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Mini Review

Plant Homologues of Components of MAPK (Mitogen-Activated Protein Kinase) Signal Pathways in Yeast and Animal Cells

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As they respond to numerous extracellular and intracellular stimuli, plants develop various morphological features and the capacity for a large variety of physiological processes during their growth. If we are to understand the molecular basis of such developments, we must elucidate the way in which signals generated by such stimuli can be transduced into plant cells and transmitted by cellular components to induce the appropriate terminal events. In yeast and animal systems, signal pathways that are known collectively as MAPK (mitogen-activated protein kinase) cascades have been shown to play a central role in the transmission of various signals. The components of these pathways include the MAPK family, the activator kinases of the MAPK family (the MAPKK family) and the activator kinases of the MAPKK family (the MAPKKK family). The members of each respective family are structurally conserved and signals are transmitted by similar phosphotransfer reactions at corresponding steps that are mediated by a specific member of each family in turn. Both cDNAs and genes that encode putative homologues of these components have recently been isolated from plant sources. Some of them have been shown to be related not only structurally but also functionally to members of the MAPK cascades of other organisms. These findings suggest that plants have signal pathways that are analogous to the MAPK cascades in yeast and animal cells but it remains to be proven that plant homologues do in fact constitute kinase cascades. Given the presence of so many homologues of MAPKs and MAPKKKs in a single plant species, namely, *Arabidopsis thaliana*, we can be fairly confident that the putative MAPK cascades are involved in various physiological processes in plants.

Key words: MAPK — MAPK cascades — MAPKK — MAPKKK — Plants — Signal transduction.

The growth and development of plants are controlled by various phytohormones, which are synthesized by the plant itself. In addition, these processes are affected by environmental conditions that include light and temperature. In spite of extensive studies by both physiological and morphological techniques, our understanding of the molecular events between the direct actions of these stimuli and the final physiological responses of plants is extremely limited. In attempt to investigate these events, efforts have been made to isolate and characterize mutants that have abnormal responses to the stimuli (for reviews, see Bowler and Chua 1994, Hobbie et al. 1995) and to clone candidate genes from plants for proteins that might be involved in the transduction of external signals into cells and in the intracellular transmission of such signals (Ma et al. 1990, Banno et al. 1993).

Protein kinases play a key role in signaling processes in many organisms including yeasts, metazoans and plants. In plants, it has been clearly demonstrated by both genetic and molecular biological approaches that genes, such as *CTR1* (Kieber et al. 1993), *ETR1* (Chang et al. 1993) and

Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor; GUS, β-glucuronidase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MBP, myelin basic protein; PCR, polymerase chain reaction; MADS, MCM1, AGAMOUS, DEFICIENS and serum-response factor (SRF).

Pto (Martin et al. 1993), which encode putative protein kinases, are involved in the transmission of signals that are generated by extracellular stimuli. Both CTR1 and ETR1 protein kinases from *Arabidopsis thaliana* have been shown to be involved in the signal pathway initiated by ethylene. CTR1 is similar in terms of amino acid sequence to protein kinases of the Raf family in animals and it is thought to have a negative regulatory effect on responses of the plant to ethylene (Kieber et al. 1993). ETR1, which acts upstream of CTR1, belongs to the family of the membrane-associated protein-histidine kinases that are involved in the two-component regulatory signal-transduction system that is conserved in a variety of prokaryotes (Chang et al. 1993). The predicted structural features of ETR1 suggest that it serves as a sensor for the ethylene signal on the plasma membrane. The Pto protein kinase from tomato, which is also predicted to be anchored to the plasma membrane, confers resistance to the pathogenic microorganism *Pseudomonas syringae* (Martin et al. 1993), and it appears to be involved in induction of the hypersensitive response by the plant to the pathogen. While it is evident that these protein kinases mediate signals generated by the respective stimuli in plant cells, little is known about the intracellular signal-transmission pathways that include these components.

Signal pathways, known collectively as mitogen-activated protein kinase [MAPK (or ERK: extracellular signal-regulated protein kinase)] cascades, each of which consists of a MAPK, the activator kinase of the MAPK [MAPKK (or MEK: MAPK and ERK kinase)] and the activator kinase of the MAPKK [MAPKKK (or MEKK: MEK kinase)], have recently been established in yeasts and animals and shown to be involved in various physiological processes (for reviews, see Marshall 1995, Herskowitz 1995). A salient biochemical feature of the MAPK cascade is the occurrence of the dual phosphorylation of both threonine and tyrosine residues in the TXY motif in kinase subdomain VIII, which is conserved in all MAPKs, and such phosphorylations are essential for the activation of MAPKs. In addition, a number of MAPKKs have a SXAXS/TFVGT motif in kinase subdomain VIII. Phosphorylation at the serine or threonine residues in this motif by upstream kinases is essential for activation of members of the MAPKK family (Alessi et al. 1994, Gotoh et al. 1994, Mansour et al. 1994, Zheng and Guan 1994). These observations suggest that signals throughout each MAPK cascade are transmitted by the essentially the same biochemical reaction in every case.

Several groups including our own have shown that plants contain homologues of the components of the MAPK cascade (Banno et al. 1993, Duerr et al. 1993, Jonak et al. 1993, Mizoguchi et al. 1993, 1994, Stafstrom et al. 1993, Wilson et al. 1993, Shibata et al. 1995). Thus, it seems likely that MAPK cascades occur in plants even though the relationships among these plant homologues have yet to be determined and the physiological processes that might be controlled by such components remain to be defined. In this review, we summarize the major features of MAPK cascades in yeast and animal cells and describe recent advances in studies of plant homologues of components of MAPK cascades. As the reader will learn, our knowledge of the plant homologues of such components is still fragmentary.

MAPK cascades in yeast and animal cells

(a) MAPK cascades in yeast cells—The yeast *Saccharomyces cerevisiae* is thought to have at least six MAPK signal pathways (for review, see Herskowitz 1995). Among them, three pathways have been shown to consist of complete sets of MAPKKK, MAPKK and MAPK (see Fig. 1). One pathway mediates the mating pheromone-responsive signal, one is involved in the process that regulates the normal growth and division of cells, and one pathway is involved in the transduction of signals related to external osmotic pressure.

In the mating pheromone-responsive pathways of *S. cerevisiae* and *Schizosaccharomyces pombe*, another yeast, the STE11 and byr2 protein kinases (yeast homologues of MAPKKK), which exhibit a high degree of structural homology to one another, act upstream of STE7 and byr1 (yeast homologues of MAPKK), respectively (see Fig. 1). STE7 and byr1, in turn, activate redundant MAPK homologues FUS3/KSS1 and spk1, respectively (Cairns et al. 1992, Gartner et al. 1992, Stevenson et al. 1992, Zhou et al. 1993). STE7 has been shown to activate FUS3 directly by phosphorylation both of threonine and of tyrosine residues (Errede et al. 1993). In *S. cerevisiae*, steps further downstream of FUS3/KSS1 have been investigated. The FUS3/KSS1 kinases phosphorylate the transcription factor STE12 (Elion et al. 1993) and the phosphorylated STE12, often together with the general transcription factor MCM1 which has a MADS box for binding to DNA (Yanofsky et al. 1990), activates the transcription of genes specific for mating types. It is worth noting here that mammalian MAPK also phosphorylates the TCF (Elk-1) protein that can, in association with the SRF that has a MADS box, induce transcription of genes in response to serum (Gille et al. 1992; for review, see Hill and Treisman 1995). It is also worth noting, parenthetically, that a number of homeotic genes in plants that are involved in flower development also contain MADS domains (Yanofsky et al. 1990, Goto and Meyerowitz 1994).

Another protein, STE5, is involved in the signaling that is mediated by the MAPK cascade that consists of STE11, STE7 and FUS3/KSS1 (Choi et al. 1994, Marcus et al. 1994, Printen and Sprague 1994). All the components of the MAPK cascade associate independently with the STE5 protein to form a large complex, which may act to inhibit

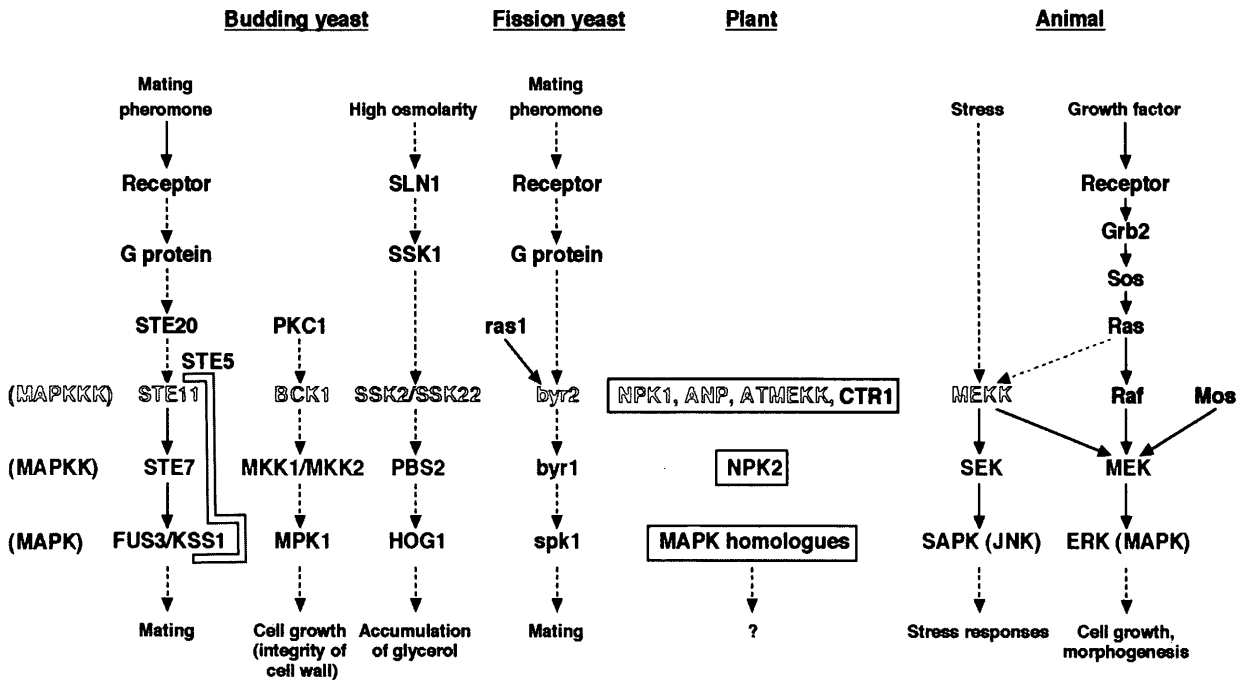

Fig. 1 Signal-transduction pathways mediated by MAPK cascades in various organisms. Six representative pathways are illustrated: the mating pheromone-responsive signal pathway, the pathway that regulates normal growth and division of cells, and the osmosensing signal-transduction pathway in budding yeast; the mating pheromone-responsive signal pathway in fission yeast; and the stress-activated signal pathway and the growth factor-stimulated signal pathway in animals. Arrows indicate the activations or the protein-protein interactions that have been proven by biochemical or genetic experiments. Dashed arrows indicate interactions that have been predicted from the results of genetic or physiological analyses. Members of the MAPKKK family (red), the MAPKK family (blue), and the MAPK family (green), respectively, are indicated in the same color.

cross-talk among different MAPK cascades. Homologues of STE5 in other organisms have not yet been identified although they probably do exist.

S. cerevisiae has another MAPK signal pathway that is regulated by PKC1 (a yeast homologue of protein kinase C, Irie et al. 1993, Lee et al. 1993) (see Fig. 1). This pathway is thought to be responsible for the integrity of the yeast cell wall and, thereby, to control the growth of yeast cells (Levin et al. 1990, 1994, Lee et al. 1993). Mutations in any one of the genes for kinases in the pathway exhibit a cell-lysis defect in conventional medium (Levin et al. 1994). The PKC1-mediated pathway includes BCK1, a protein kinase that exhibits significant similarity in terms of amino acid sequence to both STE11 and byr2. It functions upstream of the redundant MKK1/MKK2 kinases which correspond to MAPKK, kinases that are thought to activate the MAPK homologue MPK1 (Irie et al. 1993, Lee et al. 1993). Although the purified PKC1 kinase can phosphorylate the recombinant BCK1 protein in vitro (Levin et al. 1994), it remains to be demonstrated that such phosphorylation is required for activation of BCK1. Recently, a process downstream of the reaction catalyzed by PKC1 was proposed to regulate the synthesis of components of the yeast cell wall, such as (1 → 6)-β-glucan (Roemer et al. 1994) and to regulate negatively the expression of β-glucanase of yeast (Shimizu et al. 1994).

In *S. cerevisiae*, the osmosensing signal-transduction pathway also includes a cascade that is analogous to the MAPK signal pathway (Brewster et al. 1993). When yeast cells are grown under conditions of high osmolarity, the synthesis of glycerol is induced in the cells to increase their internal osmolarity. The pathway that regulates this response is mediated by the MAPKK homologue PBS2, which functions upstream of the MAPK homologue HOG1. Another protein kinases, SSK2/SSK22, which exhibits similarity in terms of amino acid sequence to MAPKKKs appears to act upstream of PBS2 (Maeda and Saito 1995). Thus, the osmosensing signal-transduction pathway also includes a complete set of components of a MAPK cascade.

A member of the MAPKK family, designated Fuz7, has been identified in *Ustilago maydis*, which is a plant pathogenic fungus. Fuz7 is necessary for various processes that are dependent and independent of the **a** mating-type locus (Banuett and Herskowitz 1994). However, it remains to be determined whether it acts upstream of members of the MAPK family.

(b) MAPK cascades in animal cells—It has been

shown that the MAPK family in animals mediates the growth factor-stimulated signal pathway and the MPF-induced kinase cascade (Blenis 1993, Egan and Weinberg 1993), a signal pathway for vulval induction during development of the hermaphroditic form of *Caenorhabditis elegans* (Lackner et al. 1994, Wu and Han 1994), as well as multiple signal pathways that are controlled by receptor tyrosine kinases, known as sevenless and torso, in *Drosophila melanogaster* (Brunner et al. 1994). Certain stresses, for example, treatment of mammalian cells with ultraviolet light or cycloheximide, have also been shown to activate a distant relative of the MAPK family which is referred to as SAPK (or JNK; Dérijard et al. 1994, Kyriakis et al. 1994).

Investigations to identify and characterize activators of MAPKs from vertebrates have revealed that their activators, such as MAPKKs from *Xenopus laevis* (Kosako et al. 1993, Yashar et al. 1993) and rat (Wu et al. 1993a, b) and MEK from mice (Crews et al. 1992), exhibit striking similarities in terms of amino acid sequence to those of the yeast MAPK activators described above. A member of the MAPKK family has also been isolated from *D. melanogaster* (Dsor1; Tsuda et al. 1993). Thus, not only the MAPK family but also the MAPKK family is conserved in yeasts and animals, a phenomenon that provides evidence for the existence of conserved kinase cascades of MAPKKs and MAPKs in eukaryotes.

In the mouse, the Raf protein kinase family (Dent et al. 1992, Howe et al. 1992, Kyriakis et al. 1992) and Mos protein kinase (Posada et al. 1993) serve as activator kinases of members of the MAPKK family. A homologue in *D. melanogaster* of the Raf protein also acts as the activator kinase of a putative protein kinase, Dsor1, that is related to STE7 and byr1 (Tsuda et al. 1993). Members of the Raf family in animals are structurally unrelated to yeast MAPKKKs, such as STE11, and homologues of Raf and Mos have not yet been identified in yeasts. The MEKK protein kinase, which is structurally similar to STE11, BCK1 and byr2, has, however, been identified as another activator in vitro of murine MEK (Lange-Carter et al. 1993). MEKK also has the ability to restore the growth defect of the *bck1* mutant of yeast (Blumer et al. 1994), as does NPK1 (a plant homologue of members of the MAPKKK family), described below (Banno et al. 1993). The existence of three different types of activator kinase (MEKK, Raf and Mos) for the MAPKK family might reflect the divergence of the MAPK cascade system in animal cells from those defined in yeast cells, while STE11-related activators have been retained for regulation of the MAPK network in both yeasts and mammals and, probably, also in plants (see below).

Although MEKK can activate MEK by phosphorylation in vitro, a substrate for MEKK in vivo is thought to be the SAPK/ERK kinase (SEK) which, in its turn, phosphorylates SAPK (or JNK; Sánchez et al. 1994, Yan et al. 1994). These results suggest that Raf and MEKK regulate distinct MAPK cascades in mammals.

(c) Upstream components of MAPK cascades—External signals are first perceived by receptors of the cell-surface and are then transduced by cytoplasmic protein factors, such as G proteins and protein kinases, to the inside of the cell. In this section, we summarize the available information about factors that have been shown to act at points upstream of various MAPK cascades.

In *S. cerevisiae*, two mating pheromones, namely, the **a**-factor and the α-factor, which are produced by **a** cells and α cells, respectively, act on cell-surface receptors, STE3 and STE2, respectively, which are members of the group of receptors with seven transmembrane domains. Activation of the receptors results in dissociation of a trimeric G protein complex into G_α and $G_{\beta\gamma}$. $G_{\beta\gamma}$ is thought to transmit a signal that activates the STE11 kinase via the STE20 protein kinase. It has yet to be determined how $G_{\beta\gamma}$ activates STE20 and whether STE20 can phosphorylate STE11 directly. A protein kinase, $p65^{PAK}$, the catalytic domain of which is 70% identical in terms of amino acid sequence to that of STE20, has been isolated from rat (Manser et al. 1994). In the case of the BCK1-mediated MAPK cascade, PKC1, a yeast homologue of protein kinase C, acts upstream of BCK1. However, components that act further upstream have not been identified.

S. cerevisiae has another membrane-anchored protein, SLN1, that acts upstream of the osmosensing MAPK cascade that includes PBS2 and HOG1 (Maeda et al. 1994). SLN1 includes a domain that is similar in terms of amino acid sequence to the catalytic domains of members of the protein-histidine kinase family which serve ubiquitously as sensors in two-component regulatory systems in prokaryotes. SLN1 is thought to regulate negatively the response-regulator protein SSK1 in the osmosensing pathway (Maeda et al. 1994). Note also that ETR1, which acts upstream of CTR1 in the ethylene-signaling pathway of *A. thaliana*, exhibits structural similarity to members of the histidine kinase family in two-component systems (Chang et al. 1993).

In mammals, the signal-transduction pathway regulated by epidermal growth factor (EGF) has been extensively analysed. EGF binds to a specific receptor, the EGF receptor (EGFR), which contains an extracellular receptor domain and a cytoplasmic catalytic domain with protein-tyrosine kinase activity (for review, see Yarden and Ullrich 1988). Activation of EGFR upon the binding of EGF results in autophosphorylation at tyrosine residues, which leads to activation of a variety of signal pathways: the Ras-Raf pathway; the Ras-PI(3)K (phosphatidylinositol-3-OH kinase) pathway (Rodriguez-Viciana et al. 1994); and the protein kinase C pathway (for review, see Schlessinger 1994). In the Ras-Raf pathway, activation of Ras to ganerate its GTP form occurs via formation of a complex

of the exchange factor SOS and the adaptor protein Grb2 that is recruited to the tyrosine-phosphorylated cytoplasmic domain of EGFR (Buday and Downward 1993, Egan et al. 1993). The role of the active form of Ras is thought to be to recruit Raf to the plasma membrane, where the Raf kinase can be fully activated by an as yet unidentified signal (Stokoe et al. 1994, Leevers et al. 1994, Fabian et al. 1994).

Not only in mammals but also in *D. melanogaster* and *C. elegans*, receptor tyrosine kinases and members of the Ras protein family act upstream of MAPK cascades (Biggs et al. 1994, Brunner et al. 1994, Lackner et al. 1994, Wu and Han 1994). In addition, stimulation of the muscarinic receptors, which have seven membrane-spanning domains and are coupled with trimeric G proteins, has been shown to result in the eventual activation of MAPK (Crespo et al. 1994).

Thus, in yeasts and animals, three types of receptor have been shown to act upstream of MAPK cascades: receptors with seven transmembrane domains, receptor tyrosine kinases, and a membrane-anchored histidine kinase.

Plant components that are homologous to members of MAPK cascades in yeast and animal cells

(a) Plant homologues of MAPKKK—Only two members of the STE11-related MAPKKK family have been reported in multicellular organisms: MEKK from mouse (Lange-Carter et al. 1993) and NPK1 from tobacco (Banno et al. 1993). The cDNA for NPK1 was isolated by use of the polymerase chain reaction (PCR) during the course of experiments in which we attempted to clone plant homologues of genes for protein kinase C (PKC) and cyclic AMP-dependent (cAMP-dependent) protein kinase (Banno et al. 1993). NPK1 cDNA encodes a protein kinase with a catalytic domain that exhibits significant similarity in terms of amino acid sequence to the members of the STE11-related MAPKKK family (Fig. 2). The predicted NPK1 protein has a large kinase-unrelated region in the C-terminal half although other members of this family, such as

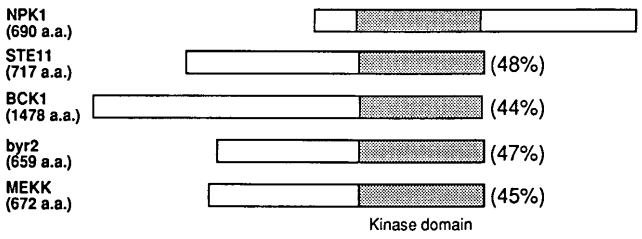

Fig. 2 Organization of the domains of the predicted NPK1, STE11, BCK1, byr2, and MEKK proteins. Shaded and open bars represent the putative catalytic domains and the kinase-unrelated domains, respectively. Numbers in parentheses on the right indicate amino acid (a.a.) sequence identities (%) between the putative catalytic domain of NPK1 protein and those of the STE11, BCK1, byr2, and MEKK proteins.

STE11, BCK1, Byr2 and MEKK, have such regions in their N-terminal halves. These regions are involved in the negative control of the respective protein kinase activities. In contrast to its kinase domain, the unrelated region of NPK1 exhibits no apparent homology to those of other members.

Overexpression of the cDNA for NPK1 in yeast cells complements mutations in genes for BCK1 and PKC1 in *S. cerevisiae* (see Fig. 1). Thus, NPK1 can replace the growth-control functions of BCK1 and PKC1 in yeast (Banno et al. 1993). The extent of complementation by a truncated form of NPK1 cDNA, in which the kinase-unrelated region has been deleted, is greater than that by the full-length NPK1 cDNA. This result indicates that the protein encoded by the truncated form is constitutively active.

Although the physiological functions of NPK1 remain to be determined, the patterns of transcription of *NPK1* gene in tobacco plants suggest that there is some correlation between the functions of NPK1 and both the proliferation of tobacco cells in meristematic regions and the growth of young organs. Northern blot analysis showed that *NPK1*-related transcript(s) is present in roots, stems, young leaves, flower buds, and tissues that include the shoot apical meristem, but they are absent from mature leaves and mature flowers (Banno et al. 1993; our unpublished data). Recent analysis of transgenic tobacco plants that carried a fusion gene composed of the *NPK1* promoter and the coding region for β-glucuronidase (GUS) revealed strong GUS activity in primodia of lateral roots, as well as in shoot and root apical meristems (our unpublished data). Although no *NPK1* transcripts were detected in mature leaves, such transcripts were observed as early as three hours after incubation of leaf discs from mature leaves with both auxin and cytokinin (our unpublished data). These results are in agreement with the hypothesis that the NPK1 protein kinase might be involved in the proliferation of tobacco cells.

We have isolated three cDNA clones from *A. thaliana* that encode homologues of NPK1 (ANPs; our unpublished data). Southern blot analysis indicated that two additional homologues are also present in the *Arabidopsis* plant. Another group has cloned three cDNAs that potentially encode members of the STE11-related MAPKKK family (ATMEKKs; personal communication from Mizoguchi and Shinozaki, Institute of Physical and Chemical Research, Tsukuba Life Science Center, Japan). These findings indicate that plants have multiple copies of genes for members of the STE11-related MAPKKK family.

The *CTR1* gene, which negatively regulates responses to ethylene of *Arabidopsis* plants, appears to encode a kinase domain that is similar in terms of amino acid sequence to the Raf protein kinase of animals (Kieber et al. 1993). In view of the structural similarity between CTR1 and Raf, CTR1 might likewise serve as a MAPKKK in the plant:

a downstream protein might be structurally similar to members of the MAPKK family. To date, however, genetic analyses of the ethylene-signaling pathway have not yet identified such a component downstream of *CTR1*.

(b) Plant homologues of MAPKK—To our knowledge, only one MAPKK-related cDNA from a plant has been reported: *NPK2* cDNA (Shibata et al. 1995). Since a protein kinase downstream of NPK1 must be structurally similar to members of the MAPKK family, we carried out cloning by PCR of cDNAs for such members from suspension-cultured tobacco cells and one cDNA clone, designated *NPK2* cDNA, was obtained (Shibata et al. 1995). As shown in Figure 3, the predicted amino acid sequence of the kinase domain of NPK2 exhibits significant similarity to those of MAPKKs (37–45% identity at the amino acid level). The recombinant NPK2 protein that was produced in *Escherichia coli* cells was able to autophosphorylate threonine and serine residues. *NPK2*-related gene(s) is transcribed in all organs of tobacco, but transcription of *NPK2* in stems seems to be more efficient than elsewhere. This pattern of transcription contrasts with that of the transcription of *NPK1*, which occurs predominantly in meristematic tissues, as mentioned above. Southern blot analysis with the *NPK2* cDNA showed that various plant species, including *A. thaliana*, tomato, potato and sweet potato, also have genes that are closely related to the *NPK2* gene of tobacco. We have isolated two types of cDNA clone from *Arabidopsis* plants that hybridize with *NPK2* cDNA (our unpublished data). Thus, the plant might have several copies, at least, of *NPK2*-related genes.

(c) Plant homologues of MAPK—Several members of the MAPK family have been identified in plants (Duerr et al. 1993, Jonak et al. 1993, Mizoguchi et al. 1993, 1994, Stafstrom et al. 1993, Wilson et al. 1993) although the physiological processes that might be mediated by the plant homologues remain to be characterized. The MAPKs are as follows: MsERK1 (or MsK7) from *Medicago sativa* (alfalfa; Duerr et al. 1993, Jonak et al. 1993); NTF3 from *Nicotiana tabacum* (Wilson et al. 1993); D5 from *Pisum sativum* (Stafstrom et al. 1993) and ATMPK1-7 from *A. thaliana* (Mizoguchi et al. 1993, 1994). Among these plant homologues, 50–90% of amino acid residues are identical (Mizoguchi et al. 1993). These plant homologues exhibit 40–50% identity in terms of amino acid sequences to MAPKs of other organisms. A recombinant MsERK1 protein, produced in *E. coli* cells, was able to phosphorylate myelin basic protein (MBP), which is known to be phosphorylated by animal MAPKs (Duerr et al. 1993). Transcription of the corresponding gene depends on the cell cycle of alfalfa cultured cells: it is not transcribed at the G1 phase but is transcribed from the S to the M phase. Such timing suggests that MsERK1 might be involved in the regulation of the cell cycle of plant cells even though such a pattern of transcription of genes for MAPKs is not seen in animal and yeast cells. Slight activation by MAPKK from *X. laevis* of the phosphorylation of MBP by ATMPK1 and ATMPK2 has been reported (Mizoguchi et al. 1994).

(d) An activity that phosphorylates myelin basic protein is induced by cutting of plant tissues and by other external stimuli—The activity has been detected in plants of a protein kinase with biochemical characteristics similar to those of known MAPKs of yeast and animal cells (Usami et al. 1995). When plants were cut, a 46-kDa protein kinase that phosphorylated MBP was rapidly and transiently activated in the wounded tissues (Fig. 4). Activation of a protein kinase with the same molecular mass was also detected

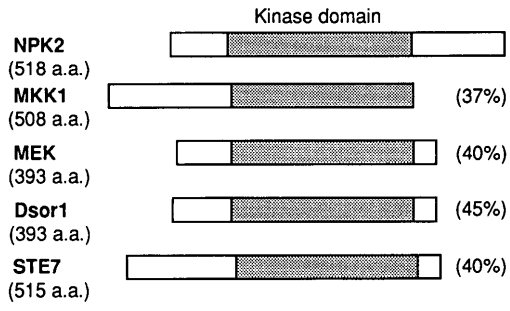

Fig. 3 Organization of the domains of the predicted NPK2, MKK1, MEK, Dsor1, and STE7 proteins. Shaded and open bars represent the putative catalytic domains and the kinase-unrelated domains, respectively. Numbers in parentheses on the right indicate amino acid (a.a.) sequence identities (%) between the putative catalytic domain of NPK2 protein and those of the MKK1, MEK, Dsor1, and STE7 proteins.

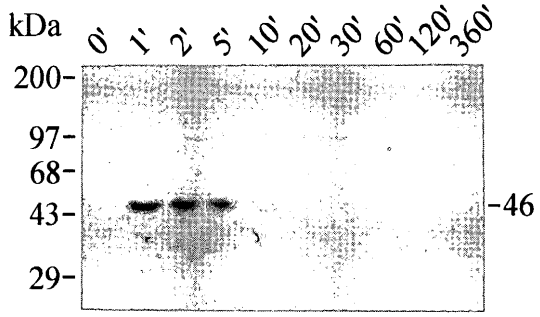

Fig. 4 Detection of the activity of the 46-kDa protein kinase in tobacco leaves on an SDS-polyacrylamide gel that contained myelin basic protein (MBP). Leaf discs were cut from mature leaves of tobacco and incubated for the times indicated. Total proteins were extracted and subjected to electrophoresis on an SDS-polyacrylamide gel that contained MBP, and then SDS was removed by successive washing of the gel with washing buffer. After denaturation and renaturation of proteins in the gel, the gel was incubated with [γ-^{32}P]ATP to allow phosphorylation reaction to occur. Then the gel was washed and autoradiographed for visualization of bands of proteins that phosphorylated MBP (Usami et al. 1995).

in a wide variety of plant species that included both dicots and monocots. Treatment of this protein kinase with either protein phophatase 2A (specific for phosphorylated serine and threonine residues) or T-cell protein phosphatase T-$CELL_R$ (specific for phosphorylated tyrosine residues) abolished the activity. These results indicate that phosphorylation of both tyrosine and serine plus threonine residues is essential for activiation of the 46-kDa kinase. Thus, the 46-kDa kinase is probably activated by phosphorylation of serine plus threonine and tyrosine residues. As mentioned in the previous section, activation of MAPK involves dual phosphorylation by MAPKK. Therefore, the 46-kDa protein kinase seems to have characteristics similar to those of a MAPK although its molecular features remain to be characterized. The relationship between activation of this kinase and wound-inducible events in plants also remains to be determined. A similar activity has been detected in tobacco cultured cells upon treatment with fungus-derived elicitors (Suzuki and Shinshi 1995).

4. Unanswered questions

At the moment, we have three basic questions about plant homologues of components in the MAPK cascade. First, what physiological and biochemical processes, including gene expression, are mediated by such homologues? It is reasonable to speculate that a number of independent signal-transmission pathways, similar to the MAPK cascade, are conserved in plants because many homologues of MAPKs, and MAPKKKs are present in *Arabidopsis* plants and the functions of the MAPK cascades in other organisms have diverged considerably (see above). However, our ability to answer this question is minimal at present. Second, do the plant homologues actually form a kinase cascade as observed in other organisms? Third, what kinds of cellular component can directly regulate the activity of homologues of MAPKKKs? We do not know what receptor molecules regulate putative MAPK pathways but two types of membrane-associated receptor-like protein have been identified in plants: one is a presumed sensor histidine kinase of the two-component regulatory systems, such as ETR1 from *A. thaliana* (Chang et al. 1993); the other is a family of receptor-like protein-serine/threonine kinases, such as ZmPK1 from maize (Walker and Zhang 1990), TMK1 from *A. thaliana* (Chang et al. 1992), and NPK15 from tobacco (Ito et al. 1994).

With regard to the first question, since members of the Raf family have been shown to be activator kinases of MAPKKs in animals, it is of interest to note that CTR1 from *A. thaliana*, which is involved in the ethylene-signaling pathway, is similar in terms of amino acid sequence to the protein kinases of the Raf family. The signal pathway activated by ethylene has been studied intensively at the genetic and molecular levels and several genes have been identified whose products act both upstream and downstream of the CTR1 kinase (Guzmán and Ecker 1990, Kieber et al. 1993). Characterization of these genes showed, however, that their putative products are structurally unrelated to known components of MAPK cascades (personal communication from Ecker, Plant Science Institute, University of Pennsylvania, U.S.A.). One possible explanation for these results is that genes for components of the putative MAPK cascade that might mediate the ethylene signal could be redundant. We note, for example, that FUS3/KSS1, MKK1/MKK2 and SSK2/SSK22 (Maeda and Saito 1995) in yeast cells are also known to have redundant functions in their respective MAPK cascades (see Fig. 1). Alternatively, a loss-of-function mutation in components of the putative ethylene-responsive MAPK cascade might result in lethality. Novel strategies may be required for the isolation of mutants of plant components of MAPK cascades. A nematode homologue of MAPK was identified, for example, from a mutation that suppressed gain-of-function phenotypes of the *Ras* homologue in the nematode (Lackner et al. 1994, Wu and Han 1994). In addition to such similar genetic approaches, further characterization of the candidate molecules described above in transgenic plants or cells might also provide clues to the nature of the putative ethylene-responsive MAPK cascade.

The third question seems to be of general importance because only the Ras protein has been unambiguously identified as a factor that acts upstream of Raf (see Fig. 1). A protein known as 14-3-3 is associated with Raf (Fu et al. 1994) and stimulates the activity of Raf (Fantl et al. 1994, Freed et al. 1994, Irie et al. 1994). Although STE20 and PKC1 were identified genetically upstream of specific MAPKKK homologues, direct relationships between these components are still unknown. To identify an activator of NPK1, we have adopted a screening system with yeast cells that carry *STE7*^{P368}, a gain-of-function mutant of *STE7* (Irie et al. 1994). STE7^{P368} has increased kinase activity but the activity is still dependent on the presence of an upstream activator kinase, such as STE11 (Irie et al. 1994). We found that a truncated (active) form of NPK1, NPK1Δ374 (Banno et al. 1993), further activated the signal pathway that involves STE7^{P368} while full-length NPK1 did not (our unpublished data). This observation allows us to predict that a tobacco cDNA clone for an activator of NPK1 should be identifiable by screening of a tobacco cDNA library with yeast cells that express STE7^{P368} and NPK1; if a cDNA for a putative activator of NPK1 from tobacco is cloned into the yeast cells, STE7^{P368} should be activated via the activation of NPK1 by the activator. A two-hybrid system, which has been used to isolate a cDNA for a protein that can interact with or bind to another protein of interest (for review, see Fields and Sternglantz 1994), might also be useful for screening factors that act upstream of MAPKKK. Investigations with such genetic systems in yeasts, together with biochemical and transgenic ap-

proaches in plants, should contribute to our understanding of signaling pathways that are mediated by the plant homologues described here, as well as the physiological functions of these pathways.

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