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Molecular Cloning and Characterization of Three cDNAs Encoding Putative Mitogen-activated Protein Kinase Kinases (MAPKKs) in *Arabidopsis thaliana*

Kazuya ICHIMURA,[1,2] Tsuyoshi MIZOGUCHI,[1] Nobuaki HAYASHIDA,[3] Motoaki SEKI,[1] and Kazuo SHINOZAKI[1,2,*]

Laboratory of Plant Molecular Biology, Tsukuba Life Science Center, Institute of Physical and Chemical Research (RIKEN), 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan,[1] Institute of Biological Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-0006, Japan,[2] and Department of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, 3-15-1 Tokida, Ueda, Nagano 386-0018, Japan[3]

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Abstract

We isolated three *Arabidopsis thaliana* cDNA clones (ATMKK3, ATMKK4 and ATMKK5) encoding protein kinases with extensive homology to the mitogen-activated protein kinase kinases (MAPKKs) of various organisms in the catalytic domain. ATMKK3 shows high homology (85% identity) to NPK2, a tobacco MAPKK homologue. ATMKK4 and 5 are closely related to each other (84% identity). Phylogenetic analysis showed that the plant MAPKKs constitute at least three subgroups. The recombinant ATMKK3 and ATMKK4 were expressed as a fusion protein with glutathione S-transferase (GST) in *Escherichia coli*. Affinity purified GST-ATMKK3 and GST-ATMKK4 proteins contained phosphorylation activity, which shows that both the ATMKK3 and ATMKK4 genes encode functional protein kinases. Northern blot analysis revealed that the ATMKK3 gene expressed in all the organs. The levels of ATMKK4 and 5 mRNAs were relatively higher in stems and leaves than in flowers and roots. We determined the map positions of the ATMKK3, 4 and 5 genes on *Arabidopsis* chromosomes by RFLP mapping using P1 genomic clones.

Key words: Protein kinase; MAP kinase cascade; MAP kinase kinase; Phosphorylation site

1. Introduction

Mitogen-activated protein kinase (MAPK) cascades are modules of signal transduction from the cell surface to the nucleus. MAPK cascades are comprised of three evolutionarily conservative kinases, a MAPKK kinase (MAPKKK, also known as MEK kinase), a MAPK kinase (MAPKK, also known as MEK, a MAPK/ERK kinase) and a MAPK (also known as ERK, extracellular signal regulated protein kinase).[1–4] In the MAPK cascades, MAPK is activated by MAPKK through dual-phosphorylation of an evolutionarily conserved TXY motif between subdomains VII and VIII. MAPKKs are regulated through the phosphorylation of the conserved phosphorylation SXXX$^{S}/_{T}$ motif between subdomains VII and VIII by several different groups of MAPKKK.[5] Substitution of the conserved serine/threonine residues by negatively charged aspartate or glutamate at the phosphorylation site causes constitutive activation of MAPKK.[6] MAPK cascades play a crucial role in the regulation of biochemical and physiological changes associated with extracellular stimuli, such as growth, differentiation and environmental stimuli, in many organisms from yeast to vertebrates.[1–4] In animals, at least three MAP kinase cascades have been defined.[1–4] In budding yeast, *Saccharomyces cerevisiae*, five MAPK cascades have been identified.[3]

In higher plants, a number of genes for MAPKKKs and MAPKs have been isolated.[7–9] Functional as well as structural similarity of MAPKKKs in plants and yeasts has been suggested.[10–13] Gene expression and/or activation of plant MAPKs or MAPK-like kinases concerned with tissue specificity, developmental regulation, hormonal regulation and abiotic stress-response have been analyzed.[7–9]

In contrast to MAPKs and MAPKKKs, there have

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* To whom correspondence should be addressed. Tel. +81-298-36-4359, Fax. +81-298-36-9060, E-mail: sinozaki@rtc.riken.go.jp

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been only three reports on plant MAPKK homologues, one from tobacco *NPK2* and two from *Arabidopsis*, *MEK1* and *ATMAP2Kα*.[14–16] The level of the *MEK1* mRNA was demonstrated to be regulated by etiolation and wounding.[15] Interactions between MAPKs and MAPKKs, and those between MAPKKs and MAPKKKs have not been analyzed in plants. In order to analyze MAPK pathways in plants, we think it important to isolate plant MAPKK homologues and characterize their structures and gene expression. In the present study, we report the isolation and characterization of three MAPKK homologues in *Arabidopsis thaliana*.

2. Materials and Methods

2.1. Preparation of DNA fragments that encode putative MAP kinase kinase

A λgt11 cDNA library[17] prepared from two-week-old *Arabidopsis* rosette plants was used as a template for the polymerase chain reaction (PCR). We carried out PCR using following primers:
NPK2-3F: GGTCGACAACTGCTTACTGAAATAAGGACATTGTG;
NPK2-9R: GGTCGACCCCAAGGCTCCAAATGTCAGCTGGGTA,
based on the cDNA sequence of tobacco *NPK2*. PCR was performed by 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. Five μl of the PCR product was subjected to the second PCR. The second PCR was carried out by 25 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. PCR was done using TaKaRa Ex Taq DNA polymerase (TAKARA, Japan). The PCR product was digested with *Sal* I and subcloned into the *Sal* I site in pBluescript vector, pSKII$^-$ (Stratagene, USA). DNA sequencing was performed with a model 373A DNA sequencer (Applied Biosystems, USA). We obtained an *Arabidopsis* expressed sequence tag (EST)[18] clone 127H15T7 (GenBank/EMBL/DDBJ accession No. T44940) from Arabidopsis Biological Research Center (Ohio State University, Columbus, OH, USA). The insert DNA of the clone 127H15T7 was excised with *Not* I and *Sal* I, and then purified by agarose gel electrophoresis.

2.2. Cloning and sequencing of cDNAs for ATMKK3, 4 and 5

We used the PCR fragment and the DNA insert of the EST clone as probes. The probes were labeled with [α-^{32}P]dCTP (Amersham, UK) using the random primer labeling kit according to a manufacturer's instruction (Boehringer Mannheim, Germany). We screened about 9×10^5 plaques of the *Arabidopsis* cDNA library in low-stringency hybridization conditions,[19] and isolated three groups of positive cDNA clones. One was obtained using the PCR fragment as a probe, named ATMKK3, and the other two, named ATMKK4 and ATMKK5, were obtained using the EST clone as a probe. We extracted the phage DNAs of the ATMKK3, 4 and 5 clones with the longest inserts according to standard protocol.[19] The DNA inserts were prepared by digestion with *Not* I for ATMKK3 and ATMKK5, or with *Eco*RI for ATMKK4, and subcloned into the *Not* I or *Eco*RI sites of pSKII$^-$ vector. We determined their nucleotide sequences with a model 373A DNA sequencer. Nucleotide and amino acid sequences were analyzed with the GENETYX (Software Development, Tokyo) and GENEWORKS (IntelliGenetics, USA) software systems.

2.3. Southern and Northern blot analysis

Southern and Northern blot analysis were performed as described.[20] The coding region [nucleotides (nt) 28–1593] of ATMKK3, a 3′ non-coding regions of ATMKK4 (nt 1251–1631) and ATMKK5 (nt 1031–1501) were used as probes for Northern blot analysis. DNA fragments corresponding to the coding regions of ATMKK3, ATMKK4 (nt 250–1353) and ATMKK5 (nt 26–1075) were used as probes for Southern blot analysis.

2.4. Expression of GST-ATMKK3 and GST-ATMKK4 fusion proteins in E. coli *and phosphorylation assay of GST-fusion proteins*

The DNA fragments corresponding to open reading frame region of cDNAs ATMKK3 (nt 28–1593) and ATMKK4 (nt 250–1353) were amplified by PCR with primers containing *Sal* I site at 5′ termini. The PCR products were digested with *Sal* I, purified by agarose gel electrophoresis and subcloned into pGEX4T1 vector (Pharmacia, Sweden). We also constructed the ATMKK3K112R (Lys112 to Arg) and ATMKK4K108R (Lys108 to Arg) that contain point mutation in the Lys residues in the kinase subdomain II by PCR. The point mutations were confirmed by sequencing. The mutagenized cDNA fragments were also cloned into pGEX4T1 vector. An *E. coli* strain JM109 was used for expression of GST-fusion proteins. Expression and affinity purification of GST-fusion proteins was performed as previously described.[21] Partially purified GST-fusion proteins (120 ng) were subjected to phosphorylation assay as described previously, except the reaction mixture contained 20 mM $MnCl_2$.[22] The samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gel was dried and autoradiographed.

2.5. Mapping of the ATMKK3, 4 and 5 genes

We identified that the P1 clones, MPO12, MEO21 and MUJ17, contained the ATMKK3 ,4 and 5 genes, respectively based on hybridization using P1 clones-blotted filter. Using these P1 clones as probes, we carried

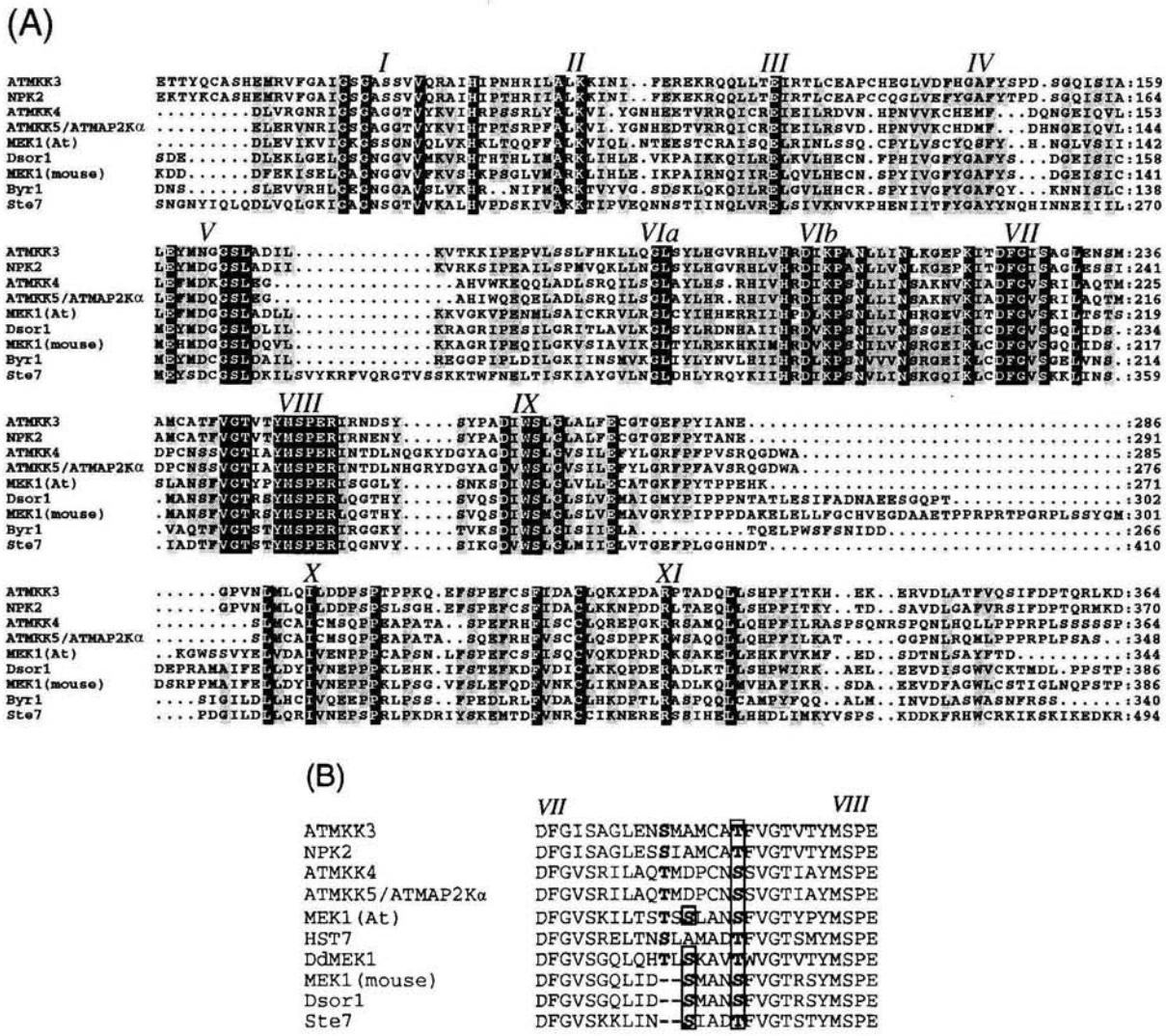

Figure 1. (A) Alignment of putative catalytic domains of ATMKK3, 4 and 5 with those of various MAPKKs. Amino acid sequence of the kinase domain of *Arabidopsis* ATMKK3, 4 and 5, tobacco NPK2 (accession number K31964), *Arabidopsis* MEK1 marked as "At" (AF000977), *Drosophila* Dsor1 (D13782), mouse MEK1 marked as "mouse" (L02526), fission yeast Byr1 (X07445) and budding yeast Ste7 (M14097) were aligned using the PILEUP program of the Wisconsin package version 8 (Genetic Computer Group, Wisconsin, USA). The putative amino acid positions are numbered on the right. The amino acids that are identical in all protein kinases are shown by solid boxes, and similar or conserved amino acids by shaded boxes. The dots indicate gaps introduced to give maximum matching. Roman numerals indicate the eleven major conserved subdomains of protein kinases identified by Hanks and Quinn.[27] (B) Comparison of the deduced amino acid sequences around kinase subdomains VII to VIII between plant MAPKK homologues and MAPKKs of other organisms. Dashes indicate gaps introduced to maximize alignment. Roman numerals indicate the major conserved subdomains of protein kinases identified by Hanks and Quinn.[27] Boxed and bold-type amino acids correspond to the regulatory phosphorylation consensus sequence "SXXX$^{S}/_{T}$." Bold-type amino acids represent those of putative phosphorylation amino acids of plant MAPKKs.

out the hybridization to the DNA gel blot filters of *Eco*RV-digested genomic DNA from the recombinant inbred (RI) lines that had been generated between two ecoytpes, *Landsberg erecta* and Columbia[23] as decribed previously.[24,25] Separation data for restriction fragment length polymorphism (RFLP) markers on the 99 RI lines were analyzed using the Mapmaker program[26] as described by Liu et al.[25] The scoring data of 67 markers (provided by Dr. C. Dean, John Innes Res. Inst., UK) and those of the 129 mi markers[25] were used for the calculation.

3. Results and Discussion

3.1. *Isolation of* Arabidopsis *MAPKK homologues*

Southern blot analysis of *Arabidopsis* genomic DNA with tobacco NPK2 as a probe showed that *Arabidopsis* included a sequence with a similarity to the *NPK2*

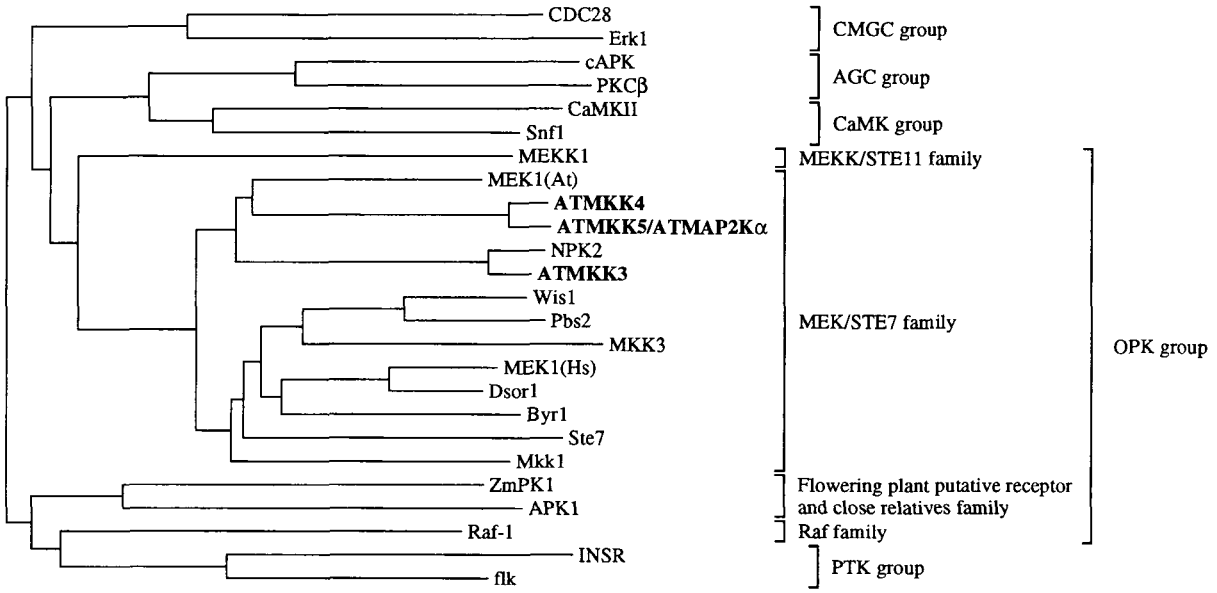

Figure 2. Relationships between eukaryotic protein kinases. The phylogenetic tree shown as a dendrogram was created by the neighbor-joining (N-J) method (the ODEN program package for molecular evolutionary analysis installed in the computer system at the National Institute of Genetics, Mishima, Shizuoka).[33] Genbank/EMBL/DDBJ accession numbers are: CDC28 (X00257), Erk1 (X65198), cAPK (P05132), PKCβ (X04439), CaMKII (D90375), Snf1 (M13971), MEKK1 (U48596), MEK1(At)(AF000977), NPK2 (K31964), Wis1 (X62631), Pbs2 (J02946), MKK3 (L36719), MEK1 (Hs)(L11284), Dsor1 (D13782), Byr1 (X07445), Ste7 (P06784), Mkk1 (D13001), ZmPK1 (X52384), APK1 (Q06548), Raf-1 (125651), INSR (M10051), flk (X13412). The classification of protein kinases is according to Hanks and Quinn.[27] ATMKK3, 4 and 5 are presented in bold type.

gene. In order to isolate the NPK2 homologue in *Arabidopsis*, we performed PCR using the *NPK2*-directed oligonucleotide primers. We amplified a 450-bp PCR product, whose nucleotide sequence showed 76% identity with cDNA for *NPK2* in the overlapping region. We screened *Arabidopsis* cDNA library using the NPK2-like PCR product as probe, and isolated cDNA clone containing nucleotide sequence corresponding to the PCR product. We designated the cDNA clone as ATMKK3 (*Arabidopsis thaliana* mitogen-activated protein kinase kinase 3).

We found that an *Arabidopsis* expressed sequence tag (EST)[18] clone 127H15T7 (GenBank/EMBL/DDBJ accession No. T44940) encoded a putative polypeptide with similarity to MAPKKs in protein kinase subdomains VII to XI. Using a partial cDNA fragment of the EST clone as probe, we isolated two full-length cDNAs which encoded putative MAPKK family protein kinases, and designated them as ATMKK4 and ATMKK5.

The cDNAs for ATMKK3, 4 and 5 were 1700, 1631 and 1501 bp in length, and encoded putative polypeptides with 520, 348 and 366 amino acids, respectively. We found in-frame stop codons in putative 5′-nontranslated regions of the ATMKK4 and 5 cDNAs, but not in the ATMKK3 cDNA. The ATMKK5 cDNA was almost identical to ATMAP2Kα.[16]

We used three strategies to isolate *Arabidopsis* MAPKK genes: 1) cloning using cDNAs for plant MAPKKs as heterologous probes; 2) a database search of the *Arabidopsis* ESTs that have extensive homology to the members of the MAPKK family; 3) PCR using degenerated primer sets for MAPKKs in animals and yeasts. Based on the strategy 3, we tried to amplify MAPKK-like fragments with several primer sets, but obtained no MAPKK-like PCR fragments.

3.2. *Structural analysis of the putative ATMKK3, 4 and 5 proteins*

We compared deduced amino acid sequences of the putative ATMKK3, 4 and 5 proteins with MAPKKs from animals, yeasts and plants (Fig. 1A). The putative ATMKK proteins showed extensive homology to MAPKKs and contained all the 11 conserved subdomains of protein kinases.[27] They also contained conserved amino acid sequences, such as $E^{Y}/_{F}MDXGSL$ in subdomain V, GLXYL in subdomain VIa, $DFG^{I}/_{V}SXXL$ in subdomain VII, VGTXXYMSPERI, in subdomain VIII, $D^{I}/_{V}WSLG$ in subdomain IX, and $F^{I}/_{V}XXCL$ in subdomain XI. Amino acid identities between ATMKK proteins and MAPKKs from animals and yeasts ranged from 27% to 37%, while those between ATMKK proteins and plant MAPKKs were 36 to 85%. ATMKK3 showed high homology to NPK2, a tobacco MAPKK homologue (85% identity). ATMKK4 and 5 were closely related (84% identity).

MAPKKs are activated by phosphorylation of the

conserved amino acids between kinase subdomains VII and VIII.[5] We compared the amino acid sequences in the putative phosphorylation site of plant MAPKKs with those of yeast and animal MAPKKs (Fig. 1B). This region is called the 'L_{12} loop,' and is thought to be involved in the regulation of multiple protein kinases including MAPK, MAPKK and many other protein kinases.[28] Most of the MAPKKs in yeasts and animals contain the $SXXX^{S}/_{T}$ consensus sequence in their phosphorylation site.[2] However, plant MAPKKs contain $^{S}/_{T}XXXXX^{S}/_{T}$ in the 'L_{12} loop' instead. The corresponding sequence of *Arabidopsis* MEK1 is TSSLANS (amino acid residues 218 to 224), which is consistent with both $SXXX^{S}/_{T}$ and $^{S}/_{T}XXXXX^{S}/_{T}$ sequences (Fig. 1B).[15] *Dictyostelium* DdMEK1 also contains both sequences in the phosphorylation site. We found the $^{S}/_{T}XXXXX^{S}/_{T}$ sequence in the catalytic domain of HST7, a MAPKK of the pathogenic fungus *Candida albicans* involved in hyphal formation.[29–31]

In order to classify these plant MAPKKs, we constructed a phylogenetic tree based on the amino acid sequences of their catalytic domains (Fig. 2). Protein kinases constitute a large gene family composed of five major groups, as classified by Hanks et al.[27,32] Plant MAPKKs formed a branch in the MEK/STE7 family of the OPK group and formed a small gene family different from those of yeasts and animals. Plant MAPKKs were classified into three subgroups: subgroup1, *Arabidopsis* MEK1; subgroup2, ATMKK4 and ATMKK5/ATMAP2Kα; and subgroup3, NPK2 and ATMKK3.

3.3. *Detection of protein kinase activity of the recombinant ATMKK3 and ATMKK4 proteins*

To examine whether ATMKKs encode functional protein kinases, the ATMKK3 and ATMKK4 cDNAs were expressed in *E. coli* as a product of a chimeric gene fused with glutathione S-transferase using the pGEX expression vector. The recombinant ATMKK3 and ATMKK4 fusion proteins (named GST-ATMKK3WT and GST-ATMKK4WT, respectively) were partially purified by affinity chromatography on Glutathione-Sepharose (Fig. 3A). We also constructed fusion proteins of GST-ATMKK3K112R and GST-ATMKK4K108R in which lysine residues in the putative ATP-binding site in kinase subdomain II were replaced by arginine residues (Fig. 3A). After incubation of these GST-fusion proteins with [γ-^{32}P]ATP and myelin basic protein (MBP) as a substrate, the proteins were fractionated by SDS-PAGE, and then autoradiographed (Fig. 3B). Two strong bands of phosphorylated proteins (70 and 23 kDa), which correspond to the GST-ATMKK4WT and MBP, respectively, were detected (lane 4). By contrast, intensity of bands corresponding to these two proteins were much reduced when GST-ATMKK4K108R was used (lane 5). This result indicates that ATMKK4 can phosphorylate itself and MBP, and that the Lys is essential for the phosphorylation activity. ATMKK3 is also capable of autophosphorylation (85 kDa in lane 2) and this ability was abolished by replacement of a lysine residue by an arginine (lane 3). The GST control showed no phosphorylation signal (lane 1). These results indicate that both ATMKK3 and ATMKK4 encode functional protein kinases, and that ATMKK4 strongly phosphorylate MBP *in vitro*.

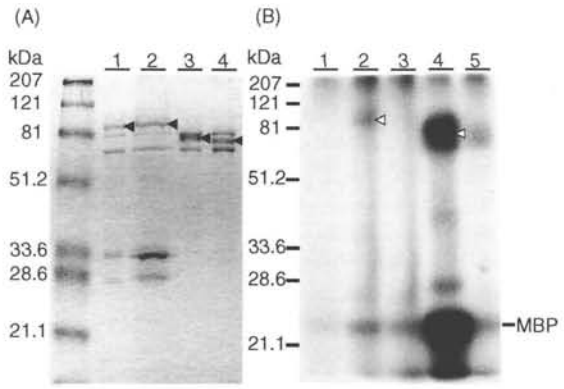

Figure 3. Detection of protein kinase activity of the recombinant ATMKK3 and ATMKK4 proteins. (A) Affinity purified GST-fusion ATMKK3 and ATMKK4 proteins. Expression and purification of GST-ATMKK3WT (lane 1), GST-ATMKK3K112R (lane 2), GST-ATMKK4WT (lane 3) and GST-ATMKK4K108R (lane 4) proteins in *E. coli* cells were carried out according to Mizoguchi et al.[21] Partially purified GST-fusion proteins (1 μg of GST-ATMKK3WT, GST-ATMKK4WT and GST-ATMKK4K108R; 1.5 μg of GST-ATMKK3K112R) were separated with SDS-PAGE and stained with Coomassie Brilliant Blue (CBB). Closed triangles indicate CBB-stained proteins corresponding to the GST-fusion product. Molecular masses of the protein standards shown on the left in kilodaltons. (B) Autophosphorylation and phosphorylation of MBP by GST-ATMKK3 and GST-ATMKK4 proteins. Glutathione sepharose-bound proteins (120 ng) were incubated with MBP and [γ-^{32}P] ATP and subjected to SDS-PAGE. The gel was dried and autoradiographed. GST, GST-ATMKK3WT, GST-ATMKK3K112R, GST-ATMKK4WT and GST-ATMKK4K108R were used in lane 1, 2, 3, 4 and 5, respectively. Open triangles in lanes 2 and 4 are phosphorylated proteins correspond to GST-ATMKK3WT and GST-ATMKK4WT, respectively. Molecular masses of the protein standards shown on the left in kilodaltons.

3.4. *Northern and Southern blot analysis of the ATMKK3, 4, and 5 genes*

To analyze the expression of the ATMKK3, 4 and 5 genes in various *Arabidopsis* organs, we carried out Northern blot analyses under high stringency conditions (Fig. 4). The ATMKK3 mRNA was detected equally in all the organs examined. The levels of the ATMKK4 and 5 mRNAs detected were relatively higher in stems and leaves than in flowers and roots. The ATMKK3, 4 and 5

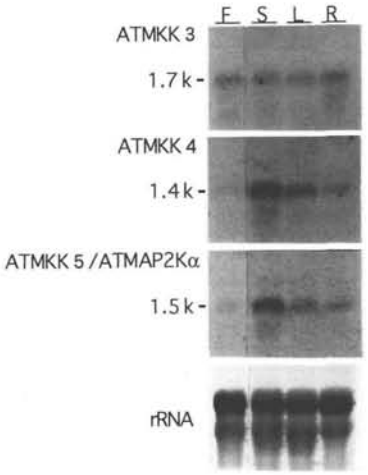

Figure 4. Northern blot analysis of ATMKK3, 4 and 5 transcripts in various *Arabidopsis* organs. Total RNA (40 μg) prepared from flowers (F), stems (S), leaves (L), and roots (R) was subjected to electrophoresis on 1% agarose gels that contained formaldehyde. A nylon membrane blotted with RNA was hybridized with the ^{32}P-labeled probes of cDNAs for ATMKK3, ATMKK4 or ATMKK5 and then autoradiographed. The sizes of the ATMKK3, 4 and 5 mRNAs in kb are indicated. The panel below labeled rRNA is a methylene-blue-stained nylon membrane blotted with total RNA. The two prominent bands are rRNAs.

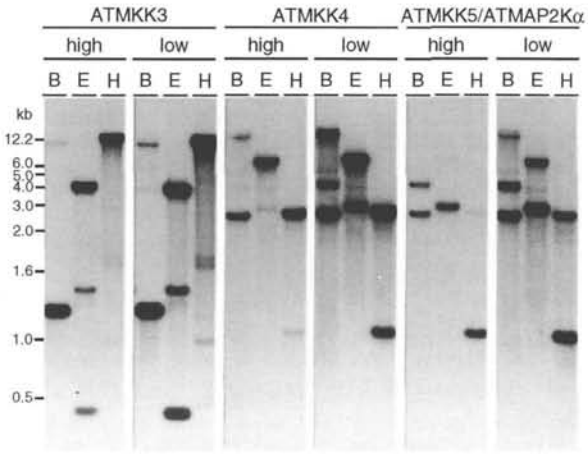

Figure 5. Southern blot analysis of ATMKK3, 4 and 5. Genomic DNA (4 μg) was digested with *Bam*HI (B), *Eco*RI (E) or *Hin*dIII (H), fractionated on 0.7% agarose gels and transferred to the nylon membranes. Filters were hybridized with the ^{32}P-labeled probes of cDNA for ATMKK3, ATMKK4 or ATMKK5 at 42°C and washed in either 0.5 × SSC/0.5% SDS at 37°C (low) or 0.1 × SSC/0.1% SDS at 65°C (high). The sizes of the DNA markers in kb are indicated.

mRNAs did not accumulate following dehydration, high or low temperature stresses, or ABA treatment (data not shown). These observations suggest that ATMKK3 may have a different biological from ATMKK4 and 5. The structures and expression patterns of ATMKK4 and 5 were very similar, which suggest their functional similarity. We found a diversity of amino acid sequences and gene expression among the three *Arabidopsis* MAPKKs. This diversity also supports the presence in plants of multiple MAPK cascades with various functions.

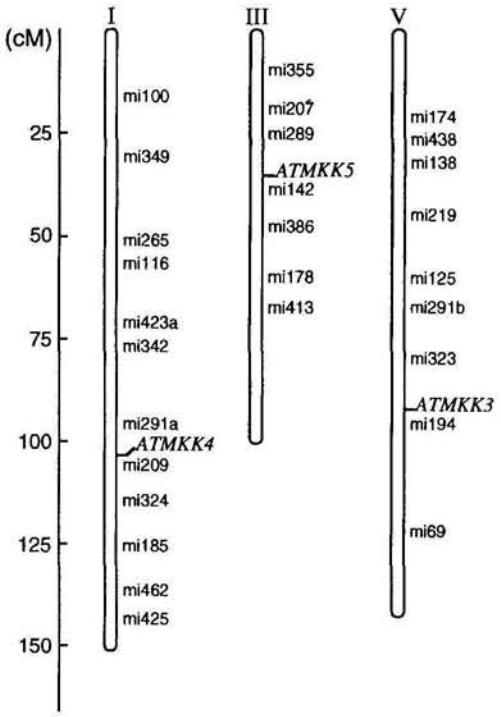

Figure 6. Chromosomal map positions of the ATMKK3, 4 and 5 genes. Map positions of ATMKK3, 4 and 5 were determined by RFLP mapping using P1 genomic clones. The scale for chromosomes of *A. thaliana* is shown on the left in centimorgan (cM).

Southern blot analyses were carried out to estimate the numbers of related genes of ATMKK3, 4 and 5 in the *Arabidopsis* genome (Fig. 5). The results suggest that *Arabidopsis* may not contain genes closely related to ATMKK3, 4 and 5; however, several MAPKK related genes may exist in the *Arabidopsis* genome because additional bands were detected in low stringency conditions in ATMKK3, 4 and 5.

3.5. Map positions of the ATMKK3, 4 and 5 genes on Arabidopsis *chromosomes*

We determined map positions of three ATMKK genes on *Arabidopsis* chromosomes by RFLP mapping using P1 genomic clones.[24] Using P1 clones as probes, we could easily obtain an RFLP pattern since a P1 genomic clone contains 70–90 kb of genomic DNA. The results of RFLP mapping of the ATMKK3, 4 and 5 genes were shown in Fig. 6. The ATMKK3 gene is located between RFLP markers, mi323 and mi194 on chromosome V. The ATMKK4 gene is located near an RFLP marker, mi106, on chromosome I. The ATMKK5 gene is located near an RFLP marker, mi142, on chromosome III. Although

there are a few mutants mapped near those three genes, the relationships between ATMKKs and the phenotypes of these mutants are not clear.

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