Agric. Chem. Biotechnol. **44**(3), 125-130 (2001)

Structural Characteristics of the Putative Protein Encoded by *Arabidopsis AtMTN3* Gene

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Received September 4, 2001

A putative protein encoded by *Arabidopsis AtMTN3* gene, a homologue of *Medicago truncatula MTN3*, consists of 285 amino acid residues, and has a predicted molecular mass of 31.5 kDa and a calculated pI of 9.1. Primary amino acid sequence analyses have revealed that the protein contains seven putative transmembrane regions with N-terminus oriented to the outside of the membrane. The AtMTN3 protein shows overall 16.4% of amino acid identity with the rat GALR3 protein, known to be a G-protein-coupled receptor. The gene is present as a single copy in the *Arabidopsis* genome, and expressed in aerial parts but not in roots of *Arabidopsis*. Therefore, *AtMTN3* appears not to be specifically involved in *Rhizobium*-induced nodule development, as was predicted for the *MTN3* gene. These proteins possibly mediate signal transmission through G-protein-coupled pathways during general interactions between plants and symbiotic or pathogenic microbes.

Key words: Arabidopsis thaliana, *G-protein, receptor, seven-transmembrane protein.*

The G-protein-coupled receptors are integral membrane proteins that contain seven hydrophobic domains representing transmembrane spanning regions of the proteins.[1] They are involved in the perception of environmental signals and transmission of the signals to the interior of the cell through interaction with heterotrimeric G-proteins.[2] The superfamily of G-protein-coupled receptors are found in a wide range of living organisms. Accumulating evidences suggest that the G-protein signaling pathway is also conserved in higher plants.[3]

Heterotrimeric G-proteins are composed of α, β, and γ subunits encoded by distinct genes.[1] Genes encoding Ga subunits have been cloned from various plants including *Arabidopsis thaliana*.[4-6] Gβ[7] and Gγ[8] cDNAs have also been cloned from *Arabidopsis* and some plant species. It remains to be demonstrated, however, whether the plant G-protein homologues are functional as signal transducers connecting receptors and the corresponding effectors.

Involvement of G-protein signaling pathways in higher plants has been implicated by intensive cell biological and biochemical studies with stomatal guard cells.[9] It has been suggested that stomatal aperture is modulated by K^+ influx channels possibly coupled with G-protein signaling pathways. In addition, evidences have accumulated for the involvement of G-proteins in phytochrome phototransduction[10,11] and plant-pathogen interactions.[12-16]

Related to the G-protein signaling pathways, a speculation has been made for the existence of seven-transmembrane receptors in higher plants.[17,18] However, there have been only a trivial number of reports supporting this speculation. For instance, a putative receptor that interacts with an *Arabidopsis* G-protein, GPα1,[4] was purified from *Zea mays* microsomal membranes.[19] Recently, an *Arabidopsis* gene (*GCR1*) that encodes a protein with seven predicted transmembrane domains was identified through sequence analysis of expressed sequence tag (EST) database.[20,21] The protein encoded by the gene exhibits similarity to G-protein-coupled seven-transmembrane receptors identified from mammalian cells. It was suggested that the GCR1 protein had a functional role in cytokinin signal transduction.[21]

We have found that an *Arabidopsis* EST clone, EST 42B2T7, contains a cDNA encoding a putative seven-transmembrane receptor. The encoded protein exhibits significant homology with mammalian galanin receptor (GALR3) that is coupled with a G-protein.[22,23] The *Arabidopsis* cDNA exhibits strong homology with the *Medicago truncatula MTN3*[24] in the deduced amino acid sequence, and thus was named as *AtMTN3*. In the present study, we analyzed and characterized the *AtMTN3* gene, and here discuss a possible role of its product in signal transmission.

Materials and Methods

Plant materials. *Arabidopsis* (Columbia ecotype) seeds were obtained from Dr. Howard Goodman (Harvard Medical School). The seeds were imbibed at 4°C for 3 days and were

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Abbreviations: BAC, bacterial artificial chromosome; DEB, DNA extraction buffer; ER, endoplasmic reticulum; EST. expressed sequence tag; FUE, far upstream element; kb, kilo base pair; MES, 2-[N-Morpholino]ethanesulfonic acid; NUE, near upstream element.

grown at 22°C for 4 weeks on commercial horticulture soil mixed with equal volume of vermiculite. The soil was watered every other day with diluted fertilizer solution (Green lab protocols: http://www.bch.msu.edu/pamgreen/vac.txt). Alternatively, the seeds were germinated and grown for two weeks on agar medium. Surfaces of the seeds were sterilized by immersing them in 70% ethanol for 2 min and in 0.5% NaOCl/0.5% SDS for 15 min, and were rinsed five times with sterile water. The media contained 4.3 mg/m*l* of MS salts (JRH Biosciences), 1% sucrose, 1 μg/m*l* of thiamine-HCl, 0.5 mg/m*l* of pyridoxine-HCl, 0.5 μg/m*l* of nicotinic acid, 0.1 mg/m*l* of myo-inositol, 0.5 mg/m*l* of MES, and 0.8% Phytagar (Sigma). The pH of the media was adjusted to 5.7 by adding a few drops of 1 N KOH. Plants grown either on soil or agar media were harvested, frozen with liquid nitrogen, and stored at −80°C.

DNA sequencing and sequence analyses. The *Arabidopsis* EST clone 42B2T7 was obtained from the Arabidopsis Biological Resources Center at the Ohio State University, USA. The pZL1 plasmids harboring the *Arabidopsis* cDNA were isolated from the stocked *E. coli* strain DH10B, following the manufacturers instruction (BRL). The plasmid DNA pellets were suspended in sterile water and separated through CsCl gradient using a Beckman ultracentrifuge.

Sequencing of the plasmid cDNA was performed using an automated sequencer (ABI). T7 and SP6 primers contained in the pZL1 vector were used for initial sequencing. For further sequencing, synthetic internal primers were designed from the nucleotide sequences obtained from the initial sequencing.

Homology search for the nucleotide sequence was performed using the BLAST programs provided by the National Center for Biotechnology Information (NCBI, USA). Primary amino acid sequence analyses were performed with the ProtScale program (http://expasy.hcuge.ch/cgi-bin/protscale.pl). The transmembrane regions were predicted using the pSORT program (http://psort.nibb.ac.jp:8800/cgi). Multiple sequence alignment was performed with the MSA program (http://www.ibc.wustl.edu/ibc/msa.html).

Genomic DNA isolation and Southern blot analysis. For genomic DNA preparation, 5 g of soil-grown plants were ground with 5 g glass beads (75-150 μm, Sigma) and 7 m*l* of 2 × DEB (DNA extraction buffer containing 0.2 M Tris-HCl, pH 8, 0.1 M EDTA, 1% Na-*N*-laurylsarkosyl, and 300 μg/m*l* of proteinase K). The residue was incubated at 48°C for 2 h and centrifuged at 4,200 rpm for 10 min using a tabletop centrifuge. DNA in the supernatant was clarified and concentrated twice through ethanol precipitation and centrifugation. The DNA was further purified using CsCl as described above for the plasmid DNA.

For blot preparation, 4 μg of the genomic DNA was digested overnight with each of restriction enzymes, *Eco*RI, *Hin*dIII, and *Xho*I. The DNA digests were electrophoresed on 0.8% agarose gel and were transferred to Hybond-N^+ membrane (Amersham, USA). The membrane was hybridized to randomly radiolabeled (^{32}P) cDNA probe at 65°C overnight in a solution containing 0.5 M $NaHPO_4$ (pH 7.2), 1 mM EDTA, 7% SDS, and 1% bovine serum albumin (BSA). The blot was washed once at room temperature with 2 × SSC for 15 min and three times at 65°C with 0.1 × SSC containing 0.1% SDS for 15 min. The blot was exposed to an X-ray film at −70°C for 1 day.

RNA isolation and Northern blot analysis. Total RNA was extracted either from aerial parts or roots of *Arabidopsis* plants using the Tri reagent (Molecular Research Center Inc., USA) following the manufactures instruction. Each RNA sample (20 μg of total RNA) was dissolved in 1 × MOPS buffer and electrophoresed on 1.5% agarose-MOPS gel containing 5.7 M formaldehyde. Uniform loading and integrity of RNAs were confirmed by examining the intensity of ribosomal RNA bands under UV light. RNAs on the gel were transferred to Hybond-N^+ membrane (Amersham, USA). The membrane was hybridized and washed as described above in Southern blot analysis.

Results

Nucleotide sequence of the *Arabidopsis* EST 42B2T7 clone. The EST 42B2T7 clone was originally isolated from the Lamda PRL2 cDNA library.[25] The cDNA inserts were cloned with *Sal*I-*Not*I arms in the λZip-Lox vector (BRL). Thus, sequencing of the insert was initiated with T7 and SP6 primers and continued with synthetic internal primers designed from the initial sequences (Fig. 1).

The cDNA consists of 1209 bp of nucleotides, including

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   1 GAAAAAACAC TTACAAAGCT GATATCTTTC TTACTACTTC GAAGAAATCT
  51 TCTTCTCCCT TCCAAAAAAA CAATCCCTAA ACCCGGAACC AAAGATCGAC
 101 ATTCATGGCT CTCTTCGACA CTCATAACAC ATGGGCCTTT GTTTTCGGCT
 151 TGCTCGGAAA CCTCATTTCC TTTGCTGTTT TCCTCTCTCC CGTGCCAACG
 201 TTCTATAGGA TTTGTAAGAA GAAAACCACA GAAGGATTTC AATCTATTCC
 251 CTATGTGGTG GCGCTCTTCA GCGCGATGCT TTGGCTCTAC TACGCTACTC
 301 AGAAGAAAGA TGTCTTCCTT CTCGTCACCA TCAACAGCTT TGGTTGCTTC
 351 ATTGAAACCA TATACATCTC CATCTTTGTT GCCTTCGCAT CCAAGAAAGC
 401 CCGAATGCTA ACGGTGAAGC TCTTGTTGCT AATGAACTTT GGAGGGTTCT
 451 GTTTGATTCT CCTCCTCTGC CAATTCTTGG CAAAAGGAAC CACACGTGCG
 501 AAGATCATTG GAGGTATCTG TGTCGGATTC TCTGTCTGCG TTTTTGCTGC
 551 GCCGCTTAGC ATTATCAGAA CGGTGATAAA GACGAAAAGT GTGGAGTACA
 601 TGCCGTTTAG CTTATCCTTG ACTCTTACCA TCAGTGCGGT CATATGGCTC
 651 CTTTATGGTC TTGCTCTTAA GGATATCTAT GTTGCTTTCC CAAACGTGAT
 701 TGGGTTTGTT CTAGGTGCAC TTCAAATGAT ACTCTATGTG GTTTACAAAT
 751 ACTGCAAAAC GCCGTCGGAT TTGGTTGAGA AAGAACTTGA GGCTGCGAAA
 801 TTGCCAGAAG TGAGCATCGA TATGGTGAAG TTAGGTACAC TCACATCTCC
 851 TGAACCAGTA GCGATCACCG TCGTCCGATC GGTGAACACA TGTAACTGTA
 901 ACGATCGAAA TGCTGAGATT GAAAATGGTC AGGGAGTTAG AAACAGTGCT
 951 GCAACTACTT GAGTTTGCCC AAGAAACCCT AATAAGAAAC CTATTTGTTG
1001 ACTATTTTGA CTTTGTACCC TTCTTGATAT ACCTATCAAT CACCATTGTC
1051 CACGTGTACT AGTTTCGTTG TAGTAATTTT CAATAGTTAT CGAAGTATGC
1101 GTATTCGAGG ATTAATTAAA TCGGGAATTA CATTTTCCTG AGTTAATTTT
1151 TTCTCTTTTT CTTTTACTTA TGGGACAATG TAATGAAACT ATCTGTTGCA
1201 TAAAAAAAA
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Fig. 1. Nucleotide sequence of *AtMTN3* cDNA. ATG translation initiation and TGA translation termination sites are boxed. Two polyadenylation signals are underlined. Intron sites obtained through the comparison of the cDNA sequence (GenBank accession number AF095641) with the genomic sequence (AB025633) are indicated by black triangles.

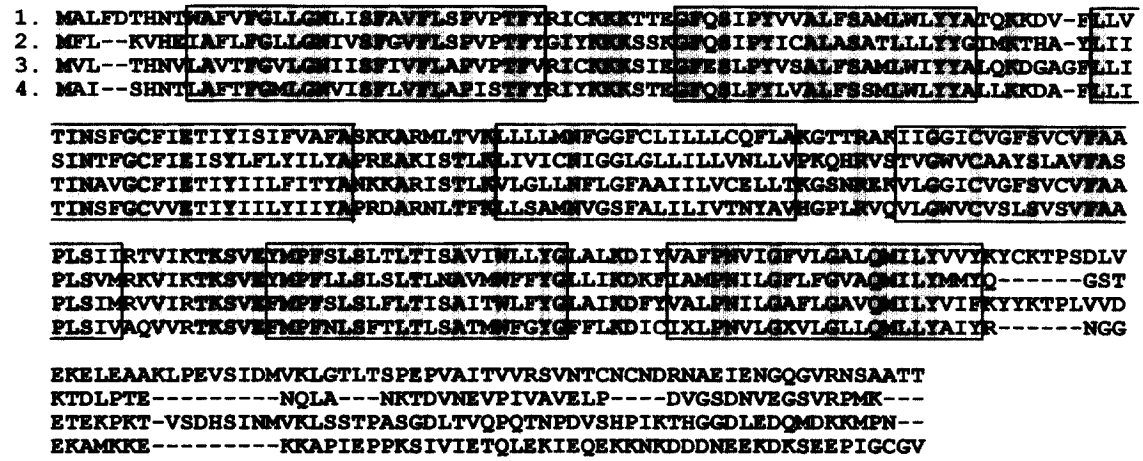

Fig. 2. Alignment of amino acid sequences of AtMTN3 homologues. Amino acid sequences were deduced from *AtMTN3* (1), *MTN3* (2), rat *GALR3* (3), and human *GALR3* (4). The sequences have been deposited in the GenBank database with accession numbers AF095641, Y08726, AF073798, and AF073799, respectively. Amino acid residues common in all four sequences are shaded. Putative transmembrane α-helix regions of the AtMTN3 protein are boxed.

104 bp of 5' untranslated region, ATG translation initiation codon (nucleotide numbers 105-107), TGA translation termination codon (960-962), and poly(A)$^+$ tail, suggesting that the cDNA contains full length coding sequence. The near upstream element (NUE), AATGAA, and the far upstream element (FUE), TTTGTA, representing polyadenylation signals[26] appear on the nucleotides 1182-1187 and 1012-1017, respectively.

BLASTN analysis revealed no known nucleotide sequence significantly homologous to the *AtMTN3* gene (data not shown). The DNA sequence of the *AtMTN3* cDNA has been deposited in the GenBank database under accession number AF095641.

Deduced amino acid sequence of the *AtMTN3*. A putative protein encoded by the *AtMTN3* consists of 285 amino acid residues (Fig. 2), and has a predicted molecular mass of 31.5 kDa and a calculated pI of 9.1. Hydropathy analysis (Fig. 3) suggests that the protein contains seven putative transmembrane regions. The putative integral regions predicted were peptides corresponding to the amino acid numbers 10-34, 44-64, 72-96, 106-126, 134-154, 165-185, and 193-214, respectively (Fig. 2). Computational analysis revealed that this protein is located to the plasma membrane or endoplasmic reticulum (ER), and N-terminus of the protein might be oriented to the outside of the membrane, and thus C-terminus to the cytosol.

BLASTX database search has revealed that the AtMTN3 protein exhibits strong amino acid sequence homology with *MTN3* gene product identified from *Medicago truncatula*.[24] The proteins have 57% identity in amino acid sequence to each other (Fig. 2). Through the database search, a number of additional genomic coding sequences encoding proteins homologous to the AtMTN3 have been identified through the *Arabidopsis* genome sequencing projects. One of the genomic sequences, for instance, was from the BAC clone T7F6 of

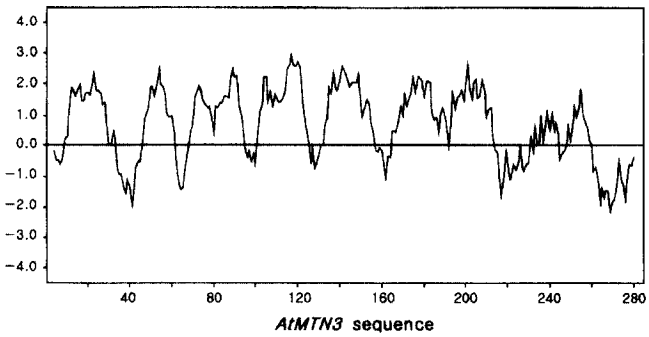

Fig. 3. Hydropathy plot of the amino acid sequence deduced from the *AtMTN3* nucleotide sequence. The plot was prepared with the ProtScale program using the method of Kyte and Doolittle.

chromosome 2 (GenBank accession number AC005770), and the other from the BAC clone F13M23 of chromosome 4 (GenBank accession number AL035523). The MTN3 and three *Arabidopsis* homologues showed high degree of amino acid homology in N-terminal side, but little homology in C-terminal side of cytosolic domains (Fig. 2, the sequences from the *Arabidopsis* BACs are not shown).

A couple of segments of the AtMTN3 protein exhibited a significant amino acid sequence homology with GALR3 proteins (Fig. 2). The *GALR3* genes were previously cloned from rat[22,23] and human.[23] They encode galanin receptors that are known to be coupled with G-protein-signaling pathways.[22,23] The rat GALR3 protein has 370 amino acid residue, 85 residues more than At MTN3. Thus, whole amino acid identity between the AtMTN3 and rat GALR3 was relatively weak (16.4%); however, the homology was observed throughout the entire sequences (Fig. 2). It is notable that the first three transmembrane regions showed significantly high homologies, 23-43% amino acid identities and 48-66% similarities.

Southern blot analysis. The *AtMTN3* cDNA insert con-

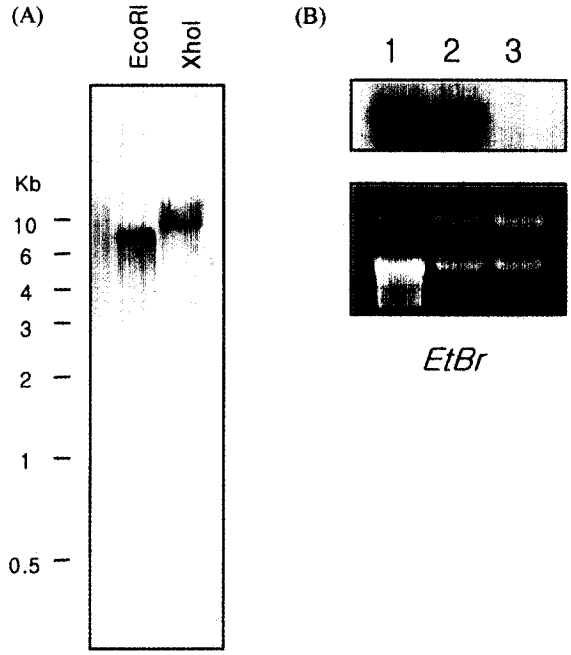

Fig. 4. Blot analyses of *AtMTN3* gene. (A) Southern blot analysis of *Arabidopsis* genomic DNA. *Arabidopsis* genomic DNA was digested with *Eco*RI or *Xho*I, and hybridized with the *AtMTN3* cDNA probe. (B) Northern blot analysis for expression of the *AtMTN3* gene in *Arabidopsis*. Lane 1, aerial parts (flowers, flower buds, leaves and stem) of 4-week-old plants grown in soil; Lane 2, aerial parts of 2-week-old plant grown on sterile agar medium; Lane 3, roots of 2-week-old plants grown on sterile agar medium. EtBr, the same RNA gel stained with ethidium bromide exhibiting ribosomal RNA bands under UV light.

tained in the EST 42B2T7 was isolated from the plasmid through the digestion with restriction enzymes, *Sal*I and *Not*I, and used as probes in genomic Southern blot analysis.

Arabidopsis genomic DNA, digested either with *Eco*RI or *Xho*I, was hybridized to the cDNA probe, resulting in the exhibition of one band on the blot (Fig. 4A), which suggests that the gene corresponding to the *AtMTN3* cDNA exist as a single copy in the *Arabidopsis* genome.

Northern blot analysis. Expression of the *AtMTN3* gene in *Arabidopsis* plant was examined through Northern blot analysis using the *AtMTN3* cDNA probe. The Lamda PRL2 cDNA library was derived from several pools of mRNA expressed in different tissues and developmental stages.[25] The cDNA sources were equal amounts of mRNA isolated from tissue culture-grown roots, 7-day-old etiolated seedlings, rosettes from staged plants of different ages, and aerial tissues (stem, flowers, and siliques) from the same plants as the rosettes. We expected that the gene might be expressed in roots, because the *MTN3* gene was reported to be expressed in nodulating roots of *Medicago truncatula*.[24] Interestingly, however, the 0.9-kb gene transcripts were detected in the aerial parts either of 2-week-old plants grown on agar medium or 4-week-old plants grown in soil, but not in roots of the plants (Fig. 4B).

Discussion

Existence of G-proteins in plants has been demonstrated through the studies of plant defense mechanism, hormonal regulation, stomatal regulation, and photo signal transduction. Thus, it has been speculated that G-protein-coupled seven-transmembrane receptors also exist in plants.[17,18] Based on this speculation, several approaches have been attempted to clone plant genes encoding this type of receptors. Cloning through PCR amplification and homologue screening through hybridization have been attempted, using primers or probes designed from amino acid sequences conserved in the mammalian receptors. In most cases, however, successful cloning of the plant receptors was hampered by low level of similarity between the primary sequences of mammalian genes and plant genes. Recently, exploitation of plant ESTs to search genes encoding proteins, which have conserved features of transmembrane receptors, has been suggested as one of the promising approaches. For instance, utilizing similarities in primary amino acid sequence and hydropathy profiles, an *Arabidopsis* gene (*GCR1*) that encodes a protein having putative seven transmembrane domains has been identified.[21] The protein showed 18-23% amino acid identity (46-53% similarity) to other known seven-trasmembrane receptors. Its highest sequence identity is with the dictyostelium cAMP receptors. Transgenic *Arabidopsis* expressing antisense *GCR1* have reduced sensitivity to cytokinins, implicating a functional role for the protein in cytokinin signal transduction.

We obtained a full nucleotide sequence of the *Arabidopsis* EST 42B2T7 (Fig. 1), and found that the protein encoded by the cDNA (named *AtMTN3*) contains seven putative transmembrane regions (Figs. 2 and 3). The AtMTN3 protein exhibited a weak but significant homology with the galanin receptors (GALR3) cloned from mammalian cells.[22,23] Galanin is a neuropeptide found in the central and peripheral nervous systems. It has been demonstrated that the GALR3 receptors are coupled with a G-protein signaling pathway.[23] In *Xenopus oocytes*, activation of the GALR3 receptors co-expressed with potassium channel subunits resulted in inward K^+ current, suggesting that the receptors activate potassium channels linked to the regulation of neurotransmitter release.

The AtMTN3 protein exhibits strong amino acid sequence homology with the *Medicago truncatula* MTN3 and two *Arabidopsis* genomic sequences. The *MTN3* gene was originally identified as one of the molecular markers associated with different stages of *Rhizobium*-induced nodule development in the legume *Medicago truncatula*.[24] This gene belongs to a group of genes corresponding to early nodulins, expressed well before the onset of nitrogen fixation, and in which transcripts are of low abundance in mature nodules. *Arabidopsis* is not a nodule-developing plant, but contains a number of *MTN3* homologues including the *AtMTN3* gene that exists as a single copy gene in the genome (Fig. 4A). Moreover, the gene transcripts were constitutively expressed in the aerial parts but not in roots (Fig. 4B). It was noted that the *MTN3* gene was also

expressed in uninfected Nar nodules.[24] Furthermore, the gene was not induced through treatment with Nod factors, lipo-oligochitin signal molecules inducing the nodule formation. Thus, it is possible that *MTN3* and *AtMTN3* play a role in general interaction between plants and microbes rather than in a specific process related to the nodule formation.

Accumulated evidence suggests that G-protein signaling pathways are involved in plant-microbe interactions. Evidence has been reported on the participation of G-proteins in the elicitation of rapid oxidative burst in cultured soybean cells.[12] Activators of G-proteins were revealed to affect the binding of fungal toxin fusicoccin to its binding protein in the oat root plasma membrane.[13] Cholera toxin elevated pathogen resistance and induced pathogenesis-related gene expression in tobacco.[14] Virus-mediated or transgenic suppression of a G-protein a subunit attenuated the fungal virulence.[15] It has been suggested that phytoalexin elicitation involves the inositol lipid signal transduction pathway.[16] Related to this suggestion, it is well known that nodule-inducing *Rhizobium* also induces general defense responses, such as phytoalexin accumulation and lignin synthesis, in plants.

The AtMTN3 and its homologues showed high degree of amino acid homology to each other in the N-terminal side transmembrane regions, but little homology in the C-terminal side (Fig. 2, not shown for the *Arabidopsis* homologues). Primary amino acid sequence analysis suggested that C-terminal side of the proteins is oriented to the cytosol. Thus, it is possible that each of the *Arabidopsis* MTN3 homologues transmits environmental signal(s) into its unique effector(s) through the cytosolic domain.

In summary, our data implicates that the AtMTN3 protein has a role in signaling between plants and some environmental stresses including microorganisms, symbiotic or pathogenic. The protein may transmit environmental signals through G-protein-coupled pathways. This prediction on the biological function of the AtMTN3 protein remains to be experimentally demonstrated.

Acknowledgments. The authors thank Drs. Yang Do Choi, Ingyu Hwang, and Jong Seob Lee of the Seoul National University for various supports. This work was supported by the Ministry of Education through the Brain Korea 21 Project. The DNA sequence of the *AtMTN3* gene has been deposited in the GenBank database under the accession number AF095641.

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