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Alternative splicing of the *OsBWMK1* gene generates three transcript variants showing differential subcellular localizations

Sung Cheol Koo [a], Hae Won Yoon [a], Cha Young Kim [b], Byeong Cheol Moon [a], Yong Hwa Cheong [c], Hay Ju Han [b], Sang Min Lee [b], Kyu Young Kang [a,b], Min Chul Kim [a,b], Sang Yeol Lee [a,b], Woo Sik Chung [a,b,*], Moo Je Cho [a,*]

[a] *Division of Applied Life Science (BK21 Program), Plant Molecular Biology and Biotechnology Research Center (PMBBRC), Gyeongsang National University, Jinju 660-701, Republic of Korea*
[b] *Environmental Biotechnology National Core Research Center, Gyeongsang National University, Jinju 660-701, Republic of Korea*
[c] *Department of Bio-Environmental Science, Sunchon National University, Sunchon 550-742, Republic of Korea*

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Abstract

In eukaryotes, mitogen-activated protein kinases (MAPKs) play important roles in various developmental processes and in environmental stress responses. Here, we show that alternative splicing of the *OsBWMK1*, a member of the rice MAPK family, generates three transcript variants, *OsBWMK1L*, *OsBWMK1M*, and *OsBWMK1S*. The *OsBWMK1L* transcript variant was highly and constitutively expressed in all rice tissues tested and its expression was not altered by various stress conditions, whereas *OsBWMK1M* and *OsBWMK1S* were normally expressed at low levels but were induced by various stresses. A transient expression assay demonstrated that OsBWMK1L::GFP and OsBWMK1M::GFP were predominantly localized in the cytoplasm, whereas most OsBWMK1S::GFP was localized in the nucleus. Moreover, treatment with defense signaling related molecules, such as H_2O_2 and SA, induced translocation of OsBWMK1 isoforms from the cytoplasm to the nucleus. Thus, our results suggest that alternative splicing of *OsBWMK1* generates three different transcript variants that produce proteins with different subcellular localizations.

Keywords: Alternative splicing; Localization; MAP kinase; *OsBWMK1* gene

Diverse extracellular stimuli are transduced into intracellular responses by a MAPK cascade that is evolutionarily conserved in many organisms, from yeast to mammals and plants [1–4]. This conserved MAPK cascade is composed of three functionally interlinked protein kinases, MAPKKK, MAPKK, and MAPK. The *OsBWMK1* cDNA (GenBank Accession No. AF177392) was the first MAPK reported from rice whose gene expression is induced by blast fungus infection and by mechanical wounding [5]. Furthermore, Cheong et al. [6] reported that constitutive expression of *OsBWMK1* (GenBank Accession No. AF194415) in tobacco plants triggered ectopic expression of pathogenesis-related (PR) genes and enhanced resistance against fungal and bacterial infections by activating the *OsEREBP1* transcription factor. To date, many additional novel rice MAPKs have been identified and their biological roles have been characterized [7–10]. These studies have greatly increased our understanding of how the MAPK signaling pathway operates in response to a wide range of environmental stimuli, as well as in development.

Alternative splicing is one of the important mechanism used to regulate gene expression and is employed by all eukaryotes [11–13]. The selection of alternative splicing

* Corresponding authors. Address: Division of Applied Life Science (BK21 Program), Plant Molecular Biology and Biotechnology Research Center (PMBBRC), Gyeongsang National University, Jinju 660-701, Republic of Korea. Fax: +82 55 759 9363.
E-mail addresses: chungws@gsnu.ac.kr (W.S. Chung), choslab@gnu.ac.kr (M.J. Cho).

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sites in the pre-mRNA of a single gene can generate multiple mRNAs that encode structurally and functionally distinct proteins. In many cases, the alternative isoforms differ by small alterations in functional elements or domains rather than by complete removal of functional elements [14]. Such changes in the protein-coding region can alter the binding properties of isoforms, influence their intracellular localization, and/or modify their enzymatic activity and/or protein stability [15,16]. In plants, many genes associated with various stress responses are alternatively spliced [17–19]. For example, the *OsMAPK5* gene, which is a water stress- and plant defense-associated MAPK gene of rice, is alternatively spliced to generate at least two splice variants [20].

Here, we report the identification of three different transcripts generated from the rice *OsBWMK1* gene. Three alternative splice variants of *OsBWMK1* showed different gene expression patterns and the isoforms were localized to different subcellular compartments. Furthermore, detailed fractionation studies showed that the OsBWMK1 isoforms translocated from the cytoplasm to the nucleus in response to stresses.

Materials and methods

Rice suspension cell culture and treatments. Suspension cell lines of rice (*Oryza sativa* L. Donjin) were cultured and maintained as described by Cheong et al. [6]. The rice blast fungus *Magnaporthe grisea* (*M. grisea*) was added to the cell cultures at a final concentration of 50 μg mL^{-1}. Salicylic acid (SA), hydrogen peroxide (H_2O_2), jasmonic acid (JA), or sodium chloride (NaCl) were added at final concentrations of 2, 2, 0.2, and 100 mM, respectively.

5′-Rapid amplification of cDNA ends (RACE). The transcription start sites of the rice *OsBWMK1* gene were mapped using the SMART RACE kit (Clontech), according to the manufacturer's protocol. The primers used were the 5′-AP2 primer (5′-ACTCACTATAG GGCTCGAGCG GC-3′) and the 3′-GSP primer (*OsBWMK1* gene) (5′-AAACTGGTAG TGCTCCGGGGTGAGGTCATC-3′). The 5′-end of the rice *OsBWMK1* was amplified using a standard polymerase chain reaction (PCR) protocol. PCR products were inserted into the pGEM-T Easy vector. Several clones were confirmed by sequencing.

Reverse transcriptase (RT)-PCR. RT-PCR was carried out using total RNA extracted from rice plants and suspension cell cultures treated with various stressors. First-strand cDNA was synthesized from 5 μg of total RNA using Superscript II RNase H-reverse transcriptase and oligo(dT) primers, as recommended by the manufacturer (Invitrogen). PCRs were carried out with the primer pair PL1 (5′-ATGGGGGGAGGGG GCAC GCTCGTCGA CGGA-3′) and P2, or PS1 (5′-GTGGTTTTGGATG ATCTCATCAGAATCGGC-3′) and P2. PCR products were analyzed on a 1.2% agarose gel.

Expression of GST-fusion proteins in Escherichia coli. For expression in bacteria, *OsBWMK1L*, *OsBWMK1M*, and *OsBWMK1S* were fused in-frame to the C-terminal sequence of glutathione *S*-transferase (GST). Full-length *OsBWMK1* cDNAs were obtained by PCR using *OsBWMK1*-specific 5′ and 3′ primers. The PCR products were inserted into the pGEX-2T vector (Amersham, Buckinghamshire, UK). The resulting constructs were confirmed by sequencing and introduced into the *E. coli* strain BL21 (pLysS) and the GST-fusion proteins were expressed and then purified for analysis.

Transient expression of GFP-Fusion proteins in arabidopsis rotoplasts. To examine the subcellular localization of OsBWMK1L, OsBWMK1M, and OsBWMK1S, the coding regions of the transcript variants were fused to the N-terminus of GFP under the control of the CaMV 35S promoter. Full-length *OsBWMK1* cDNAs were obtained by PCR using *OsBWMK1*-specific 5′ and 3′ primers. The PCR products were inserted into the pUC::GFP expression vector. A fusion construct consisting of the nuclear localization signal from the SV-40 large T antigen and the red fluorescent protein (NLS::RFP) served as a positive control. To transiently express the fusion proteins in protoplasts isolated from *Arabidopsis* leaves, PEG-mediated transformation [6] was used to co-transfect the protoplasts with each OsBWMK1::GFP construct and NLS::RFP. GFP expression was monitored at various times after transfection using an Axioplan 2 fluorescence microscope.

Isolation of cytosolic and nuclear proteins. Cytosolic and nuclear proteins from rice suspension cell cultures were prepared as described by Busk and Pages [21]. Rice suspension cell cultures treated with SA and H_2O_2 were harvested at various time points after treatment.

Production of antibodies. The N-terminal 74 amino acids of the OsBWMK1L protein and the C-terminal 182 amino acids of the OsBWMK1 protein were fused to the C-terminus of GST (GST::OsBWMK1L-Nt and GST::OsBWMK1-Ct). Purified recombinant GST::OsBWMK1L-Nt and GST::OsBWMK1-Ct were used to raise antibodies in rabbit (anti-Nt and anti-Ct, respectively). The antisera were affinity-purified using the corresponding immobilized 6xHis-fused OsBWMK1L-Nt and OsBWMK1-Ct proteins, respectively.

Western blot analysis. After the fractions were isolated from rice suspension cell cultures, they were separated by 10% or 12% SDS–PAGE and analyzed by Western blot analysis using the appropriate antibodies. Western blot analysis was performed as described [6]. The anti-ACaM2 (*Arabidopsis* Calmodulin2) antibody was used to identify the cytosolic protein fraction and the anti-AcH4 (Acetylated Histone) antibody was used to identify the nuclear protein fraction.

Results and discussion

Identification of three alternative splice variants of OsBWMK1

To determine the transcript start sites of the rice *OsBWMK1* cDNA isolated from our previous study [6], we conducted a RACE analysis. The 5′-RACE generated two PCR products that were approximately 800 and 1000 bp in size (Fig. 1A). The 5′-RACE products were cloned and sequenced. From the sequencing of 30 different clones, we identified three different transcript variants and referred to these as *OsBWMK1L* (long), *OsBWMK1M* (middle), and *OsBWMK1S* (short), based on the lengths of their open reading frames (Fig. 1B). When the nucleotide sequences of three transcript variants were compared to the rice genomic sequence of the *OsBWMK1* gene obtained from the TIGR database (http://www.tigr.org), we found that the *OsBWMK1* gene consists of 11 exons and 10 introns and that the *OsBWMK1L* transcript corresponds to the full-length mRNA (Fig. 1C). Compared to *OsBWMK1L*, the other two transcript variants start at the first intron (+483 and +517 positions for *OsBWMK1M* and *OsBWMK1S*, respectively), and thus, lack the first exon of the *OsBWMK1* gene. Furthermore, *OsBWMK1M* had undergone an additional splicing event in its 5′-untranslated region (UTR) that eliminates nucleotides +903 to +1111. As a result, the *OsBWMK1M* transcript lacks 209 nucleotides found in the *OsBWMK1S* transcript. The *OsBWMK1L* transcript appears to have been

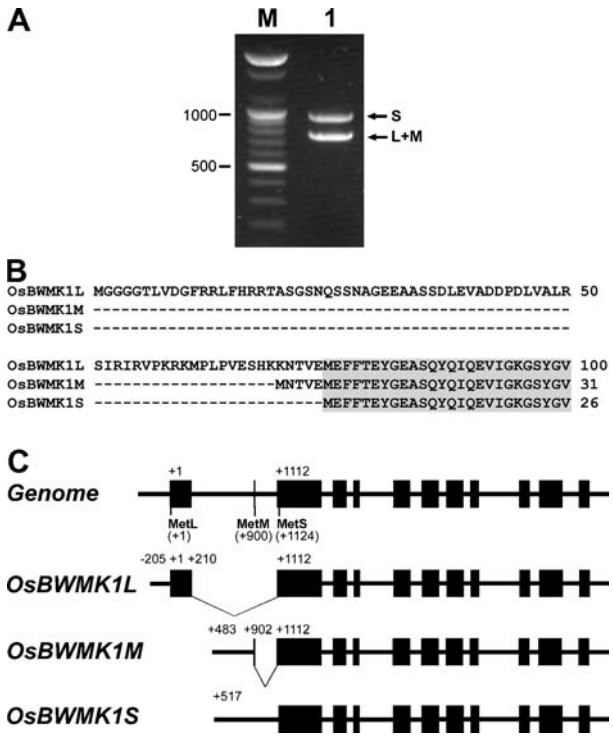

Fig. 1. RACE-PCR analysis and *OsBWMK1* gene structure. (A) Detection of alternative transcripts of *OsBWMK1* by RACE-PCR analysis. M, DNA size markers. L, M, and S indicate the *OsBWMK1L*, *OsBWMK1M*, and *OsBWMK1S* transcripts, respectively. (B) Alignment of the N-terminal deduced amino acid sequences of *OsBWMK1L*, *OsBWMK1M*, and *OsBWMK1S*. The gray boxes indicate the amino acid residues that are common to the three OsBWMK1 splice variants. (C) Schematic depiction of the genomic structure of *OsBWMK1* and alternative splicing of the three transcript variants. The exons and introns are represented by boxes and lines, respectively. The numbering of the nucleotides in all sequences is based on the first translation start site in *OsBWMK1L* (numbered +1). Upstream sequences are indicated by negative numbers. MetL, MetM, and MetS under the genome sequences indicate the first translation start codon of each of the alternative transcripts.

generated from another transcription start site which gives rise to another exon in addition to the 10 exons shared with the other two transcripts. Moreover, *OsBWMK1L* has also undergone alternative splicing. Compared to splicing of *OsBWMK1M*, splicing of *OsBWMK1L* uses the same 3′-acceptor splice site (at position +1112) but a different 5′-donor splice site (position +211 versus position +903). Many alternative splicing events of the eukaryotic genes containing multiple promoters use the same 3′-splice site but a different 5′-splice site to generate transcript variants that have different first exons and different transcription start sites [22–25]. Thus, these results suggest that the *OsBWMK1* transcript variants could be generated by the use of different promoters, as well as by alternative splicing.

The deduced protein sequences of the three transcript variants were identical, with the exception that OsBWMK1L and OsBWMK1M have extended N-termini comprised of additional 74 and 5 amino acids, respectively (Fig. 1B). The deduced amino acid sequence of the *OsBWMK1S* transcript is same as that of the *OsBWMK1* cDNA isolated previously.

Primer extension and RT-PCR experiments verified the differential transcription initiation sites and alternative splicing of the *OsBWMK1* gene (Supplementary Fig. 1B and C). This result confirms that three transcript variants are indeed present in suspension cultured rice cells. We also confirmed that the three transcript variants could be generated from the same genomic DNA by genomic PCR analysis (Supplementary Fig. 1D). Our observations suggest that the rice *OsBWMK1* gene produces three different transcript variants through alternative splicing as well as the selection of different transcription start sites.

Expression profiles of the OsBWMK1 transcript variants

To investigate expression patterns of three *OsBWMK1* transcripts, we analyzed their expression in whole rice plants at the three-leaf, young plant (3 week old), and mature flowering plant (10 week old) stages by RT-PCR (Fig. 2A). We found that *OsBWMK1L* was the most highly expressed of the three transcripts and was constitutively expressed at all developmental stages. In contrast, *OsBWMK1M* and *OsBWMK1S* were transcribed at lower levels and were only detected in young and mature plants. Moreover, the *OsBWMK1M* transcript was expressed at significantly lower levels than the *OsBWMK1S* transcript. We next examined the expression of the three transcripts in various tissues from young and mature plants by RT-PCR. We found that the *OsBWMK1L* transcript was abundantly expressed in all tissues, regardless of the developmental stages of the plants. In contrast, the *OsBWMK1M* and *OsBWMK1S* transcripts were expressed at moderate to very low levels in most tissues (Fig. 2B). Moreover, these two transcripts differed in their expression patterns as well. *OsBWMK1S* was expressed at moderate levels in the roots and leaves of young plants and in the leaves and flowers of mature plants, whereas *OsBWMK1M* was expressed at very low levels only in young leaf tissues. These results indicate that three *OsBWMK1* transcript variants are differentially expressed in various tissues and developmental stages. To determine whether various plant stressors affect the expression patterns of the *OsBWMK1* transcript variants, we treated rice suspension cell cultures with fungal elicitor from *M. grisea*, H_2O_2, JA, SA, or NaCl. The total RNAs of the cells was then subjected to RT-PCR (Fig. 2C). *OsBWMK1L* was abundantly expressed regardless of the treatment. In contrast, the *OsBWMK1M* and *OsBWMK1S* transcripts were upregulated by all treatments. Thus, the *OsBWMK1M* and *OsBWMK1S* transcript variants are induced by various stressors, whereas the *OsBWMK1L* transcript remains abundantly and constitutively expressed. These results suggest that *OsBWMK1* splicing variants may participate in different signaling pathways in rice through their different gene expressions.

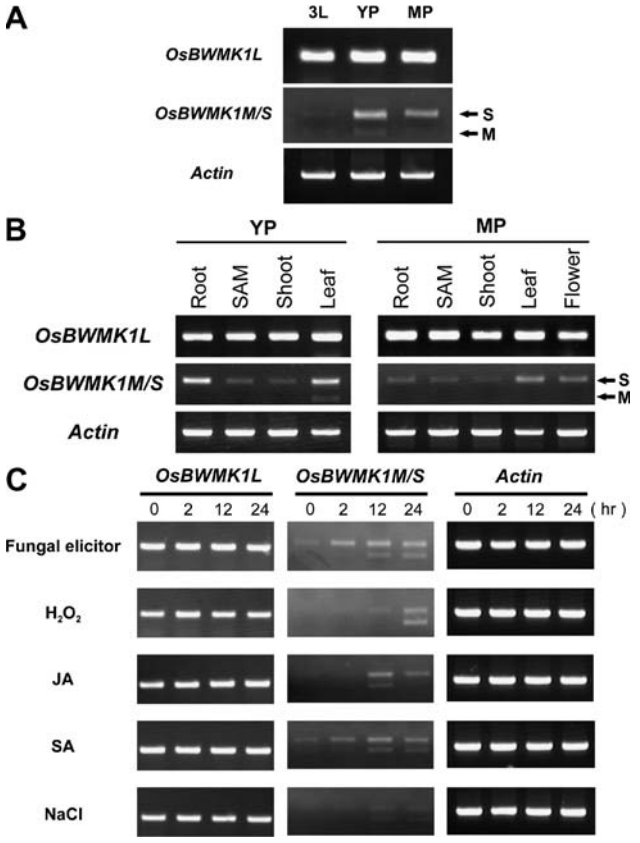

Fig. 2. Expression patterns of the *OsBWMK1* transcript variants. (A) Expression patterns of *OsBWMK1* transcript variants at different developmental stages: 3L, plants with 3 leaves; YP (young plants), plants grown for approximately 3 weeks; MP (mature plants), flowering plants grown for approximately 10 weeks. (B) Expression patterns of *OsBWMK1* transcript variants in different tissues: roots, shoots, shoot apical meristem (SAM) and leaves of young (YP), and mature (MP) plants. (C) Effect of various plant stressors on the expression of *OsBWMK1* transcript variants in suspension cultured rice cells. Total RNAs were isolated from suspension cultured cells treated with the fungal elicitor, H_2O_2, SA, JA, or NaCl, at the indicated times. RT-PCRs were performed with the PL1/P2 primer pair (*OsBWMK1L*) and PS1/P2 primer pair (*OsBWMK1M/S*) using first-strand cDNA as a template. Actin was also amplified to verify equal amounts of cDNA used for RT-PCR.

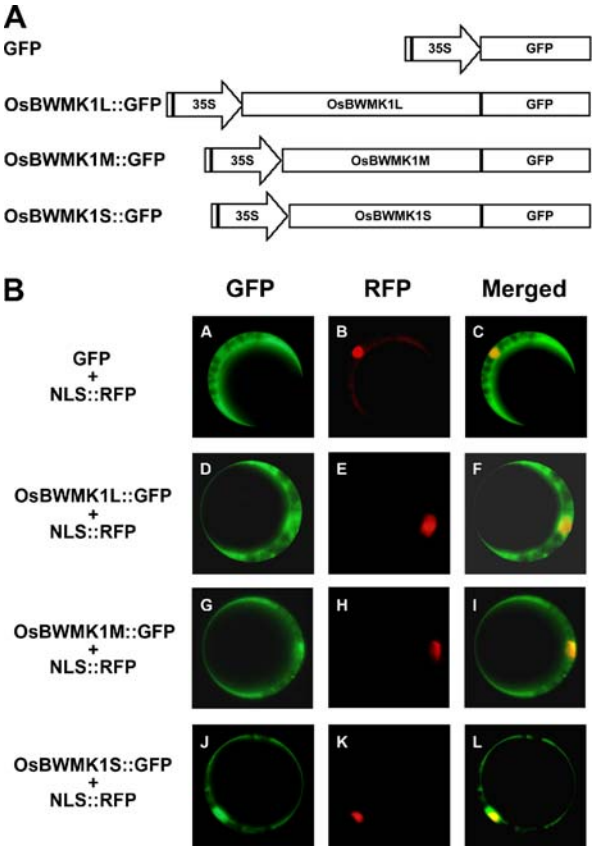

Fig. 3. Different subcellular localization patterns of the three OsBWMK1 isoforms. (A) Schematic depiction of the GFP, OsBWMK1L::GFP, OsBWMK1M::GFP, and OsBWMK1S::GFP fusion constructs. The constitutive 35S CaMV promoter was used to express these fusion proteins in *Arabidopsis* protoplasts. (B) Subcellular localization of OsBWMK1L::GFP, OsBWMK1M::GFP, and OsBWMK1S::GFP. The fluorescent images of the GFP fusion proteins are shown in the left panels (A, D, G, and J), the fluorescent images of NLS::RFP in the nucleus are shown in the middle panels (B, E, H, and K), and the merged fluorescence images of GFP and RFP are shown in the right panels (C, F, I, and L).

Subcellular localization of the OsBWMK1 isoforms

To determine the subcellular localization of the OsBWMK1 isoforms, OsBWMK1L::GFP, OsBWMK1M::GFP, and OsBWMK1S::GFP were expressed in *Arabidopsis* protoplasts. NLS::RFP was coexpressed with each construct to compare localization in individual cells. OsBWMK1S::GFP was primarily localized in the nucleus, although a portion of OsBWMK1S::GFP was localized in the cytoplasm. In contrast, OsBWMK1L::GFP and OsBWMK1M::GFP were localized predominantly in the cytoplasm (Fig. 3B). The differential localization of the OsBWMK1 isoforms suggests that the proteins play different roles in cell signaling. OsBWMK1L differs from OsBWMK1S by an additional 74 N-terminal amino acids (Fig. 1B). Although we did not detect any signal sequences-related protein localization, database analysis of this additional N-terminal region revealed the presence of interaction motifs with several proteins such as 14-3-3, cyclin, TRAFT2, and CK1, suggesting that the N-terminus of OsBWMK1L could be involved in protein–protein interactions with regulatory proteins or specific substrates that are responsible for its localization in the cytoplasm.

Translocation of OsBWMK1 isoforms in response to extracelluar stimuli

To detect endogenous OsBWMK1 isoforms, we raised polyclonal antibodies against the N-terminal region (amino acids 1–74) of OsBWMK1L (anti-Nt) and the C-terminal region (amino acids 415–506) of OsBWMK1S (anti-Ct). The specificities of these two antibodies were tested by Western blot analysis using recombinant GST::OsBWMK1L and GST::OsBWMK1S (Fig. 4A). The anti-Ct antibody recognized both GST-OsBWMK1L and GST-OsBWMK1S. In contrast, the anti-Nt antibody specifically reacted only with GST-OsBWMK1L which

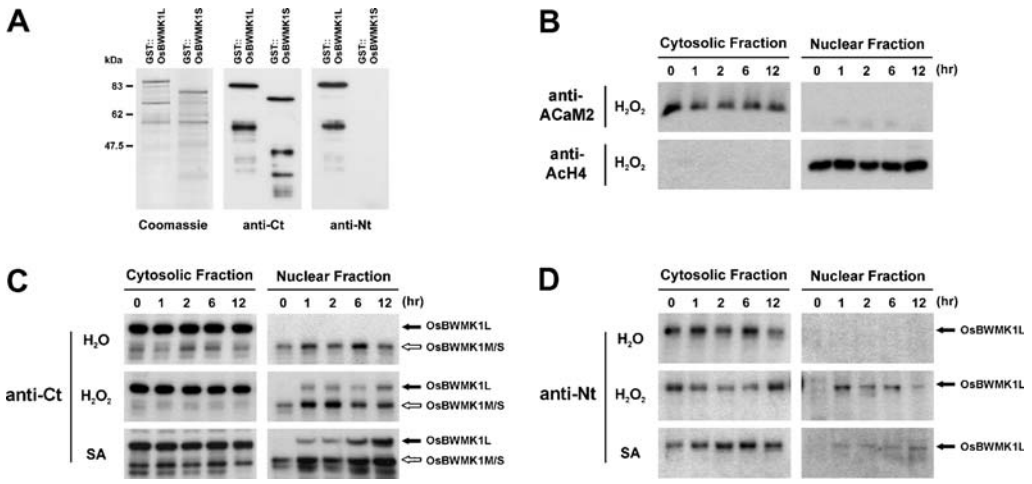

Fig. 4. Translocation of OsBWMK1 proteins from the cytoplasm into the nucleus in response to H_2O_2 or SA treatment. (A) Western blot analysis demonstrating the specificity of polyclonal antibodies against the N-terminus of OsBWMK1L (anti-Nt) or the C-terminus of OsBWMK1 (anti-Ct). Recombinant GST fusion OsBWMK1L and OsBWMK1S proteins expressed in *E. coli* were purified and used for assay. (B) Fractionation of cytosolic and nuclear proteins. Anti-ACaM2 and anti-AcH4 antibodies were used as cytosolic and nuclear protein markers, respectively. (C) Western blot analysis of OsBWMK1 proteins using the anti-Ct antibody. The cytosolic and nuclear protein extracts from rice suspension cell cultures treated with H_2O, H_2O_2, and SA were analyzed at various times by Western blotting. The filled and empty arrows indicate the OsBWMK1L and OsBWMK1M/S proteins, respectively. (D) Western blot analysis of OsBWMK1 proteins using the anti-Nt antibody. The same samples as shown in (C) were analyzed by Western blotting using the anti-Nt antibody.

contains the additional N-terminal region. This result shows that these two antibodies specifically could recognize different OsBWMK1 isoforms. Several plant MAP kinases have been reported to translocate from the cytoplasm to the nucleus in response to various stresses, such as fungal elicitors or ozone [26,27]. To examine whether the subcellular localization of the OsBWMK1 isoforms is altered in response to various stimuli, we treated rice suspension cell cultures with the plant defense-related signaling molecules, H_2O_2 and SA, and examined the localization of the proteins using subcellular fractionation and Western blot analysis. Isolation of cytosolic and nuclear fractions was confirmed by probing with anti-ACaM2 and anti-AcH4 antibodies as the cytosolic and nuclear protein markers, respectively [28,29] (Fig. 4B). The anti-Ct antibody cross-reacted with two bands, which correspond to the sizes of the OsBWMK1L (65.9 kDa) and OsBWMK1M/S (58.5 and 57.9 kDa, respectively) proteins (Fig. 4C). We could not separate OsBWMK1M and OsBWMK1S by electrophoresis, due to the similar molecular weights of two proteins. Regardless of H_2O_2 and SA treatments, OsBWMK1M/S isoforms were detected in both the cytosolic and the nuclear fractions. In contrast, most of the OsBWMK1L isoform was present in the cytoplasm before treatment, but the OsBWMK1L isoform was translocated to the nucleus after treatment with H_2O_2 and SA (Fig. 4C). To confirm translocation of the OsBWMK1L isoform into the nucleus after treatment, fractionated proteins were subjected to Western blot analysis using the anti-Nt antibody (Fig. 4D). Nuclear translocation of OsBWMK1L was detected by the anti-Nt antibody, consistent with results using the anti-Ct antibody. These data strongly indicate that OsBWMK1L isoform can move from the cytoplasm to the nucleus in response to external stresses, suggesting that OsBWMK1L isoform may regulate stress response pathways. In plants, changes in subcellular localization of MAPKs under various stresses have been reported [26,27,30]. The cytoplasmic retention of ERK5 is achieved by the binding between the N- and C-terminal halves and the activating phosphorylation of the N-terminal half cause nuclear import of ERK5 [31]. The OsBWMK1L protein was able to autophosphorylate itself and to phosphorylate the MBP and OsEREBP1 substrates as functional kinase (Supplementary Fig. 2). Thus, the cytoplasmic retention and translocation of OsBWMK1L could be achieved by phosphorylation dependent control mechanism, similar to ERK5.

Therefore, we can speculate that OsBWMK1L may sense external stimuli as the target of upstream regulators in the cytoplasm and then translocate into the nucleus to interact with its downstream target molecules. Conclusively, our observations indicate that the different OsBWMK1 protein isoforms generated from the three *OsBWMK1* transcript variants may respond to environmental stresses via distinct regulatory mechanisms.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.06.052.

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