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Molecules and Cells

Overexpression of *BrMORN*, a Novel 'Membrane Occupation and Recognition Nexus' Motif Protein Gene from Chinese Cabbage, Promotes Vegetative Growth and Seed Production in *Arabidopsis*

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Proteins that contain membrane occupation and recognition nexus (MORN) motifs regulate various aspects of cellular metabolism by localizing proteins in different cellular organelles. The full-length *Brassica rapa* MORN motif protein (*BrMORN*) cDNA consists of 1,510 bp encoding 502 deduced amino acids with a predicted molecular mass of 55.8 kDa and an isoelectric point of 9.72. *Br*MORN is a novel protein composed of two N-terminal transmembrane helices and seven C-terminal MORN motifs and it appears to be localized on the plastid envelope. *BrMORN* expression was relatively high in actively-growing tissues, but low in mature tissues and under some abiotic stresses. *Arabidopsis thaliana* plants overexpressing *BrMORN* showed an enhanced rate of growth, hypocotyl elongation, and increases in the size of vegetative organs and seed productivity under normal growth conditions. In addition, cell size in *Arabidopsis* plants overexpressing *BrMORN* was 24% larger than that of wild-type plants, implying that the increase in the size of vegetative organs is due to cell enlargement. The increased size of the vegetative organs also led to increased seed production. Our data suggest that the MORN motif of *Br*MORN may act at the plastid envelope and facilitate plant growth *via* cell enlargement.

INTRODUCTION

Membrane occupation and recognition nexus (MORN) motifs consist of 14 highly-conserved amino acids in the pattern YQ/EGE/QTXNGKXHGYG, which was first described in mammalian junctophilin (JPH1) (Takeshima et al., 2000). The presence of the motifs has been reported in MORN 1 of the parasite *Toxoplasma gondii* (Gubbels et al., 2006), human amyotrophic lateral sclerosis 2 (ALS2) (Hadano et al., 2007), mammalian meichroacidin (MCA) (Tokuhiro et al., 2008; Tsuchida et al., 1998), MORN motif-containing male-specific axonemal protein (MSAP) of *Cyprinus carpio* (Ju et al., 2004), human MCA (RSP44) (Shetty et al., 2007), *Drosophila* retinophilin (Mecklenburg, 2007) and human JPH2 (Landstrom et al., 2007). The number of MORN motifs varies; retinophilin has four, MCA has six, RSP44 and MSAP have seven, and JPH1 and 2 and ALS2 have eight. The positions of the MORN motifs also vary, occurring at the N-terminus in JHP1, JHP2, RSP44, and MCA; at the C-terminus in ALS2; in the middle in retinophilin; and throughout the protein in *Toxoplasma* MORN1 and MSAP. MORN motif proteins also have diverse functions, including membrane fusion (JHP1, HHP2, ALS2, MSAP, and retinophilin), fission (*Toxoplasma* MORN1), and mobility (RSP44, MCA).

Two types of MORN motif-containing proteins have been reported in plants, phosphatidylinositol phosphate kinases (PIPKs, also known as PIP5Ks) (Ma et al., 2006; Mueller-Roeber and Piscal, 2002) and accumulation and replication of chloroplast 3 (ARC3) (Shimada et al., 2004). In contrast to animal PIPKs, plant PIPKs contains MORN motifs at their N-termini. Nine *Arabidopsis* PIPKs (*At*PIPK1-9) and five rice PIPKs (*Os*PIPK1, 3-5, and 7), which harbor seven to nine MORN motifs, have been reported (Ma et al., 2006; Mueller-Roeber and Piscal, 2002). Among them, *At*PIPK 1, 3, 4, and 9 and *Os*PIPK1 have been intensively studied with respect to localization, binding properties, and physiological function. The number of MORN motifs has been shown to be critical for determining cellular localization of the protein, which included the plasma membrane, nucleus, and cytosol (Ma et al., 2006). Localization of MORN motif proteins could be mediated by lipid-binding activity, specifically to phosphatidic acid, phosphatidylinositol 3,5-bisphosphate, and phosphatidylinositol 3,4,5-triphosphate (PtdInsPs) (Im et al., 2007). *At*PIPK1 (*At*PI5K1) is thought to be localized at the endomembrane (Im et al., 2007) together with F-actin (Tan and Boss, 1992). *At*PIPK3, *At*PIPK4, and *Os*PIPK1 regulate root hair growth (Kusano et al., 2008; Stenzel et al., 2008), pollen tube polarity (Sousa et al., 2008), and rice heading (Ma et al., 2004), respectively. ARC3, a chloroplast division factor, is a chimera with its N-terminal half derived from prokaryotic FtsZ (filamentous temperature-sensitive Z) and its

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C-terminus homologous to eukaryotic phosphatidylinositol-4-phosphate 5-kinase. Two MORN repeats are present in the PIPK-homologous region. It has been suggested that ARC3 is localized at the site of division in the chloroplast envelope and that its function is essential for organelle fission involving protein scaffolding on the outer chloroplast envelope.

A Chinese cabbage clone (GenBank accession No. FJ 460465), the expression of which is decreased by abiotic stresses, shows 87% and 82% identity with *Arabidopsis* At4g 17080 at the level of nucleotide and amino acid sequences, respectively. The predicted amino acid sequence of FJ460465 consists of two transmembrane domains at the N-terminus and seven MORN motifs at the C-terminus. With the exception of the MORN motifs, this structure is quite different from that of the PIPKs. This type of MORN-motif protein has not been previously reported in plants and we designated the Chinese cabbage clone *BrMORN*.

To determine the function of *Br*MORN we examined its expression in Chinese cabbage tissues. *BrMORN* was expressed in all tissues examined, with the exception of pistil and petal. When we transformed *BrMORN* into *Arabidopsis* under the control of the strong Cauliflower Mosaic Virus (CaMV) promoter (*35S::BrMORN*), the transgenic *Arabidopsis* plants showed increases in vegetative organ growth and seed mass (number and weight) of up to 40% compared to wild type plants.

MATERIALS AND METHODS

Plant materials and growth conditions

Wild-type *Arabidopsis thaliana* ecotype Columbia (Col-0), *Br MORN*-overexpressing *Arabidopsis*, Chinese cabbage (*Brassica rapa* ssp. *pekinensis*), and *Nicotiana benthamiana* were grown in a growth chamber at 22°C with a photon flux density of 150 $\mu mol \cdot m^{-2}s^{-1}$ for a 16 h light/8 h dark photoperiod. For plate culturing, *Arabidopsis* seeds were sterilized in a solution of 50% bleach containing 0.1% Triton X-100, followed by germination on plates containing 0.5X Murashige and Skoog (MS) salts, 0.05% MES, 1% sucrose, and 0.8% agar. Measurement of hypocotyl length was performed by spreading seeds evenly on MS medium followed by cold-treatment at 4°C for 4 d in the dark and then by incubation at 22°C for 4 d. Lengths were directly measured using ImageJ 1.40 software (NIH-Image, http://rsbweb.nih.gov/ij/index.html).

Stress treatment

Light-chill treatment was conducted by exposing 1 cm^2-leaf discs of Chinese cabbage grown in chambers to 100 μmol photons $m^{-2}s^{-1}$ at 4°C for 1 to 12 h. Heat shock at 42°C was applied to 10 leaf discs under the same light conditions. Salt stress was carried out by floating 10 leaf discs on a solution of 250 mM NaCl under the same light conditions.

Protein sequence analysis

The amino acid sequences of *Br*MORN and related MORN repeat protein families were examined using BLASTP (http://www.ncbi.nlm.nih.gov/BLAST) and ClustalW (http://www.ebi.ac.uk/clustalw). *Br*MORN protein domain structures were analyzed using the software available in the ExPASY database (http://us.expasy.org/tools/). Transmembrane regions of the *Br*MORN protein were predicted using the ARAMEMNON membrane protein database (http://aramemnon.botanik.uni-koeln.de/) (Schwacke et al., 2003).

Plasmid construction and transgenic plant analysis

The coding region of *BrMORN* was amplified from the full-length *BrMORN* cDNA sequence in pBluescriptII KS (+) (Stratagene, USA) by PCR using the forward primer 5′-AGACCCCGGGT-GTAAATGCA-3′ and the reverse primer 5′-GCCGGAGCTCT-AACTACTCACAAG-3′. The *BrMORN* gene was expressed in *Arabidopsis* under the control of the CaMV 35S promoter (35S-P) by inserting the 1.5-kb *Sac*I/*Sma*I fragment of the *BrMORN* coding sequence between the CaMV 35S-P and the nopaline synthase terminator (nos-ter) of the pCAMBIA3300 binary vector. The recombinant plasmid (*35S::BrMORN*) was introduced into *Agrobacterium tumefaciens* strain GV3101. Transformed *Arabidopsis* (*Arabidopsis thaliana* ecotype Columbia) was generated by standard floral-dip methods (Clough and Bent, 1998). Homozygous T_3 generation plants with a high level of *BrMORN* expression were maintained at 22°C for a 16-h photoperiod and phenotypes were analyzed using the method described by Boyes et al. (2001).

Phenotype analysis

Phenotype analysis was conducted with at least 20 wild-type plants and three independent transgenic *Arabidopsis* lines that overexpress *BrMORN* (#1, #2, and #3), each harboring a single insertion. The analysis was performed three times at 3-month intervals.

RT-PCR analysis

Total RNA (5 μg) from each sample was combined with random hexamer primers in a SuperScript first-strand cDNA synthesis system according to the manufacturer's instructions (Invitrogen Life Technologies, UK). Complementary DNA was generally diluted 10-fold and 1 μl of diluted cDNA was used in a 20-μl PCR mixture. RT-PCR primers were as follows: 5′-CAGAG-TCGTCATCATCTGACGG-3′ (forward) and 5′-TCATCCCTT-GCCTGTATTGACC-3′ (reverse) for *AtMORN*; 5′-ACGTACC-GAAGCGCCATTGGA-3′ (forward) and 5′-TCATCCCTTGCC-TGTATTGACC-3′ (reverse) for *BrMORN*; 5′-GTCTTGACCT-TGCTGGACGTGA-3′ (forward) and 5′-CCTTTCAGGTGGTG-CAACGAC-3′ (reverse) for *AtACT1*; and 5′-GAACCGGGTG-CTCCTCAGGA-3′ (forward) and 5′- ATGGTACCGGAATGGT-CAAGGC-3′ (reverse) for *BrACT1*. In general, PCR was performed with a 5-min denaturation at 94°C, followed by 25 cycles of 94°C for 30 s, 55 °C for 30 s, and 72°C for 90 s. PCR products were analyzed following electrophoresis through a 1% agarose gel.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed for precise analysis of transcript levels. Primers that targeted *BrMORN* and *BrACT1* and produced fragments of 80 to 90 bp were designed using the Primer Quest computer program (http://eu.idtdna.com/Scitools/Applications/Primerquest/); *BrMORN* primers were 5′-ACGAAGGAGATTGGGTTGATGGGA-3′ (forward) and 5′-TTCCATGTCTCATCCCTTGCCTGT-3′ (reverse) and *BrACT1* primers were 5′-ACACCATGATGTCTTGGCCTAC-CA-3′ (forward) and 5′-AATGGTACCGGAATGGTCAAGGCT-3′ (reverse). Complementary DNA was synthesized using the iScript Select cDNA Synthesis Kit (Bio-Rad, UK) according to the manufacturer's instructions. Quantitative real-time PCR was performed using a SYBR® Green RT-PCR kit (IQ Sybr Green Super Mix; catalog no. BR170-8880) on a MiniOption detection system (Bio-Rad, UK). Results were analyzed using Bio-Rad software (GeneXpression Macro Chromo4) and the comparative threshold cycle (Ct) method according to the manufacturers' instructions for data normalization.

In situ hybridization

Tissue preparation and pretreatment, hybridization, and washing

of slides were performed according to Hejatko et al. (2006). An antisense RNA probe was prepared from linearized plasmids using digoxigenin (DIG)-11-rUTP (Roche, Germany). Hybridization was performed at 42°C for at least 16 h. Following post-hybridization treatment and incubation with anti-digoxigenin conjugated to alkaline phosphatase, substrate was added and color was allowed to develop in the dark for 1 d. Light microscopy was carried out using an Olympus model CX21 (Olympus, Japan).

Protoplast isolation and transfection

BrMORN-GFP was constructed by PCR amplification of the *BrMORN* cDNA with primers 5′-AGCGAGTCGACATGCATC-TAAAG-3′ (forward) and 5′-TGTCTCCCGGGTACAAGGGAT-3′ (reverse), which contain the *Sal*I and *Sma*I restriction sites, respectively, followed by cloning into a pUC19-GFP3-containing vector. A green fluorescent protein (GFP) fusion construct that was fused in-frame to the 3′ end of the gene was introduced into *Arabidopsis* protoplasts prepared from 2-week-old seedlings by polyethylene glycol-mediated transformation as previously described (Kang et al., 1998). Isolated protoplasts were transformed with 30 μg plasmid DNA. The cells were examined by bright field and fluorescence microscopy after incubation in the dark at 22°C for 24 h.

Transient expression of *BrMORN-GFP* in *N. benthamiana*

BrMORN cDNA fragments were amplified by PCR with the primers 5′-CATGGTAGATCTGTAAATGCATCTAAAGA-3′ (forward) and 5′-TAGCTAGATCTACCATGCACAAGGGATC-3′ (reverse), which contain the *Bgl*I restriction site, and cloned into pCAMBIA1302 between the 35S promoter and the GFP gene. The *BrMORN-GFP* construct was transformed into *Agrobacterium tumefaciens* C58C1 and the cells were then suspended in 10 mM $MgSO_4$, 10 mM Mes, and 100 μM acetosyringone and infiltrated into the fourth leaf of 3-week-old *N. benthamiana* plants using a syringe (Cho et al., 2008). After 3 days of incubation the infiltrated leaves were removed and the chloroplasts isolated.

Purification and western analysis of chloroplast proteins

Intact chloroplasts were purified from 10 g *BrMORN-GFP*-expressing *N. benthamiana* leaves using the method described by Kubis et al. (2008). Membrane-bound and soluble protein fractions were separated as follows (Salvi et al., 2008). The intact chloroplasts were suspended in hypotonic medium (10 mM MOPS, pH 7.8, containing 1 mM PMSF, 1 mM benzamidine, and 0.5 mM ε-amino capronic acid) in nine volumes of cold chloroform/methanol (5:4, v/v). The mixture was incubated on ice for 15 min and centrifuged for 15 min at 18,000 rpm with a Beckman JA14 rotor. The pellet containing insoluble membrane-bound proteins was resuspended with gel loading buffer and the acetone-precipitated soluble proteins and intact chloroplast proteins were quantitated using the BCA kit (PIERCE, USA). Western analysis was carried out with anti-GFP antibody and NBT/BCIP substrates for color development.

Determination of cell size

For microscopic analysis of transverse sections of roots, seedling samples were prepared as described by Reidel et al. (2009). Root tissue from 1-week-old plants was taken 1 cm from the root tip, where elongation should be complete. Tissue was cross-sectioned, fixed in 2% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 70 mM sodium cacodylate buffer, pH 7.0, for 1 h at room temperature, washed with the same buffer, and postfixed in 1% (v/v) osmium tetroxide. The tissue was dehydrated by passage through an acetone series and embedded in Spurr's epoxy resin (Electron Microscopy Sciences, USA) according to the manufacturer's instructions. Unstained root sections were dried onto glass slides and stained with toluidine blue. The stained tissue was observed with an E-600 microscope (Nikon, Japan) and photographed with a SPOT camera and software.

The fifth rosette leaf of each plant was dissected in preparation for microscopic analysis. Epidermal tissue was peeled from the central half of the leaf between the tip and base. At least five epidermal fragments from the abaxial surface of two to three leaves were placed on a microscope slide, stained with 1% Safranin O, and observed with a compound microscope. Leaf and epidermal cell areas were measured using the public domain NIH Image 1.61 program. Experiments were three times repeated.

RESULTS

***BrMORN* gene**

The *BrMORN* gene (GenBank accession No. FJ460465) was initially identified in a microarray experiment (data not shown) and isolated from a cDNA library constructed for an EST project. The coding sequence of the gene consists of 1,510 bp and encodes 502 deduced amino acids, with a predicted molecular mass of 55.8 kDa and isoelectric point of 9.72. *Br*MORN harbors two transmembrane domains located at the N-terminus and seven repeated MORN motifs at the C-terminus (Fig. 1A). The two transmembrane domains exhibit high levels of hydrophobicity (0.73 and 0.79) as calculated by the ARAMEMNON membrane protein database (http://aramemnon.botanik.uni-koeln.de) (Schwacke et al., 2003) and the ExPASY database (http://us.expasy.org/tools/) (Fig. 1B). The amino acid glycine (Gly, G) is highly-conserved among the seven MORN motifs (Fig. 1C), which is found in all MORN motif-containing proteins. In contrast to plant PIPKs, *Br*MORN does not contain a kinase domain and the MORN motif is located at the C-terminus rather than at the N-terminus. This structural difference may imply that the function of *Br*MORN differs from that of PIPKs, with the exception of the role of MORN repeats.

Expression of *BrMORN* in Chinese cabbage

The level of the *BrMORN* transcript was high in roots, hypocotyls, and young leaves of Chinese cabbage, but was not detectable in the pistil or petal. In addition, transcript levels were low in mature leaves, filaments, and anthers (Fig. 2A). These expression data suggest that *Br*MORN activity may be required in almost all growing tissues. The expression of *BrMORN* gradually decreased as hypocotyl elongation progressed (Fig. 2B). Gendreau et al. (1997) reported that elongation occurred throughout the hypocotyls under light, whereas, in the dark, hypocotyl basal cells elongated first followed by expansion of the more apical cells. Since we assessed the entire hypocotyl region, the decreased transcript level may be due to the increase of the basal region of the hypocotyls. This conclusion was supported by the data in Fig. 2C, which shows a gradient of transcript levels from the top to the basal part of a hypocotyl. This finding indicates that *Br*MORN activity is high in actively growing regions. Abiotic stresses such as chilling and salt treatment markedly decreased the expression of *BrMORN* (Fig. 2D), suggesting that *Br*MORN activity is not required under these stresses or that the stresses inhibit *BrMORN* expression. However, *BrMORN* transcription increased slightly during the initial 4 h of heat-shock treatment and declined rapidly thereafter, suggesting that *Br*MORN activity might be related to the heat-shock response.

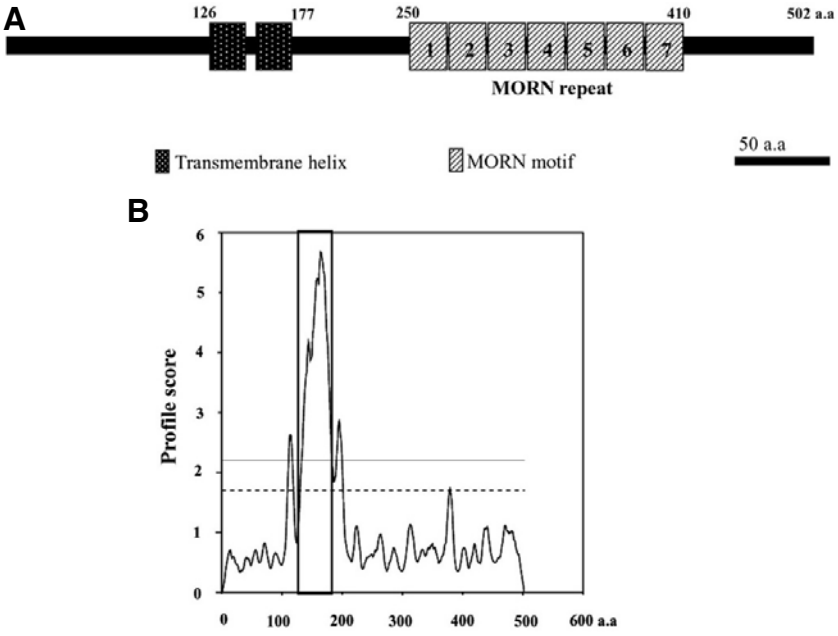

Fig. 1. Structure and amino acid sequence of the *Br*MORN protein. (A) Functional domains present in *Br*MORN. Amino acid sequences were analyzed using the Pfam (http://pfam.sanger.ac.uk/search) and SOSUI (http://bp.nuap.nagoyau.ac.jp/sosui/sosui_submit.html) databases. Transmembrane helices and MORN motifs are indicated on the linear diagram. Numbers at the top represent amino acid positions. (B) Hydrophobicity analysis of *Br*MORN. The hydrophobicity profile was generated using DAS in the ExPASY database (http://us.expasy.org/tools/). The box represents transmembrane motifs. (C) Sequence alignment of MORN motifs (amino acid [a.a.] 250 to 410) in *Br*MORN. The dark-shadowed boxes indicate conserved glycine residues.

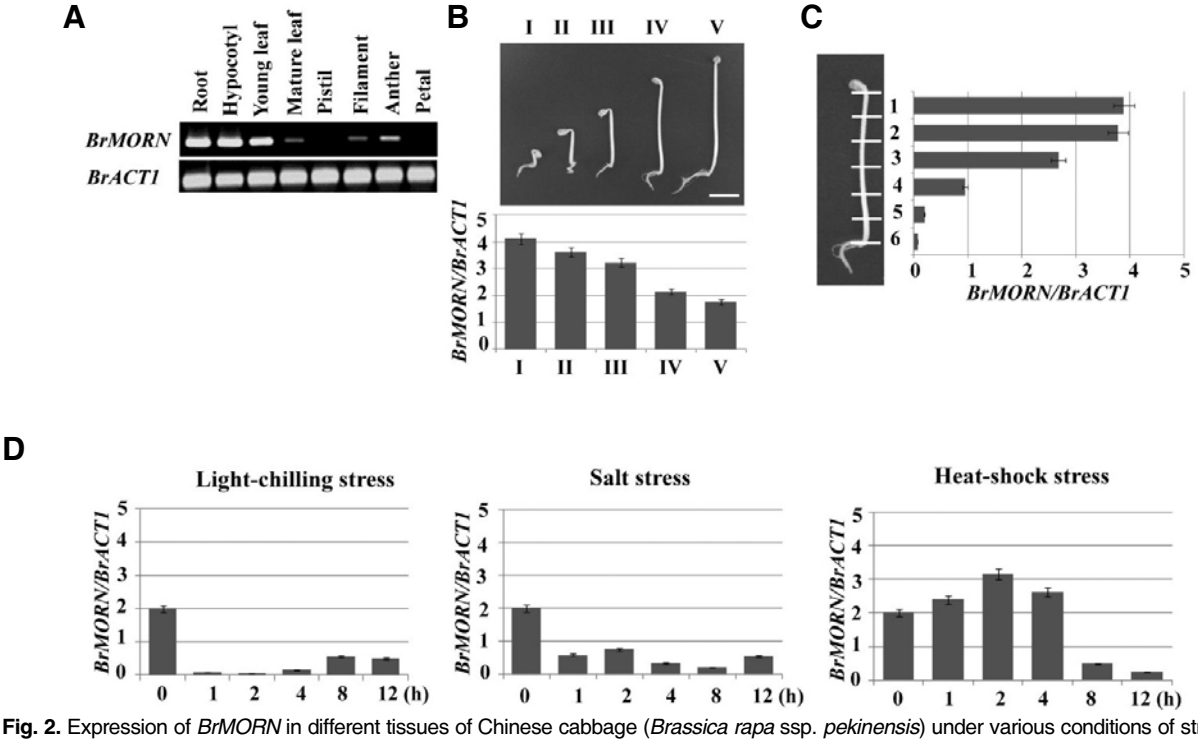

Fig. 2. Expression of *BrMORN* in different tissues of Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) under various conditions of stress. (A) Expression pattern of *BrMORN* in different tissues analyzed by RT-PCR. The *BrACT1* gene was used as a loading control. (B) qRT-PCR results (bottom panel) at five stages of hypocotyl growth in Chinese cabbage (upper panel). Hypocotyl length at Stage I, 0.5 cm; Stage II, 1 cm; Stage III, 2 cm; Stage IV, 3 cm; Stage V, 4 cm. Bars = 1 cm. (C) qRT-PCR results from six segments of a stage IV Chinese cabbage hypocotyl (see Fig. 2B). A hypocotyl (30 mm in length) was cut into 5 mm segments from top to bottom (1, 2, 3, 4, 5, and 6, respectively). Total RNA was extracted from 30 individual hypocotyls. (D) qRT-PCR results of *BrMORN* under light chilling (4°C), salt stress (250 mM NaCl), and heat-shock stress (42°C).

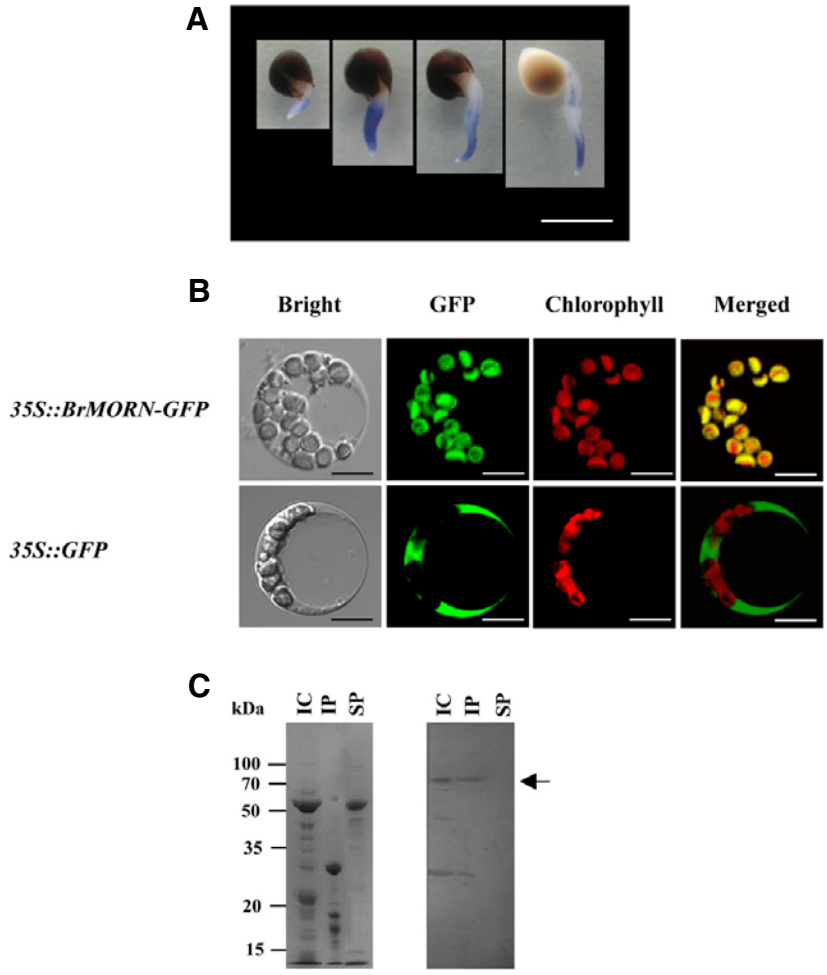

Fig. 3. (A) *In situ* hybridization of *BrMORN* transcripts from germinating Chinese cabbage seeds. Samples were taken at different stages of germination over the course of 2 days and hybridized with antisense DIG-labeled RNA probes, followed by incubation with alkaline phosphatase-conjugated anti-digoxigenin and colorimetric detection of transcripts. Bar = 1 cm. (B) Subcellular localization of *Br*MORN. A *BrMORN-GFP* fusion construct was introduced into *Arabidopsis* protoplasts. Images are optical photomicrographs (Bright field), show GFP fluorescence (GFP; green pseudocolor), or show chlorophyll fluorescence (Chlorophyll; red pseudocolor). Bars = 10 μm. (C) Western analysis of chloroplast proteins. Proteins from intact chloroplasts (IC), membrane fraction (insoluble proteins, IP), and soluble fraction (SP) were separated by SDS-PAGE through a 10% gel (left panel) and transferred to nitrocellulose membrane. *Br*MORN-GFP was detected using anti-GFP antibody and NBT/BCIP as substrate (right panel). Arrow indicates the predicted size of *Br*MORN-GFP protein.

In situ hybridization and localization of *Br*MORN

To examine the transcript levels of *BrMORN* in tissues, *in situ* hybridization was performed with germinating seedlings. High levels of *BrMORN* transcripts were detected in the elongation region, but not in meristematic tissues of the primary root (Fig. 3A). While an intermediate signal was also detected in the hypocotyl region, no signal was detected in root hairs. These results suggest that *Br*MORN activity may be related to cellular growth.

To elucidate the subcellular localization of *Br*MORN, a *BrMORN-GFP* fusion construct was introduced into *A.thaliana* protoplasts (Fig. 3B) and *N. benthamiana* followed by western analysis with anti-GFP (Fig. 3C). The fusion protein appeared to be localized in the plastid envelope and western blotting showed that *Br*MORN-GFP protein was present in the insoluble membrane fraction, suggesting the possibility that *Br*MORN can carry out its function *via* MORN motifs in the plastid membrane.

Arabidopsis plants overexpressing *BrMORN*

To elucidate *Br*MORN function, the *BrMORN* gene was introduced into *Arabidopsis* plants under the control of the CaMV 35S promoter. Sixteen independent transgenic lines were selected using Basta spray followed by genomic PCR and RT-PCR. To select transgenic plants harboring a single transgene, T_1 plants were self-fertilized and 10 T_2 lines demonstrating 3:1 segregation were obtained by Basta selection (Katiyar-Agarwal et al., 2003). Three homozygotes overexpressing *BrMORN* (*35S::BrMORN*) were selected from the T_3 generation and their phenotypes were analyzed three times at 3-month intervals.

As shown in Figs. 4A, 4C, and 4D, growth of hypocotyls and roots in *35S::BrMORN Arabidopsis* plants was enhanced by 30% and 39%, respectively, compared to wild-type plants. This growth correlated well with the increase in *BrMORN* expression levels (Fig. 4B), suggesting that *Br*MORN stimulates growth in *Arabidopsis* plants. Elongation of hypocotyls from etiolated seedlings was also enhanced by 31% (Figs. 5A and 5B), which is similar to that of light-grown seedlings. This suggests that stimulation of hypocotyl elongation by *Br*MORN is not light-dependent.

To obtain detailed information on the function of the *BrMORN* gene, the phenotype of *35S::BrMORN Arabidopsis* plants was carefully observed and analyzed for an entire life cycle (Fig. 6, Tables 1 and 2). Both growth rate and plant size were greatly increased in plants overexpressing *BrMORN* (Fig. 6). However, there was no difference in germination rate or responses to cold and heat shock between wild-type and transgenic plants (data not shown). Several physical properties of the fifth rosette leaf were measured and are summarized in Table 1 because this leaf was proposed to be the most representative for phenotypic analysis (Tsuge et al., 1996). Most factors measured were enhanced in *BrMORN*-overexpressing plants compared to wild-type plants. Blade width and petiole length were only slightly increased but blade length, leaf area, and weight were greatly enhanced. The leaf area in *Arabidopsis* overexpressing *BrMORN*

Table 1. Measurements of rosette leaves from *Arabidopsis* plants overexpressing *BrMORN*

Measurement	Wild-type	*35S::BrMORN*		
		#1	#2	#3
Length of leaf blade (cm)[a]	1.7 ± 0.3	2.1 ± 0.4	2.4 ± 0.3	2.5 ± 0.4
Width of leaf blade (cm)[a]	1.1 ± 0.2	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3
Length/width ratio[a]	1.5 ± 0.3	1.6 ± 0.4	1.8 ± 0.5	1.9 ± 0.4
Length of petiole (cm)[a]	1.4 ± 0.3	1.5 ± 0.3	1.7 ± 0.4	1.6 ± 0.4
Weight of leaf (mg)[a]	91.7 ± 4.5	107.4 ± 4.8	121.9 ± 6.0	120.3 ± 6.0
Average leaf area (mm^2)[b]	141 ± 7	156 ± 7	181 ± 9	189 ± 9
Average epidermal cell area ($\times 10^{-3}$ mm^2)[c]	1.8 ± 0.1	2.1 ± 0.1	2.3 ± 0.1	2.3 ± 0.1

Plants were grown in 2.5-inch pots under long-day (16 h light/8 h dark) conditions for 30 days
All values represent the mean ± SD. SD, standard deviation
[a]Measurements taken from the fifth rosette leaf. $n = 20$
[b]Measurements taken from all expanded rosette leaves. $n = 20$
[c]Measurements taken from 30 epidermis cells of the fifth rosette leaf from five plants

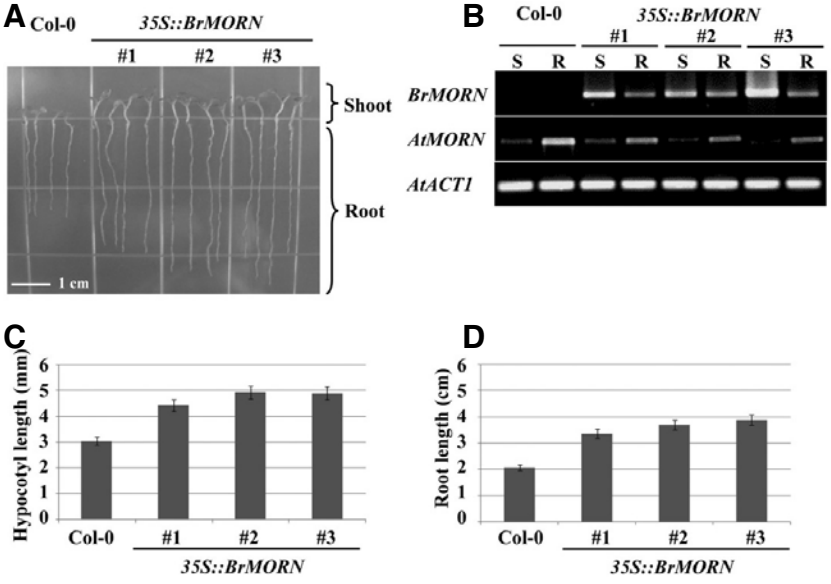

Fig. 4. Growth of *Arabidopsis* plants overexpressing *BrMORN* at an early stage of growth. (A) Plate-grown wild-type and transgenic *Arabidopsis* plants. Plants were grown on MS-agar plates for 4 days after germination. Bar = 1 cm. (B) RT-PCR analysis of *BrMORN* expression. S and R indicate shoot and root, respectively. *AtMORN* indicates the transcript level of the endogenous *Arabidopsis* homolog of *BrMORN* (At4g 17080). *AtACT1* (*Arabidopsis actin 1*) was used as a loading control. (C, D) Lengths of hypocotyls and roots, respectively. Measurements were performed with 50 plants from each line. Error bars represent the standard deviation of three independent experiments. S, shoot; R, root.

was 24% larger than that of the wild type.

Because growth of hypocotyls and roots in *35S::BrMORN Arabidopsis* plants was enhanced, transverse sections of roots from 1-week-old plants were examined (Fig. 7A). The size of cells in the epidermis, vascular bundle, and pericycle was increased, but the number of cells remained unchanged. This result indicated that root and hypocotyl growth may be related to the increase in cell size induced by *Br*MORN. In addition, the size of epidermal cells in *BrMORN*-overexpressing plants was 24% larger in the wild-type (Fig. 7B), clearly suggesting that the observed increase in leaf size is due to a corresponding increase in cell size.

To examine the relationship between vegetative growth and seed production several vegetative and reproductive elements were measured (Table 2). No differences in primary shoot length or mature silique length were seen between *BrMORN*-overexpressing and wild-type plants. However, the number of leaves present before the reproductive stage and the number of lateral shoots were greatly increased in transgenic *Arabidopsis*, but the length of vegetative period was not affected. This had the effect of facilitating the rate of vegetative growth and increasing the number of total siliques by up to 40%. In addition, while the seed number per silique was similar, the weight per seed was increased by 23% due to the increase in its size, particularly seed width (Table 2). These increases in the total number of siliques and weight per seed led to vastly improved seed production. Taken together, we can conclude that the ectopic expression of *BrMORN* enhances both vegetative growth and seed production by increasing cell size.

DISCUSSION

MORN motif repeats are found in various proteins derived from both animals and plants. The number of MORN motif repeats varies between two and 14 and the position of the motifs varies as well; motifs are found at the N-terminus, the C-terminus, or throughout a protein. Functions of the MORN motif have been identified for several proteins. The motif is involved in the endoplasmic reticulum (ER)- or sarcoplasmic reticulum (SR)-to-plasma membrane attachment of junctophilins (Landstrom et al., 2007; Takeshima et al., 2000); in contractile ring formation for *Tg*MORN1 (Gubbels et al., 2006); in plastidial fission for *Arabidopsis* ARC3 protein (Maple et al., 2007; Shimada et al., 2004); and in subcellular localization of the PIPKs (Ma et al., 2006).

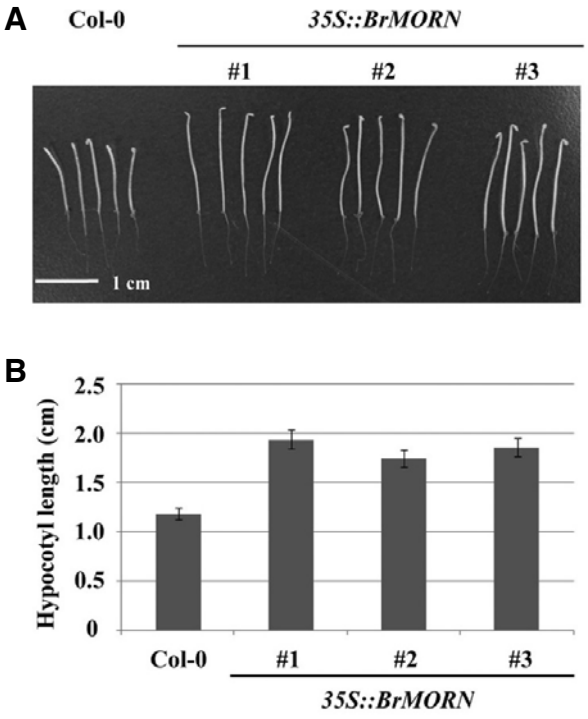

Fig. 5. Hypocotyl growth of etiolated wild-type and *BrMORN*-overexpressing *Arabidopsis* plants. (A) Etiolated seedlings. Sterilized and chilled seeds were sown on 0.5× MS medium containing 0.8% agar and grown for 4 d at 22°C in the dark. Bar = 1 cm. (B) Hypocotyl length measured in at least 50 seedlings. Lengths were measured with the NIH-Image program. Data are expressed as the mean ± standard deviation of three replicates.

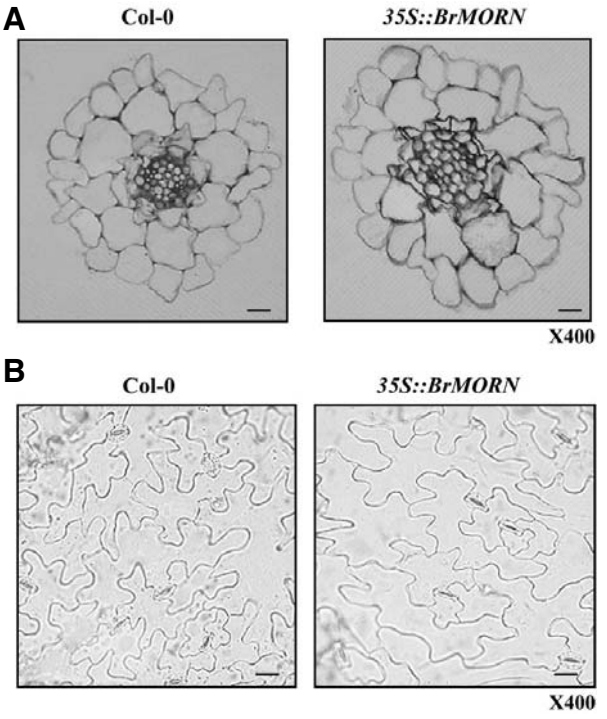

Fig. 7. Transverse sections of roots (A) and micrograph of epidermal cells (B) from wild type and *BrMORN*-overexpressing *Arabidopsis* plants. (A) Transverse sections from the roots of 1-week-old plants grown on plates containing 0.5× MS medium were prepared, fixed, stained with toluidine blue, observed with an E-600 microscope (Nikon Instruments), and photographed with a SPOT camera and software. Bars = 10 μm. (B) Epidermal tissues were peeled from the fifth rosette leaf of 4-week-old plants grown under long-day conditions. The tissue was stained with 1% Safranin O.

Generally, the function of MORN-containing proteins is to bind to membranes and/or facilitate the formation of a protein scaffold involved in tight membrane adhesion or organelle fission.

However, only two classes of MORN motif proteins, i.e., human

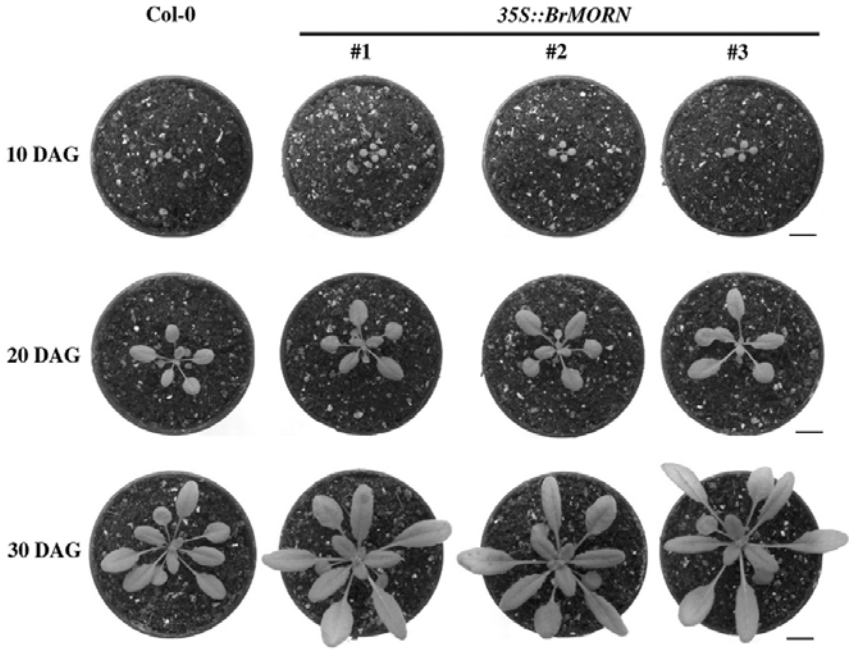

Fig. 6. Phenotypes of *Arabidopsis* plants overexpressing *BrMORN* compared with wild-type plants. Photos were taken 10, 20, and 30 days after germination (DAG). Three independent transgenic lines are indicated as #1, #2, and #3 at the top. Bars = 1 cm.

Table 2. Summary of vegetative growth and seed production in soil-grown *Arabidopsis* plants overexpressing *BrMORN*

Measurement	Wild type	*35S::BrMORN*		
		#1	#2	#3
Primary shoot length (cm)[a]	38.3 ± 0.9	37.4 ± 1.8	37.3 ± 1.8	36.9 ± 1.7
No. of lateral shoots[a]	6.0 ± 0.2	7.0 ± 0.3	6.8 ± 0.3	6.6 ± 0.3
Total no. of leaves[a]	12.5 ± 1.2	16.2 ± 1.2	15.8 ± 1.4	15.3 ± 1.3
Final no. of siliques[a]	114 ± 19	153 ± 10	169 ± 8	154 ± 6
Mature silique length (cm)[b]	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1
No. seeds per silique[b]	61 ± 3	62 ± 3	62 ± 3	63 ± 3
Weight of 150 seeds (mg)	13.3 ± 0.6	15.6 ± 0.7	17.1 ± 0.8	16.4 ± 0.8
Seed length (mm)[c]	1.1 ± 0.05	1.2 ± 0.05	1.2 ± 0.05	1.1 ± 0.06
Seed width (mm)[c]	0.7 ± 0.03	0.8 ± 0.04	0.9 ± 0.04	0.9 ± 0.05

All values represent the mean ± SD. SD, standard deviation
[a]Data documented at maturity. $n = 20$
[b]Measurements taken from five siliques/plant. $n = 20$
[c]Measurements of mature seeds. $n = 50$

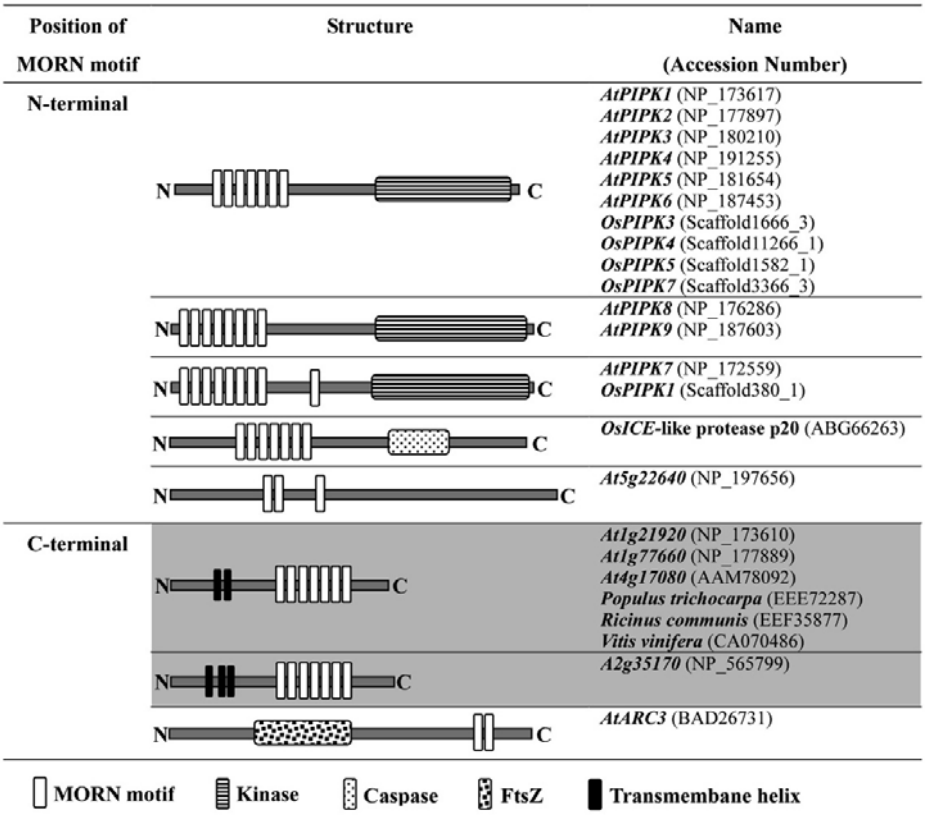

Fig. 8. MORN motif-containing proteins in plants. The shaded region contains *Br*MORN homologs present in the *Arabidopsis* genome and reported in three plant species.

ALS2 and plant PIPKs, have an enzymatic function; ALS2 is a Rab5 guanine nucleotide exchange factor (GEF) that controls endosomal membrane trafficking (Kunita et al., 2004) and plant PIPKs have multiple regulatory effects mediated by a kinase domain (Im et al., 2007; Ma et al., 2006). All of this evidence implies that MORN motif proteins possess diverse functions.

In plants, several MORN motif-containing proteins have been reported in *Arabidopsis* and rice or are found in the NCBI database (Fig. 8). For example, plant PIPKs, rice ICE-like protease p20, and At5g22640 all harbor MORN motifs at the N-terminus, whereas *At*ARC3 and four PIPK-related proteins contain MORN motifs at their C-termini. In addition to MORN motifs, rice ICE-like protease p20, *At*ARC3, and all PIPKs possess other functional domains, including, a putative caspase domain at the C-terminus, a prokaryotic FtsZ domain in the N-terminal half, and a kinase domain at the C-terminus respectively. Only At5g22640 does not have any other functional domain.

The *Arabidopsis* genome contains two groups of MORN-motif proteins - those with the motif at the N-terminus and those with C-terminal motifs (Fig. 8). PIPKs comprise the majority of N-terminal MORN motif proteins, but PIPK-related proteins are C-terminal MORN motif proteins. Four *Arabidopsis* PIPK-related proteins

and three other plant proteins contain transmembrane domains other than MORN motifs (Fig. 8). These *Br*MORN homologs (At4g17080, At2g35170, At1g21920, At1g77660, EEE72287, EEF35877, and CA070486) do not possess any functional domain other than the MORN motif; however, the function of these novel MORN motif proteins has not been studied to date. At4g17080 (AAM78092) shows the highest level of identity with *Br*MORN, with 82% identity at the amino acid level, and At2g35170 (NP_565799) shares approximately 66% identity. At1g21920 (NP_173610) and At1g77660 (NP_177889) show less than 50% identity with *Br*MORN. However, the seven MORN motifs are well-conserved among the four *Arabidopsis* proteins and *Br*MORN, despite the fact that *Br*MORN possesses a variable N-terminal region (data not shown). At2g35170 has one additional transmembrane domain that the other three proteins lack (Fig. 8). In addition to possessing transmembrane domains, *Br*MORN and its homologs are similar in that their size is small relative to other known plant MORN motif proteins. These physical properties may imply that these types of proteins exert their function in a different manner from those of PIPKs.

The number of MORN motifs is the primary factor controlling the localization of proteins to either the plasma membrane or organelles (Ma et al., 2006; Takeshima et al., 2000). Indeed, MORN motif proteins play roles in membrane binding, localization of the resulting complexes, and control of the activity of various components, as suggested by variations in the number and position of MORN motifs as well as the presence and absence of enzymatic domains. In this study, overexpression of *BrMORN* resulted in increased plant size and seed production as well as stimulation of the growth rate (Figs. 4-7, Tables 1 and 2). However, the expression of *BrMORN* in Chinese cabbage was highest in growing tissues and suppressed by some abiotic stresses (Figs. 2 and 3). *BrMORN* overexpression in *Arabidopsis* plants did not confer resistance to abiotic (cold and heat shock) stresses (data not shown), indicating that *Br*MORN may function primarily under normal growth conditions. Therefore, we propose that *Br*MORN bound to the plastid envelope may interact with other membranes or cellular components under normal growth conditions, thereby facilitating cellular processes related to plant growth. However, we could not rule out the possibility that *Br*MORN stimulates plant growth by interaction with the cytoskeleton. To extend our research on *Br*MORN, we are currently examining the phenotype of *Arabidopsis* knockout mutants corresponding to four *BrMORN* homologs and we are preparing and analyzing double mutants as well as complementation mutants.

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