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The TMK1 Gene from Arabidopsis Codes for a Protein with Structural and Biochemical Characteristics of a Receptor Protein Kinase

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Genomic and cDNA clones that code for a protein with structural and biochemical properties similar to the receptor protein kinases from animals were obtained from Arabidopsis. Structural features of the predicted polypeptide include an amino-terminal membrane targeting signal sequence, a region containing blocks of leucine-rich repeat elements, a single putative membrane spanning domain, and a characteristic serine/threonine-specific protein kinase domain. The gene coding for this receptor-like transmembrane kinase was designated TMK1. Portions of the TMK1 gene were expressed in *Escherichia coli*, and antibodies were raised against the recombinant polypeptides. These antibodies immunodecorated a 120-kD polypeptide present in crude extracts and membrane preparations. The immunodetectable band was present in extracts from leaf, stem, root, and floral tissues. The kinase domain of TMK1 was expressed as a fusion protein in *E. coli*, and the purified fusion protein was found capable of autophosphorylation on serine and threonine residues. The possible role of the TMK1 gene product in transmembrane signaling is discussed.

INTRODUCTION

Intercellular signaling is an integral component of growth and development in all eukaryotes. In many cases, signal perception in animals and fungi is mediated by well-characterized cell-surface receptors (Pawson, 1991). Whereas these receptors come in a variety of biochemical forms, most are membrane spanning proteins with an extracellular ligand binding domain and a signal-transducing cytoplasmic domain. In animals, a large family of cell-surface receptors, the receptor protein kinases, contain a single transmembrane domain and a cytoplasmic protein-kinase domain, which is regulated by the extracellular ligand binding domain (reviewed in Yarden and Ullrich, 1988; Ullrich and Schlessinger, 1990). Members of this family include the epidermal growth factor receptor, the insulin receptor, and the platelet-derived growth factor receptor.

Intercellular communication is also fundamental to the growth and development of higher plants. However, virtually nothing is known about the molecular mechanisms that mediate cellular responses to such signals as plant hormones (Napier and Venis, 1991), foreign and endogenous carbohydrate elicitors (Farmer et al., 1989), and even peptides (Pearce et al., 1991). A growing body of evidence suggests that responses to many of these signals are mediated by cell-surface receptors (Barbier-Brygoo et al., 1989; Farmer et al., 1989; Hooley et al., 1991; Lomax and Hicks, 1992; Reymond et al., 1992) and may involve kinase-mediated phosphorylation (Farmer et al., 1989; Reymond et al., 1992).

Recent reports on the cloning of genes from maize (Walker and Zhang, 1990) and Brassica (Stein et al., 1991) that code for proteins that are structurally similar to the receptor kinases from animals have provided the first evidence that this family of cell-surface receptors may be present in higher plants. Both of these genes code for proteins with single transmembrane domains and carboxy-terminal domains that are characteristic of eukaryotic protein kinases (Hanks and Quinn, 1991). Both genes also encode amino-terminal domains that show sequence homology to the S-allele protein from Brassica (Walker and Zhang, 1990; Stein et al., 1991). Whereas no biochemical function has been assigned to the products of either of these genes, physical linkage of the putative receptor kinase gene from Brassica to the Brassica S-locus provides strong circumstantial evidence that this protein may play a role in mediating the incompatibility interaction between pollen tube and stigma (Stein et al., 1991).

We report here the molecular cloning of an Arabidopsis gene, designated TMK1, coding for a receptor-like protein kinase. The coding sequence of this gene predicts a protein with topological features similar to the maize and Brassica receptor kinases but with a completely divergent amino-terminal domain. We present biochemical evidence that the product of this gene is expressed in a variety of tissue types and that the

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protein is a functional protein kinase capable of autophosphorylation on serine and threonine residues.

RESULTS

Isolation and Characterization of the TMK1 Gene

The transmembrane kinase (TMK1) gene was identified serendipitously in the course of a chromosome walk to the ethylene response (ETR1) locus in Arabidopsis (~7 cM from AP1 and 3 cM from CLV2 on chromosome 1) (Chang et al., 1992). DNA fragments of a cosmid clone that is genetically linked to the ETR1 locus were used as probes to screen an Arabidopsis leaf cDNA library. The LC5 cDNA clone represented one of three independent cDNA families detected by the cosmid DNA. A full-length cDNA of the TMK1 gene was obtained as a composite clone comprised of the LC5 clone and a cDNA clone, derived from polymerase chain reaction, representing the 5′ end of the TMK1 mRNA. The full-length cDNA consists of 3243 nucleotides; this is consistent with the estimated size of the message that is detected on RNA gel blots (RNA blots not shown). A restriction map based on sequence analysis of the cDNA and genomic clones is shown in Figure 1. Hybridization of the TMK1 gene sequences to Arabidopsis genome blots indicated that the gene has no extremely close relatives in the Arabidopsis genome, as shown in Figure 2. Additional weakly hybridizing bands were detected under low stringency (data not shown).

DNA sequence analysis of the TMK1 gene was initiated to investigate the possibility that TMK1 represented the ETR1 gene. However, comparison of the TMK1 sequences in ETR1 mutant and wild-type DNA revealed no nucleotide differences. More recently, high-resolution restriction fragment length polymorphism mapping of the ETR1 locus has confirmed that the TMK1 gene lies outside the genetically defined region containing the ETR1 locus (data not shown).

Sequence Analysis of the TMK1 Gene

The derived amino acid sequence for the TMK1 gene, demonstrated in Figure 3A, contains 942 residues and has a calculated molecular mass of 102.4 kD. A comparison of genomic and cDNA sequences (on file with GenBank, accession number L00670) indicates that a single 84-bp intron is located between the first and second nucleotides of the codon corresponding to Val 766.

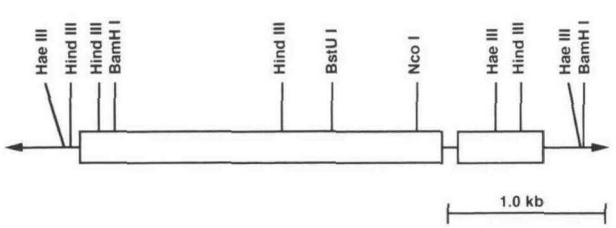

Figure 1. Restriction Map of the TMK1 Gene.

The 5′ end is on the left. The restriction sites are based on DNA sequence analysis of genomic and cDNA clones. Boxes indicate positions of the two exons.

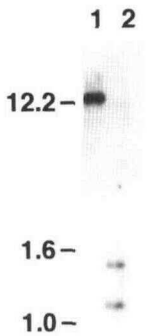

Figure 2. Genome Blot of Arabidopsis DNA Using the Two Internal HindIII Fragments of the TMK1 Gene as a Probe.

Lane 1, EcoRI digest; lane 2, HindIII digest. There are no EcoRI restriction sites within the TMK1 gene.

Hydropathy analysis (Kyte and Doolittle, 1982) of the derived TMK1 protein sequence revealed two hydrophobic domains. A hydrophobic domain near the amino terminus is indicative of a leader sequence associated with membrane targeting (von Heijne, 1990). A second hydrophobic domain between amino acids 479 and 504 is characteristic of a membrane spanning domain (McCrea et al., 1988). The carboxy-terminal side of the putative membrane spanning domain contains positively charged residues that potentially form a stop transfer sequence (Weinstein et al., 1982). These topological features of the predicted TMK1 gene product closely resemble those of the receptor class of animal protein kinases that are targeted to the plasma membrane, and that have a single membrane spanning domain, an extracytoplasmic amino-terminal domain, and a cytoplasmic carboxy-terminal kinase domain (Ullrich and Schlessinger, 1990). Consistent with this possible location and orientation for the TMK1 gene product is the presence of six characteristic N-linked glycosylation sites on the putative amino-terminal side of the membrane spanning domain. Such glycosylation sites are often associated with extracytoplasmic domains of membrane proteins including the animal receptor protein kinases (Yarden and Ullrich, 1988; Ullrich and Schlessinger, 1990). The topological features of the TMK1 gene product are summarized in Figure 3B.

Computer analysis of the primary amino acid sequence revealed homology to protein sequences in the Protein Identification Resource data base (version 32.0) for both the putative cytoplasmic and extracytoplasmic domains, as shown in Figure 4. The putative cytoplasmic domain contains all 11 conserved subdomains of eukaryotic protein kinases in the proper order (Hanks and Quinn, 1991). The kinase domain of the TMK1 is

A

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MKKRRTFLLFSFTFLLLLSLSKADSDGDLSAMLSLKKSLNPPSSFGWSDP  50
DPCKWTHIVCTGTKRVTRIQIGHSGLQGTLSPDLRNLSELERLELQWNNI 100
SGPVPSLSGLASLQVLMLSNNNFDSIPSDVFQGLTSLQSVEIDNNPFKSW 150
EIPESLRNASALQNFSANSANVSGSLPGFLGPDEFPGLSILHLAFNNLEG 200
ELPMSLAGSQVQSLWLNGQKLTGDITVLQNMTGLKEVWLHSNKFSGPLPD 250
FSGLKELESLSLRDNSFTGPVPASLLSLESLKVVNLTNNHLQGPVPVFKS 300
SVSVDLDKDSNSFCLSSPGECDPRVKSLLLIASSFDYPPRLAESWKGNDP 350
CTNWIGIACSNGNITVISLEKMELTGTISPEFGAIKSLQRIILGINNLTG 400
MIPQELTTLPNLKTLDVSSNKLFGKVPGFRSNVVVNTNGNPDIGKDKSSL 450
SSPGSSSPSGGSGSGINGDKDRRGMKSSTFIGIIVGSVLGGLLSIFLIGL 500
LVFCWYKKRQKRFSGSESSNAVVVHPRHSGSDNESVKITVAGSSVSVGGI 550
SDTYTLPGTSEVGDNIQMVEAGNMLISIQVLRSVTNNFSSDNILGSGGFG 600
VVYKGELHDGTKIAVKRMENGVIAGKGFAEFKSEIAVLTKVRHRHLVTLL 650
GYCLDGNEKLLVYEYMPQGTLSRHLFEWSEEGLKPLLWKQRLTLALDVAR 700
GVEYLHGLAHQSFIHRDLKPSNILLGDDMRAKVADFGLVRLAPEGKGSIE 750
TRIAGTFGYLAPEYAVTGRVTTKVDVYSFGVILMELITGRKSLDESQPEE 800
SIHLVSWFKRMYINKEASFKKAIDTTIDLDEETLASVHTVAELAGHCCAR 850
EPYQRPDMGHAVNILSSLVELWKPSDQNPEDIYGIDLDMSLPQALKKWQA 900
YEGRSDLESSTSSLLPSLDNTQMSIPTRPYGFAESFTSVDGR 942
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B

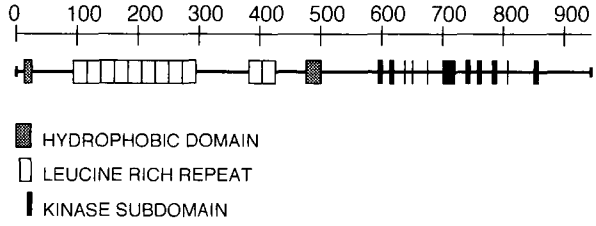
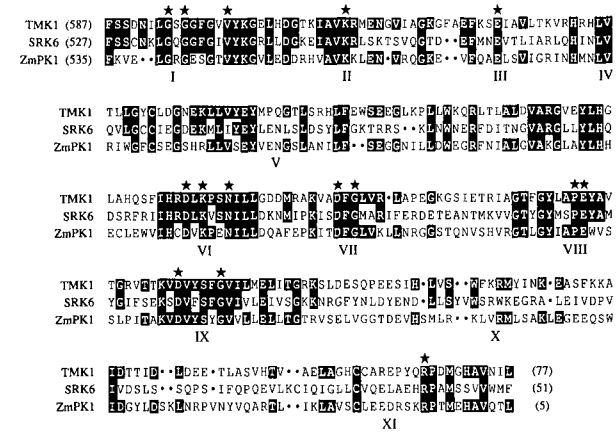


Figure 3. Structural Characteristics of the TMK1 Gene Product.

(A) Deduced amino acid sequence of the TMK1 protein. The putative signal sequence and transmembrane domain are indicated by the underlines. The potential N-linked glycosylation sites are indicated by superior dots. Diagnostic sequences for serine/threonine protein kinases are shown with dashed underlines. LRRs are indicated in bold lettering. The nucleotide sequence has been submitted to GenBank and assigned accession number L00670.

(B) Topological features of the derived amino acid sequence of the TMK1 gene are shown.

A



B

Leucine Rich Repeat (LRR) Consensus Sequence	Number of Repeats	Protein
L * * L * * L * * L * L * * N * α * G * αP	11	*Arabidopsis* TMK1[1]
P * * - α * * L * * L * * L * L * * N * α * * α	26	yeast CYR1[2]
P * * L L * * L * * L * * L * L * * N * L * * L	7	human GpIb[3]
P * * L L * * * * * L * * L * L * * N * L * * L	9	human LRG[4]
P * * L F * H * * N L * * L * L * * N * L * * L	15	*Drosophila* Toll[5]
P * * * F * * L * * L * * L D L S * N *I/L* * I	41	*Drosophila* Chaoptin[6]
L * * L * α * * N * L * * α	3	human trk[7]

Figure 4. Conserved Features of the TMK1 Amino Acid Sequence.

(A) Sequence similarity of the kinase domain of the TMK1 gene to the kinase domains of the SRK6 gene from Brassica and the ZmPK1 gene from maize is shown. Residues identical to those in TMK1 are indicated by reverse type. The positions of the 11 protein kinase subdomains are shown in Roman numerals, and the canonical highly conserved kinase residues are indicated by stars (Hanks and Quinn, 1991). The numbers in parentheses are the amino- and carboxy-terminal residues not included in the alignment.

(B) Comparison of LRR consensus sequence of TMK1 to consensus sequences of other LRR-containing proteins. Nonconserved amino acids are indicated by an asterisk. Superscripts refer to references for the proteins: (1) present study; (2) Kataoka et al. (1985); (3) Lopez et al. (1988); (4) Takahashi et al. (1985); (5) Hashimoto et al. (1988); (6) Reinke et al. (1988); and (7) Schneider and Schweiger (1991).

most closely related to the predicted receptor-like kinase proteins of maize ZmPK1 (39% identity) (Walker and Zhang, 1990) and Brassica SRK (35% identity) (Stein et al., 1991). The amino acid sequence alignment for all three plant genes is shown in Figure 4A. Unlike the receptor kinases from animals, which generally show a substrate specificity for tyrosine residues (Yarden and Ullrich, 1988; Hunter, 1991), all three receptor kinase-like genes identified in plants code for the consensus amino acids (subdomains VIb and VIII) that are characteristic of serine/threonine substrate specificity (Hanks and Quinn, 1991).

The putative extracytoplasmic domain contains 11 imperfect repeats of a 22-amino acid leucine-rich motif. These repeats are arranged in two blocks: one block of nine repeats and one block of two repeats (Figure 3B). A consensus

sequence for this leucine-rich repeat (LRR) is found in a number of diverse proteins from mammals (Takahashi et al., 1985; Lopez et al., 1988; Schneider and Schweiger, 1991), Drosophila (Hashimoto et al., 1988; Reinke et al., 1988), and yeast (Kataoka et al., 1985) (Figure 4B). These different LRR-containing proteins share two features: most are membrane-associated proteins, and in many cases the LRR domains are implicated in protein–protein interactions (Rothberg et al., 1990). Only one of these proteins, the human *trk* gene product, contains a protein kinase domain (Schneider and Schweiger, 1991). It should be noted that the LRR-containing domain of the Arabidopsis TMK1 gene does not resemble the S-protein homologous domains of the maize and Brassica receptor-like kinase genes (Walker and Zhang, 1990; Stein et al., 1991).

Immunological Detection of the TMK1 Gene Product

To obtain more direct information about the protein product of the TMK1 gene, antibodies against the LRR domain, the kinase domain, and the carboxy-terminal portion of the TMK1 gene were prepared. Domain-specific sequences were cloned into the pGEX-2T expression vector (Pharmacia) and expressed in *Escherichia coli.* Serum obtained from rabbits immunized with the purified recombinant proteins was cleared with *E. coli* proteins and used to immunodecorate protein blots of PAGE-fractionated protein extracts from Arabidopsis. Immunoblots of plant membrane preparations using polyclonal antibodies against either the LRR or the kinase domain showed a single immunodecorated band at ~120 kD, as indicated in Figure 5. Using the LRR-specific antibodies, the 120-kD band was immunodetectable in crude extracts from roots, stems, leaves, and flowers of Arabidopsis, indicating that antigen of the expected molecular weight is present in all organ systems of the Arabidopsis plant, as shown in Figure 6.

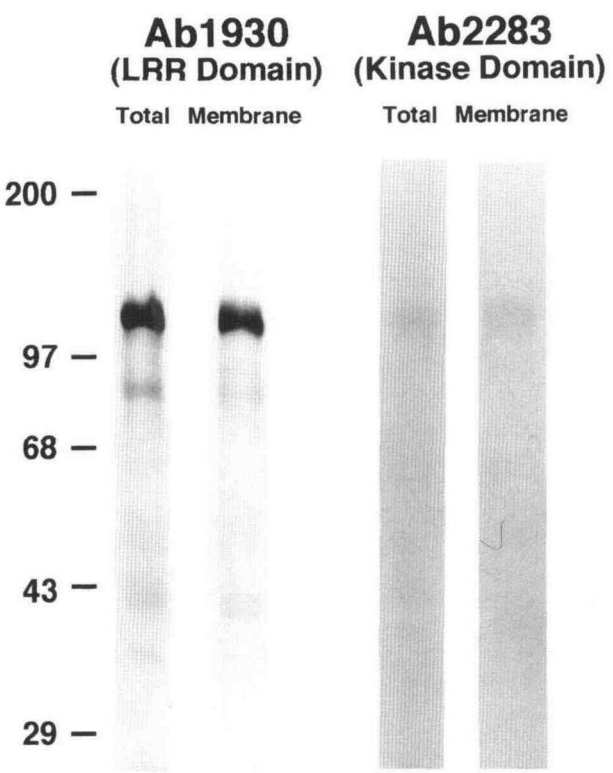


Figure 5. Immunodetection of the Native TMK1 Protein in Crude Extracts and Membrane Preparations of Arabidopsis with Domain-Specific Antibodies.

Antibodies raised against the LRR domain (Ab1930), or against the kinase domain (Ab2283) were used to immunodecorate protein blots of fractionated whole plant extracts and Triton X-100 solubilized membrane preparations. Migration positions of molecular mass markers are indicated in kilodaltons.

Protein Kinase Activity of the Kinase Domain of TMK1 Expressed in *E. coli*

To investigate the protein kinase activity of the TMK1 gene product, the carboxy-terminal half of the protein was expressed as a fusion protein in *E. coli.* The carboxy-terminal half of the TMK1 gene was cloned into the pMAL-c vector (New England Biolabs) that generates a fusion protein in which the kinase domain of TMK1 is fused to the carboxy terminus of the maltose binding protein (Maina et al., 1988). Using this vector, a recombinant fusion protein of the expected molecular weight (88 kD) was expressed in *E. coli* under inducing conditions, as shown in Figure 7A. The fusion protein was affinity purified by binding to amylose resin (Maina et al., 1988), and a protein kinase assay using γ-^{32}P-ATP was carried out on the affinity-purified protein. Autoradiography of the SDS-PAGE–fractionated protein (Laemmli, 1970) indicated that a protein band of 88 kD was labeled with ^{32}P. These results provide direct biochemical evidence that the kinase-like domain of the TMK1 gene is a functional protein kinase that is capable of autophosphorylation. To determine the amino acid specificity of the autophosphorylating activity, the ^{32}P-labeled band was transferred to nylon membrane and subjected to acid hydrolysis for 1 or 4 hr. Analysis by thin-layer electrophoresis indicated the presence of labeled phosphoserine and phosphothreonine but not of phosphotyrosine (Figure 7B).

DISCUSSION

The polypeptide encoded by the TMK1 gene from Arabidopsis has many structural features that indicate a receptor-like function for the gene product. The membrane targeting amino terminus, the putative protein–protein interaction domain (LRR), the single transmembrane domain, and the protein kinase domain present an overall structure that is very similar to the large

class of tyrosine receptor kinases found in the animal kingdom (Yarden and Ullrich, 1988; Ullrich and Schlessinger, 1990). The biochemical properties of the native TMK1 polypeptide and its recombinant derivatives—the membrane association, solubility in 1% Triton X-100, reversible binding to lectin columns (data not shown), and autophosphorylating activity of the kinase domain—are also consistent with a biological function analogous to that of the animal receptor protein kinases.

The enzymatic function of the kinase domain of the TMK1 gene product was originally inferred from its similarity to the large number of protein kinase sequences available in the data base. Amino acid sequence comparisons indicated that the TMK1 kinase domain is most closely related to the kinase-related domains of the two known receptor kinase-like genes from plants: ZmPK1 from maize (39% identity) and the SRK gene from Brassica (35% identity). All three of these genes contain the subdomains that are diagnostic of protein kinases with serine/threonine substrate specificity (Hanks and Quinn, 1991). The results reported here provide direct evidence that the TMK1 kinase activity is specific for serine and threonine, at least with respect to autophosphorylation. On the other hand, comparison of the TMK1 kinase domain sequence to the sequences of animal protein kinases indicated that the highest degree of identity is with the class of kinase-like transforming proteins related to members of the c-src family (27% identity to c-fgr) (Katamine et al., 1988). Members of this class of kinases are membrane associated, although not membrane spanning, and all show a substrate specificity for tyrosine. The plant receptor-like kinases show a much lower degree of identity to the animal serine/threonine-specific receptor kinases such as the activin receptor (16% identity) (Mathews and Vale, 1991). Thus, these plant genes may represent a distinct class of protein kinases. They show sequence similarity to a class of tyrosine kinases but autophosphorylate on serine and threonine residues.

The presence of the LRRs in the putative extracytoplasmic domain of the TMK1 gene presents some intriguing possibilities concerning the biochemical mechanism of action of the TMK1 gene product. The LRR motif is represented in a variety of proteins with a diversity of functions. Two common features for many of these proteins are a plasma membrane localization and an implication of the LRR motifs in strong

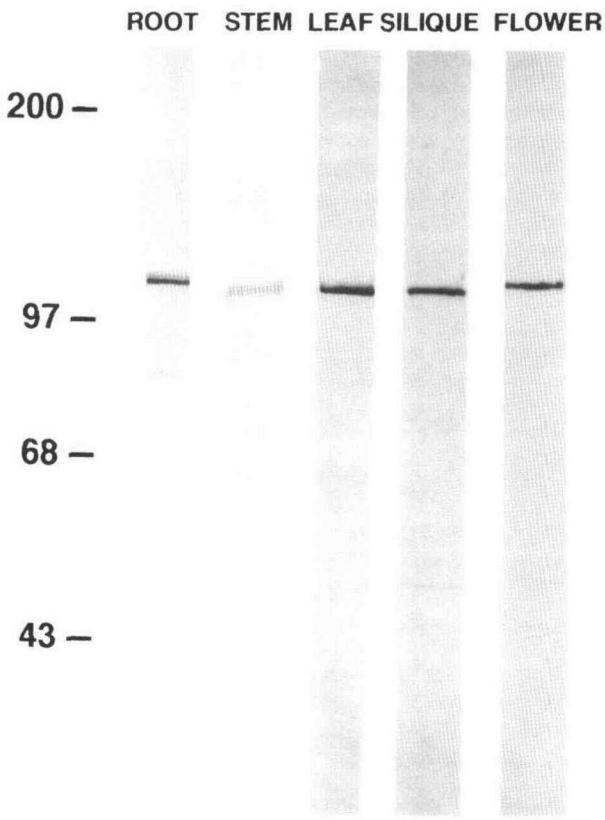


Figure 6. Immunodetection of the TMK1 Protein in Different Tissues of Arabidopsis.

Crude extracts of individual tissues were fractionated on SDS-PAGE, blotted to nitrocellulose, and immunodecorated with the anti–LRR antibody 1930. Migration positions of molecular mass markers are indicated in kilodaltons.

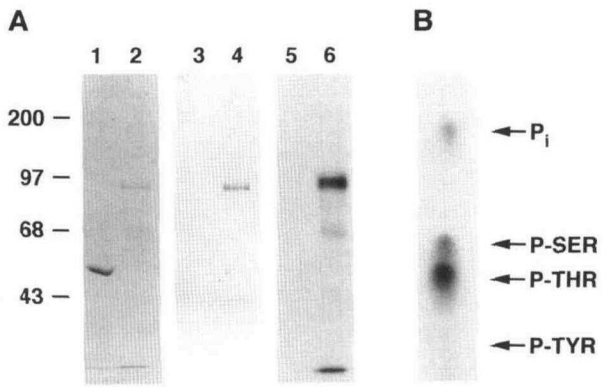


Figure 7. Autophosphorylation of the TMK1 Kinase Domain.

(A) Evidence for autophosphorylating activity of the TMK1 kinase domain expressed in *E. coli*. Fusion proteins consisting of the maltose binding protein fused at the carboxy terminus to either β-galactosidase-a (lanes 1, 3, 5) or to the kinase domain of TMK1 (lanes 2, 4, 6) were affinity purified by binding to amylose resin. The bound fusion protein was then assayed for kinase activity and subjected to SDS-PAGE. Lanes 1 and 2 represent the Coomassie-stained polypeptide profile; lanes 3 and 4 are from an immunoblot using the carboxy-terminal–specific antibody 2513; and lanes 5 and 6 are from an autoradiogram of phosphorylated polypeptides.

(B) Phosphoamino acid analysis of autophosphorylated fusion protein. Fusion protein labeled with γ-^{32}P-ATP was hydrolyzed for 4 hr, and the phosphoamino acids separated by thin-layer electrophoresis and visualized by autoradiography. Arrows denote the positions of inorganic phosphate (P_i), phosphoserine (P-SER), phosphothreonine (P-THR), and phosphotyrosine (P-TYR), as determined by standards.

protein–protein interactions (Rothberg et al., 1990). Of particular interest are the *Toll* gene of Drosophila (Hashimoto et al., 1988), human platelet glycoprotein Ib (Lopez et al., 1988), and the human proto-oncogene *trk* (Schneider and Schweiger, 1991). The protein products of all three genes are targeted to the plasma membrane and are composed of an extracytoplasmic domain with LRR motifs, a single transmembrane domain, and a carboxy-terminal cytoplasmic domain containing recognized signal transduction–related sequences (a tyrosine kinase domain in the case of the *trk* gene). Genetic, physiological, and/or structural evidence has indicated that all three of these gene products function as cell-surface receptors.

The LRR blocks located on the putative extracytoplasmic domain of the TMK1 polypeptide may play a functional role in transmembrane signaling. In the case of the tyrosine receptor kinases, there is biochemical evidence that transmembrane signaling occurs by a mechanism in which ligand binding causes a conformational change that favors dimerization of the extracytoplasmic domains. According to theory, this dimerization results in the juxtaposition of the cytoplasmic kinase domains that are thought to cross-activate each other (Ullrich and Schlessinger, 1990). The activated kinases phosphorylate substrate proteins within the cell, resulting in transduction of the signal. The LRR domains in the TMK1 gene product could play a role in dimerization either through direct self-interaction or through interaction with an unidentified protein.

The biological function of the TMK1 gene product is unknown. Protein gel blots indicate that the protein is expressed in all major organ systems at a level sufficient to be immunodetectable in crude protein extracts. Immunocytochemical localization studies currently underway may provide some clues as to the function of the protein. A more direct approach toward determining function will be the expression in transgenic plants of altered forms of the TMK1 gene. Truncated forms of the tyrosine receptor kinases that lack the extracytoplasmic domain often show constitutive kinase activity, commonly resulting in cellular transformation (Ullrich and Schlessinger, 1990). In contrast, over-expressed receptor kinases with truncated kinase domains may act as dominant inhibitors of the native gene products (Ueno et al., 1991, 1992).

The molecular mechanisms by which plant cells perceive and transduce external signals have yet to be elucidated. There is a growing body of evidence that suggests rapid phosphorylation of membrane-associated proteins is coupled to these early signaling events (Farmer et al., 1989; Reymond et al., 1992). The biochemical analysis of recombinant products of genes such as TMK1, which are obtained via molecular cloning techniques and identified strictly on the basis of their primary sequences, provides an additional tool with which to study signal transduction processes in plants. The work reported here on the TMK1 gene provides both structural and biochemical data that are consistent with a function for this gene product in transmembrane signaling similar to that of the receptor protein kinases in animals. The identification of at least two different classes of receptor kinase-like proteins, those containing Brassica S-allele homology and those containing LRRs, indicates that these transmembrane kinases may play multiple roles in signal transduction in plants.

METHODS

Plant Material

Arabidopsis thaliana ecotype Columbia was used for all experiments. Plants were grown under continuous light (combined incandescent and fluorescent, 150 $\mu E\ m^{-2}\ sec^{-1}$) at 22°C and 70% relative humidity in a controlled environment chamber. Alternatively, for some biochemical studies, plants were germinated and grown in liquid culture in MS medium (Murashige and Skoog, 1962) (Sigma) containing 2% sucrose, 100 mg/L inositol, 1 mg/L nicotinic acid, 1 mg/L pyridoxine HCl, and 10 mg/L thiamine HCl on a rotary shaker.

Cloning and Sequencing of the TMK1 Gene

A genomic DNA cosmid clone (designated n11) containing the TMK1 gene was obtained as a result of a chromosome walk to the ETR1 locus in Arabidopsis. DNA fragments of n11 were subcloned into plasmid vector pGEM-7Zf(+) (Promega). Using a 1.4-kb HindIII subclone of n11 as a probe, multiple copies of a single cDNA clone, designated LC5, were obtained from an amplified leaf cDNA library provided by Nigel Crawford (University of California at San Diego). The 5′ end of the TMK1 mRNA was obtained by polymerase chain reaction (PCR) extension (Frohman et al. 1988) using first-strand cDNA synthesized from seedling poly(A)$^+$ RNA. The amplified PCR fragments were cloned into pGEM-7Zf(+). Nucleotide sequences of the cDNA and genomic subclones were obtained according to Sanger et al. (1977) using Sequenase (version 2.0) (U.S. Biochemical) and synthetic oligonucleotide primers. Both strands of the genomic DNA clones were sequenced, including 318 bp upstream of the mRNA start site and 140 bp downstream of the polyadenylation site. The cDNA sequence was obtained on both strands, except for 15% of one strand. Finally, 14 of the 5′ PCR clones were sequenced; two of these clones extended 8 bases further than the other 12 clones and were assumed to represent the 5′ end of the mRNA.

DNA Gel Blot Analysis

Genomic DNA blot analysis was performed according to Sambrook et al. (1989). Hybridization was carried out for 16 hr at 65°C in 5 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4) buffer. For normal stringency, the final wash was 20 min in 0.1 × SSPE at 65°C; for low stringency, hybridization and washes were performed at 42°C.

Construction of Expression Plasmids

The region of TMK1 containing the leucine-rich repeat (LRR) domains was obtained as a 1.15-kb HindIII subclone of the LC5 cDNA clone. This HindIII fragment was end filled to create blunt ends (Sambrook et al., 1989) and 10-mer EcoRI linkers (Promega) were added by blunt-end ligation (Sambrook et al., 1989). The modified fragment was then cloned into the EcoRI site of vector pGEX 2T (Pharmacia). The resultant

construct, designated pGST-LRR, was expected to express a fusion protein containing that portion of the TMK1 gene corresponding to amino acids 37 to 420. A plasmid expressing a portion of the kinase domain was constructed in the same manner—taking a 538-bp BstUI/NcoI fragment, end filling, adding 10-mer EcoRI linkers, and then ligating into the EcoRI site of pGEX 2T. This construct, designated pGST-PK, was expected to express a fusion protein containing that portion of the TMK1 gene corresponding to amino acids 525 to 705. A plasmid expressing the carboxy-terminal region of the TMK1 gene was generated from a 551-bp HaeIII genomic subclone. After end filling to create blunt ends, BamHI 8-mer linkers (Promega) were added, and the resulting fragment was ligated into the BamHI site of the pGEX 2T vector. The resulting construct, designated pGST-CT, was expected to express a region of the TMK1 gene corresponding to amino acids 844 to 942 (the carboxy terminus). All of the above pGEX 2T-derived constructs expressed fusion proteins in which the TMK1 sequences were fused to the carboxy terminus of the glutathione-S-transferase enzyme.

For the expression of the enzymatically active protein kinase domain, the following construct was used. The 1.4-kb HindIII fragment of the LC5 cDNA clone was cloned into the HindIII site of the pGST-CT construct described above. From this construct, a 1.5-kb BstU1-EcoRI fragment containing the carboxy-terminal portion of TMK1, from Arg527 through the carboxy terminus and including 3′ untranslated sequences, was isolated and a 10-mer EcoRI linker added to the BstU1 site. This fragment was cloned into the pMAL-c expression vector (New England Biolabs), which had been previously modified by insertion of 8-mer EcoRI linkers into the StuI site of the polylinker. The resultant construct, designated pMAL-c-KCT, encoded an 88-kD fusion protein consisting of an amino-terminal maltose binding protein region of 42 kD and a carboxy-terminal TMK1 kinase domain of 46 kD. The amino acid sequence at the junction is Arg-Gly-Ile-Pro-Arg (the first Arg of this junction sequence represents the last residue of the maltose binding protein, and the last Arg of this sequence represents the first residue of the TMK1 cytoplasmic domain).

Expression of Recombinant Proteins and Preparation of Antibodies

Fusion proteins were expressed in *Escherichia coli* according to Smith and Johnson (1988). The LRR fusion protein and the partial kinase domain antibodies were obtained as inclusion bodies and partially purified according to the method of Sambrook et al. (1989). The carboxy-terminal fusion protein was expressed as a soluble protein and affinity purified on glutathione-Sepharose (Sigma) according to Smith and Johnson (1988). The recombinant proteins were sent to Promega for immunization of rabbits. Antisera were collected and tested after four immunizations. Antiserum Ab1930 was prepared from the LRR recombinant protein, and Ab2283 and Ab2513 were prepared from the kinase domain and the carboxy terminus, respectively. Antisera were cleared by passage through a column of crude *E. coli* proteins containing expressed glutathione-S-transferase that were covalently bound to cyanogen bromide-activated-Sepharose (Pharmacia). For all subsequent experiments, the cleared antisera were used.

Preparation, SDS-PAGE Fractionation, and Immunoblotting of Plant Proteins

Plant samples for total protein extracts were prepared by homogenizing frozen, ground tissue with an equal volume of lysis buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 100 mM NaCl, 1% NP40, 0.1% SDS, 0.1% Triton X-100, 0.7% β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride [PMSF]). Samples were vortexed and an equal volume of 2 × loading buffer (125 mM Tris, 1% β-mercaptoethanol, 4% SDS, 20% glycerol, and 0.01% bromophenol blue) was added. Samples were microcentrifuged briefly to remove debris and then boiled for 5 min. These samples were used immediately or stored frozen at −80°C. Protein samples were fractionated on 8% or 10% acrylamide gels in the presence of SDS (Laemmli, 1970). Proteins were electrotransferred (12 V, 1.5 hr) from gels to nitrocellulose according to the method of Towbin and Gordon (1984) and blocked in Tris-buffered saline (TBS), 1% BSA, 1% goat serum, and 4.5% nonfat dry milk (Carnation). Immunodecoration with primary antibody (1:2000) was carried out for 1 to 2 hr in TBS with 0.1% BSA and 0.1% goat serum. Filters were washed for 1 hr (Towbin and Gordon, 1984) and incubated with goat anti-rabbit alkaline phosphatase (Vector Laboratories) for 1 hr. Filters were washed and then developed with NBT/BCIP according to Knecht and Dimond (1984).

For the experiment described in Figure 5, Arabidopsis was germinated and grown in liquid culture. Leaves (2 g) were harvested after 2 to 4 weeks and homogenized with 2 mL of extraction buffer (20 mM Tris, pH 8.5, 150 mM NaCl, 1 mM EDTA, 20% glycerol, and 1 mM PMSF, 1 unit/mL macroglobulin, 1 μg/mL pepstatin, 10 μg/mL aprotinin, and 10 μg/mL leupeptin as protease inhibitors). The homogenate was strained through Miracloth and centrifuged at 100,000*g* for 30 min. The pelleted membrane fraction was resuspended in 500 μL solubilization buffer (10 mM Tris, pH 7.3, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100) with the protease inhibitors listed above, and centrifuged at 100,000*g* for 30 min. This supernatant fraction is referred to as the Triton X-100 solubilized membranes and was used as a source of partially purified TMK1 protein. Samples were subjected to SDS-PAGE, electroblotted to nitrocellulose, and the TMK1 protein visualized using anti-LRR antibody 1930 and anti-kinase domain antibody 2283.

Autophosphorylating Protein Kinase Activity of the TMK1 Kinase Domain Expressed in *E. coli*

Expression of the maltose binding protein/TMK1 kinase fusion protein in *E. coli* was induced with 0.05 mM isopropyl β-thiogalactoside. Cells were then sonicated in a buffer containing 10 mM Tris, pH 7.3, 150 mM NaCl, 1 mM DTT, 1 mM PMSF, and 0.1% Tween 20. The solubilized fusion protein was affinity purified by binding to amylose resin (New England Biolabs), as described by Maina et al. (1988). The protein kinase assay was carried out directly on the fusion protein bound to the amylose resin. The assay mixture contained 50 mM Tris, pH 7, 10 mM $MnCl_2$, 1 mM DTT, 20 μM ATP, and 20 μCi γ-^{32}P-ATP in a volume of 50 μL. The mixture was incubated for 15 min at room temperature. Proteins were eluted from the resin with SDS-containing buffer, fractionated by SDS-PAGE, and subjected to either autoradiography or immunoblotting.

For phosphoamino acid analysis, fusion protein labeled with 150 μCi γ-^{32}P-ATP was subjected to SDS-PAGE and electroblotted to Immobilon-P membrane (Millipore). This membrane, containing the fusion protein, was incubated at 110°C in 200 μL of constant-boiling 6N HCl under vacuum. Portions of supernatant were removed at 1 and 4 hr, evaporated to dryness, and resuspended in 7 μL of the high-voltage electrophoresis buffer (945 mL H_2O, 50 mL glacial acetic acid, 5 mL pyridine) containing 2 μg each of phosphoserine and phosphothreonine and 4 μg of phosphotyrosine as standards. Samples were subjected to thin-layer electrophoresis on 20-cm-long plastic-backed

cellulose sheets (Kodak) for 2 hr at 500 V, followed by ninhydrin (0.5% in acetone) visualization of standards, and autoradiography.

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