmoplast MTs; (ii) fusion of the vesicles in the interdigitating regions of MTs; and (iii) outward redistribution of phragmoplast MTs. Inhibition of any one of these processes, by specific drugs or by mutations in genes that are required for the processes, as described above, prevents the expansion of the cell plate (Yasuhara *et al.* 1993; Lukowitz *et al.* 1996; Yasuhara & Shibaoka 2000; Assaad *et al.* 2001). For example, mutations in the *KNOLLE* gene and *KEULE* gene of *Arabidopsis*, which encode homologues of syntaxin and Sec1, respectively, proteins that are involved in vesicle fusion in other systems, result in the generation of multi-nucleate cells with incomplete cell walls. Since we also observed similar abnormal phenotypes when kinase-negative NPK1 was overexpressed in tobacco cells, it seems likely that the NACK1–NPK1 system might be involved in one of these processes.

Although we cannot yet specify the process(es) in which NPK1 is involved, the ring-like distribution of NPK1 in the equatorial zone of the phragmoplast might provide a clue. In addition, aberrant cell plates created upon expression of kinase-negative NPK1 contained callose and the deposition is shown to be detected only after fusion of vesicles (Samuels *et al.* 1995). This observation suggests that such expression still allows occurrence of vesicle fusion. Therefore, it is likely that the system that includes NACK1 KLP and NPK1 MAPKKK might function in the translocation or redistribution of phragmoplast MTs. Further experiments are, however, needed to identify the process(es) that is/are directly controlled by the NACK1–NPK1 system.

Our recent examination of NACK1-interacting molecules revealed that the stalk region of NACK1 can interact with various proteins in addition to NPK1 (M. Ishikawa, R. Nishihama and Y. Machida, unpublished data). However, NQK1 and NRK1 were not associated with NACK1 but might have associated with NPK1. In addition, transgenic tobacco plants that overproduced the stalk region of NACK1 exhibited more severe changes in phenotype than those that overproduced kinase-negative NPK1. These observations imply that NACK1 might play a role in cytokinesis in addition to its NPK1-related function.

Cytokinesis in animal cells is achieved by contraction of the cell cortex (for reviews, see Field *et al.* 1999; Hales *et al.* 1999). Thus, animal and plant cells have apparently

developed different strategies for cytokinesis. However, the demonstration that a syntaxin is required for cytokinesis in the nematode suggests that membrane-vesicle trafficking might be involved in animal cytokinesis as well as in plant cytokinesis (Jantsch-Plunger & Glotzer 1999). In addition, the mid-zone of the spindle has been shown to play an important role in furrow ingression in animal systems (Adams *et al.* 1998; Raich *et al.* 1998; Field *et al.* 1999). In plants, the phragmoplast is formed in the spindle mid-zone. There are several reports suggesting that MAPK cascades might be involved in animal cytokinesis. Biochemical assays have demonstrated that MAPKs are activated in cells as they progress from the M to the G1 phase (Edelmann *et al.* 1996). Active MAPKs and MAPKKs are found in the spindle mid-zone and mid-body (Shapiro *et al.* 1998; Zecevic *et al.* 1998). Taken together, the available data suggest that a MAPK cascade might regulate the progression of cytokinesis in animal and plant cells by similar mechanisms that include membrane fusion and the action of mid-zonal MTs, in spite of the opposite directions involved. In animals, two other families of protein kinases, the PLKs and the Aurora kinase family, are required for cytokinesis (for reviews, see Glover *et al.* 1998; Bischoff & Plowman 1999). Protein kinases in both families interact with KLPs in the MKLP1 family in the mid-zone of the spindle and in the mid-body (Lee *et al.* 1995; Adams *et al.* 1998; Severson *et al.* 2000). Thus, the subcellular localization of NPK1 and its ability to interact with a KLP resemble those of PLKs and Aurora kinases. Structural similarities between NPK1 and PLKs or Aurora kinases are, however, not obvious. The systems involving NPK1 and these kinases control cytokinesis in plants and other organisms, respectively, but they might play different roles in cytokinesis in the respective organisms.

The partitioning of a cell by the formation of a cell plate (or septum) is unique to cells with cell walls. This mitotic process must involve various characteristic macromolecules that are not involved in animal cytokinesis, in addition to the factors that are needed in common by both systems. The system that includes NACK1 and NPK1 might be one of several unique aspects of the division of plant cells because no homologues of these proteins have yet been found in animal and yeast cells.

The mechanism of transcription of plant genes that are specific to the G2-M phase of the cell cycle, such as the gene for B-type cyclin and NACK1, is different from that of G2-M phase-specific genes in yeast and animal cells (Ito *et al.* 1998, 2001). Transcription of genes for B-type cyclin and NACK1 is positively controlled by a single type of *cis* element known as MSA, which is necessary and sufficient for the activation of G2-M phase-specific promoter (Ito *et al.* 1998). A novel group of plant Myb proteins, which are structurally similar to animal c-Myb proteins with three Myb repeats, is involved in transcription of the G2-M phase-specific genes via binding to the MSA element. In contrast to plant Myb proteins, animal Myb proteins are involved in transcription of G1-S phase-specific genes, such as genes for cyclin A (Müller *et al.* 1999) and DNA polymerase α (Venturelli *et al.* 1990). The mechanisms that control transcription of G2-M phase-specific genes in animal cells remain controversial (Hwang *et al.* 1998). In yeast cells, G2-M phase-specific

transcription of genes for B-type cyclin is controlled by the transcription factors MCM1 and Swi5 (Althoefer *et al.* 1995; Maher *et al.* 1995; Breeden 2000). Thus, a unique mechanism for transcription of genes is apparently required for progression through the M phase of the plant cell cycle. Plant genes whose transcription is controlled by such a distinct mechanism might control the 'inside-out' mode of cytokinesis in plant cells. Our present results suggest that the MSA-Myb-controlled transcription system might be responsible, at least in part, for one of processes that activate the NACK1-NPK1-mediated MAPK cascade.

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GLOSSARY

- DEX: dexamethasone
 GFP: green fluorescent protein
 KD: kinase domain
 KLP: kinesin-like protein
 MAPK: mitogen-activated protein kinase
 MAPKK: MAP kinase kinase
 MAPKKK: MAP kinase kinase kinase
 MBP: myelin basic protein
 MSA: M-phase-specific activator
 MT: microtubule
 NLS: nuclear localization signal
 NPK1: nucleus- and phragmoplast-localized protein kinase 1
 PLK: polo-like family of protein kinase
 RD: regulatory domain