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Original Paper

Enhanced translation of the downstream ORF attributed to a long 5′ untranslated region in the *OsMac1* gene family members, *OsMac2* and *OsMac3*

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Abstract The 5′-untranslated region (5′UTR) of mRNAs often affects the translational efficiency of the downstream open reading frames (ORFs), and some of its regulatory elements are involved in the initiation of translation. We found that the 5′UTR of the rice *OsMac1* mRNA, which consisted of more than 500 nucleotides, yielded a significant enhancement of the translational efficiency of the downstream ORF. In the rice genome, *OsMac1* represents a conserved gene family with two homologues, *OsMac2* and *OsMac3*, which contain DUF300 (domain of unknown function 300) domains with predicted transmembrane regions. Similarly to the *OsMac1* mRNA, the *OsMac2* and *OsMac3* mRNAs possess long 5′UTRs consisting of 312 and 318 nucleotides, respectively, that precede the main ORFs, which allow the elevation of the translational efficiency of the downstream ORF. The estimation of the translational efficiency of the *GUS* gene, which is located after the 5′UTRs, in suspension cultures of rice protoplasts showed that it was significantly greater than that of the control. These results suggest that 5′UTRs of *OsMac2* and *OsMac3* enhance the translation of the downstream ORF. Our results indicate that these 5′UTRs play a role of novel translational enhancer elements that enable the efficient translation of the downstream ORF.

Key words: Rice (*Oryza sativa*), 5′-untranslated region, enhancer of translation, downstream ORF, conserved gene family member.

The process of mRNA translation is an important step in the regulation of protein accumulation. An mRNA contains 5′- and 3′-untranslated regions (UTRs), as well as a protein-coding region. Recent studies have suggested that the 5′UTRs of mRNAs are often involved in posttranscriptional regulatory pathways, which control mRNA localization, stability, and translation efficiency (Chatterjee and Pal 2009; Pesole et al. 2001). The initiation of translation in eukaryotic cells involves a large number of factors, some of which are involved in the binding of the initiator tRNA to the 40S ribosomal subunit, to form a 43S preinitiation complex (43S PIC), and the initial association of 43S PIC with the capped 5′ end of the mRNA results in formation of the 48S preinitiation complex (48S PIC). After the initial binding, 43S PIC scans the mRNA until it recognizes the first initiation codon, AUG. After codon–anticodon complex formation, some initiation factors are released and the 60S ribosomal subunit joins the complex to start protein synthesis (Kozak 1999, 2007).

Many 5′UTRs of mRNAs contain multiple AUG codons, which suggests that the first AUG rule is not followed in a remarkable fraction of mRNAs (Grillo et al. 2010; Pesole et al. 2001). The regulation of the translation of specific eukaryotic mRNAs is sometimes mediated by small upstream open reading frames (uORFs) that limit the access of the small ribosomal subunit to a downstream ORF (Holcik and Pestova 2007). Translational control by uORFs located within the 5′UTRs has also been demonstrated for mRNAs that encode the transcription factors GCN4, bZIP11, and the yeast AP1-like transcription factor Yap2, and for the plant *S*-adenosylmethionine decarboxylase mRNAs (Hanfrey et al. 2005; Hinnebusch et al. 2004; Nielsen et al. 2004; Rahmani et al. 2009; Vilela et al. 1999). It has been shown in plants that the translation of the downstream ORF within uORF-containing mRNAs depends on the target-of-rapamycin (TOR)

Abbreviations: CaMV, cauliflower mosaic virus; GUS, β-glucuronidase; nt, nucleotide(s); RT-PCR, reverse transcription polymerase chain reaction.
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protein kinase, in which active TOR is required for successful reinitiation after uORF translation in plants (Schepetilnikov et al. 2013).

Some mRNAs contain long 5′UTRs that function to reduce the efficiency of translation of the downstream ORF. We have demonstrated that the rice *OsMac1* mRNA has a 5′UTR of more than 500 nucleotides (nt) that contains a CU-rich region and three uORFs preceding the downstream ORF, which is involved in the enhancement of the translation of the downstream ORF. The 5′UTR of this mRNA contained three splicing variants that were generated by alternative splicing, and the longest one (termed UTRc) showed a significant ability for the efficient translation of the downstream ORF, which suggests that the additional 38 nt sequence unique to this 5′UTR variant may be involved in the increase of the translational efficiency of the downstream ORF (Teramura et al. 2012).

The rice genome comprises two homologues of *OsMac1*, *OsMac2*, and *OsMac3*, which also contain long 5′UTRs located upstream of the protein-coding ORFs, whose functions are unknown, although they show local homology to a MAP-kinase activating protein and conserve DUF300 (domain of unknown function 300) domains. Here, we describe the characteristics of the 5′UTRs of *OsMac2* and *OsMac3*, and their effects on the translation of downstream ORFs.

Materials and methods

Plant materials

Rice (*Oryza sativa* L. cv. Nipponbare) was used as the wild-type plant and was grown in a greenhouse. Cultured cells were established from rice calluses according to Fujimura et al. (1985).

Construction of reporter genes

The *GUS* gene was used as a reporter and was obtained from pBI221 (Jefferson et al. 1987). The coding region was amplified using primers that corresponded to the regions located between the initiation and termination of the ORF (5′-CAC CCT CGA GAG ATT AGC CTT TTC AAT TTC-3′ and 5′-TGA ATT CCC GAT CTA GTA ACA T-3′). The amplified fragment was inserted into pENTRTM/D-TOPO (Invitrogen, Carlsbad, CA, USA) to generate pENTR-35S::GUS. An expression plasmid containing each UTR followed by the *GUS* gene was obtained as follows: the 5′UTRs of *OsMac2*, and *OsMac3* were amplified from the corresponding cDNAs using the PCR primer sets 5′-CCC GGA TCC ACT CTC CTC G-3′ and 5′-GGG GGA TCC GAC CCT CAT GAC AGC TGG TAA-3′ for *OsMac2*, 5′-CCC GGA TCC ACA GGG AAG G-3′, and 5′-GGG GGA TCC CAT TGC CAT ATT GCG AGA CAG-3′ for *OsMac3*. These sequences were inserted into the *Bam*HI site preceding the *GUS* gene in pENTR-35S::GUS, after digestion of the amplified fragments with *Bam*HI. Binary plasmids were produced using the resultant plasmids, pENTR-OsMac2-5′UTR and pENTR-OsMac3-5′UTR, respectively, and pGWB1 (Nakagawa et al. 2007) via an LR clonase (Invitrogen) reaction. The fragments in the resultant plasmids, pGWB1-OsMac2-5′UTR and pGWB1-OsMac3-5′UTR, were replaced in another binary vector, pDONR 221 (Invitrogen), by the BP clonase reaction. The resultant plasmids were used for transformation of the protoplast cells. The expression plasmid containing UTRc of *OsMac1* mRNA followed by the *GUS* gene, termed OsMac1-UTRc::GUS (Teramura et al. 2012) was used as a control.

Transient expression of the GFP fusion gene in onion epidermal cells

Reporter genes including the green fluorescent protein (*GFP*) gene fused with the protein encoded by the downstream ORF in the *OsMac1*, *OsMac2*, and *OsMac3* genes were constructed as described previously (Teramura et al. 2012). They were driven by the CaMV 35S-promoter (termed 35S::OsMac1-GFP, 35S::OsMac2-GFP, and 35S::OsMac3-GFP, respectively). The regions covering the 5′UTRs and following the downstream ORFs of *OsMac2* and *OsMac3* were amplified using the primer sets 5′-CAC CAT GAG GGT CAA TCC TGC GCT C-3′ and 5′-TGA TTT CTT GAT TTC CCA GCG GC-3′ for *OsMac2* and 5′-CAC CAT GGC AAT GAA AAA TGT TGT CCG-3′ and 5′-CTG GAA CCA TCT ACC TAA TCT G-3′ for *OsMac3* from the corresponding full-length cDNAs of *OsMac2*, and *OsMac3* (accession numbers AK073148, and AK069607, respectively), which were obtained from the Rice Genome Resource Center (Tsukuba, Japan). The amplified fragments were introduced into pENTRTM/D-TOPO (Invitrogen). Subsequently, they were replaced in pGWB5 (Nakagawa et al. 2007) via an LR clonase (Invitrogen) reaction, to express the chimera genes encoding the desired proteins fused to GFP. 35S::GFP and 35S::OsMac1-GFP (Teramura et al. 2012) were used as the control. The *GFP* fusion genes 35S::GFP, 35S::OsMac1-GFP, 35S::OsMac2-GFP, and 35S::OsMac3-GFP were introduced into onion epidermal cells via particle bombardment (Bio-Rad, Richmond, CA, USA), and transient expression was detected according to von Arnim et al. (1998) using a confocal laser scanning microscope (LSM 510 META; Carl Zeiss AG, Oberkochen, Germany).

RT-PCR and real-time quantitative RT-PCR

Total RNA was prepared from each tissue as described previously (Imamura et al. 2007). First-strand cDNA was synthesized from 1 μg of total RNA using the ReverTra-Ace cDNA synthesis kit (Toyobo) with an oligo-dT (20) primer. The resultant cDNA was used for the detection of the transcripts of the *OsMac1*, *OsMac2*, and *OsMac3* genes by RT-PCR and real-time quantitative PCR, as described previously (She et al. 2010). RT-PCR was performed using the set of primers 5′-AGT TTT AAC GAT CAG TTC GC-3′ and 5′-GTT TTC TTG CCG TTT TCG TC-3′, which were synthesized based on the nucleotide sequence of the *GUS* gene. Real-time quantitative PCR was performed using an ABI PRISM 7000 Sequence Detection

System (Applied Biosystems, Foster City, CA, USA) with a SYBR Green real-time PCR mix (Toyobo) and using the primer sets 5′-ATC CAG ATG AAG TTA AGG ACA GTG A-3′ and 5′-TCA CAA TAT ATT CTC CTC CTC CAA G-3′ for *OsMac1*, 5′-GGC AGT GGA GAA TAT GTG ATA AAA G-3′ and 5′-GAG GTA CTC ACC CAA TTG TCA TC-3′ for *OsMac2*, and 5′-ACA ATG TTT TCG GAG GTA GTG TTA G-3′ and 5′-GAA CCA TCT ACC TAA TCT GAA CTC G-3′ for *OsMac3*, via which the amounts of the *OsMac1*, *OsMac2*, and *OsMac3* transcripts were measured. The amount of the *Actin1* mRNA (accession number AK100267) was measured as a positive control, and was used for normalization of the data. The primer set 5′-CCC TCC TGA AAG GAA GTA CAG TGT-3′ and 5′-GTC CGA AGA ATT AGA AGC ATT TCC-3′ was used for the detection of the *Actin1* transcript.

Preparation of rice protoplasts, transformation, determination of GUS activity, and estimation of translational efficiency

Rice protoplast cells were prepared from suspension-cultured cells and were transformed by introduction of the desired plasmid DNA using the PEG method according to Yoo et al. (2007). The cells were incubated for 16 h at 26°C in WI buffer (4 mM MES (pH 5.7), 0.5 M mannitol, and 20 mM KCl), then harvested by centrifugation. The resultant protoplast cells were suspended in GUS extraction buffer (0.5 mM Tris-HCl (pH 7.0), 10 mM EDTA, 1% TritonX-100, and 1% NoniDet P-40 (Sigma-Aldrich, St. Louis, MO, USA)). The aliquots were immediately taken for *GUS* reporter gene assays. GUS activity was measured using the fluorometric assay method described in Pooggin et al. (2000). In parallel, 35S::GFP was introduced into protoplast cells. The amount of fluorescence signals derived from the introduced GFP gene was measured. Translational efficiency was estimated as the relative ratio of GUS activity to the amount of GFP in cells.

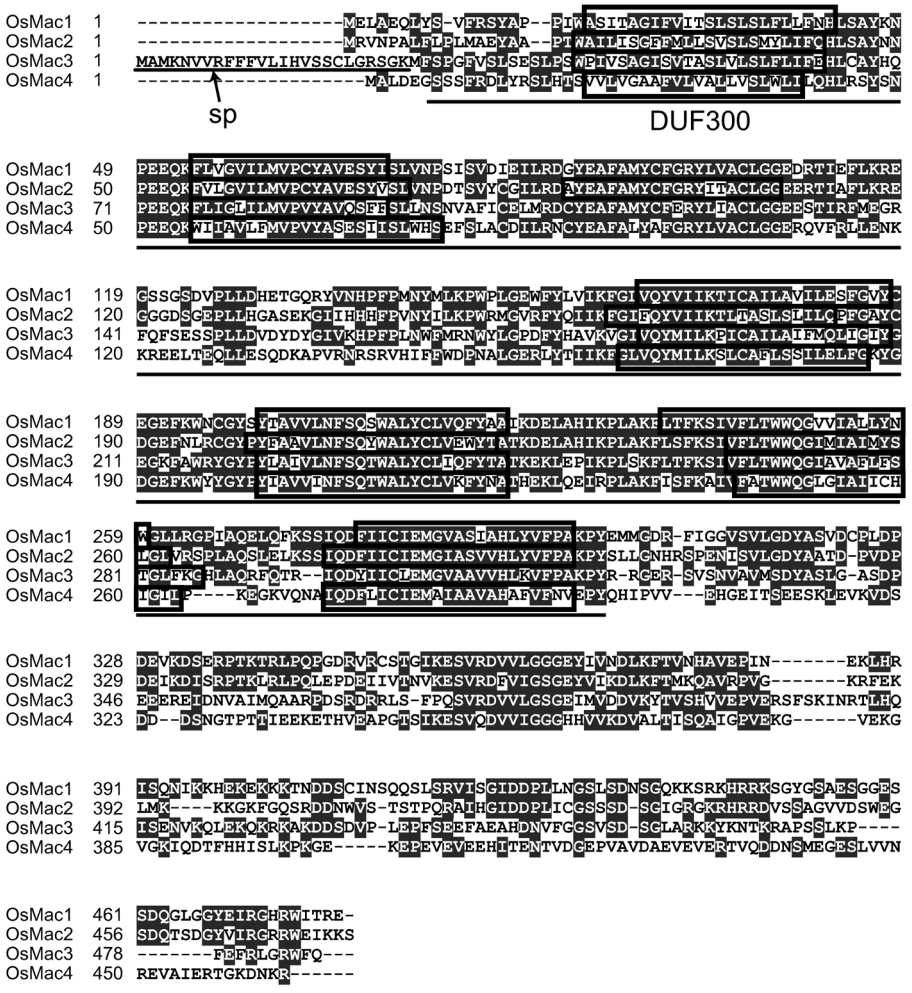

Figure 1. Alignment of the deduced amino acid sequences of *OsMac1*, *OsMac2*, *OsMac3*, and *OsMac4*. Gaps are introduced to optimize the homology. The number of amino acid residues is indicated on the left-hand side. Conserved amino acid residues are shown by reversed letters. The position corresponding to the predicted signal peptide sequence in the N-terminal region of *OsMac3* is underlined. A conserved DUF300 domain region is indicated by a line below, and *trans*-membrane regions are shown in open boxes, which are predicted by TMHMM program (http://www.cbs.dtu.dk/services/TMHMM/). sp: predicted signal peptide.

Results

Rice has OsMac1 homologues as members of a gene family

We analyzed homologue genes of the *OsMac1* gene in the rice genome. There were three homologous genes of *OsMac1* in the rice genome, designated *OsMac2* (Os02g0670000), *OsMac3* (Os05g0516900), and *OsMac4* (Os03g0406900). The predicted proteins encoded by *OsMac2*, *OsMac3*, and *OsMac4* consist of 479, 475, and 488 amino acid residues and showed 57.2%, 50.5%, and 42.1% similarity in amino acid sequence to *OsMac1*, respectively. These homologous proteins contained the conserved DUF300 domain of unknown function, within which transmembrane regions were predicted to exist (Figure 1). *OsMac2* and *OsMac3* showed higher similarity throughout the entire region, whereas *OsMac4* showed a local homology around the DUF300 region. An analysis that was performed using the Signal-P program (http://www.cbs.dtu.dk/services/SignalP/) predicted that *OsMac3* possessed a transit peptide in the N-terminal region.

OsMac1 orthologous genes and its homologues were also found in other plant genomes, such as in maize (*Zea mays*), brachypodium (*Brachypodium distachyon*), grape (*Vitis vinifera*), Arabidopsis (*Arabidopsis thaliana*), and moss (*Physcomitrella patens*). Phylogenetic tree analysis indicated that these homologues in higher plants were classified into four groups, which contained *OsMac1*, *OsMac2*, *OsMac3*, and *OsMac4*, respectively, whereas the moss homologues diverged from these groups. Homologues of *OsMac1*, *OsMac2*, and *OsMac3* exhibited an evolutionarily close relation with each other compared with the members of the *OsMac4* group (Figure 2). These results suggest that *OsMac1* homologues constitute a conserved gene family in plants. Among them, *OsMac2* and *OsMac3* showed higher similarity than *OsMac4*, and we further analyzed on *OsMac2* and *OsMac3*.

Expression patterns of OsMac2 and OsMac3

We analyzed the spatial expression of *OsMac2* and *OsMac3* by real-time RT-PCR. The transcripts of these genes were abundantly detected in mature leaves and developing seeds, but poorly detected in seedlings and roots (Figure 3).

OsMac1 is expressed at the cell membrane (Teramura et al. 2012). To determine the localization of the expression of *OsMac2* and *OsMac3* in cells, we generated fusion genes composed of the *GFP* gene, which was placed after each of the main ORFs (35S::Mac2-GFP and 35S::Mac3-GFP) and was driven by the CaMV 35S-promoter (Figure 4A). Transient expression of the

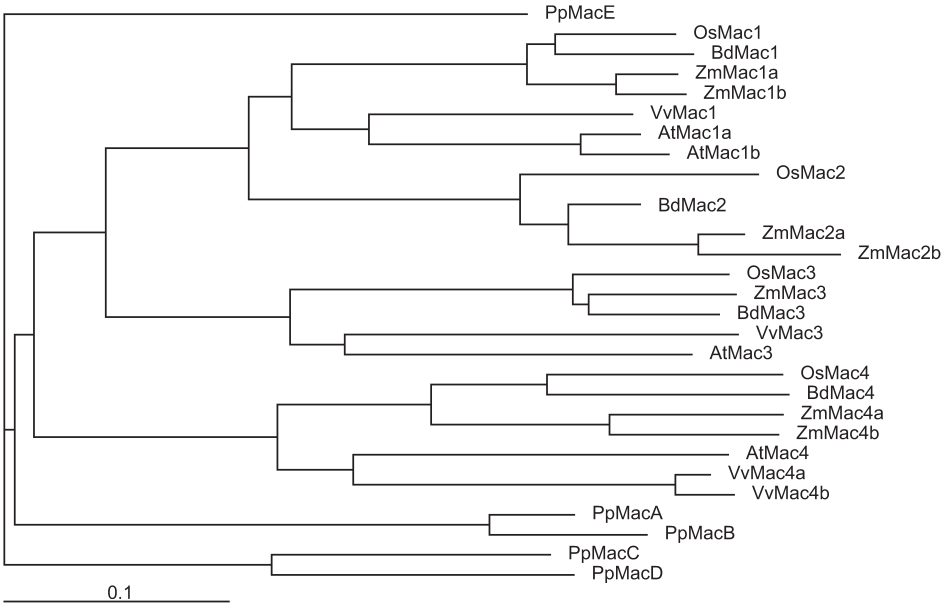

Figure 2. Phylogenetic tree of the homologues of *OsMac1*. Protein names are shown in the figure, and they indicate the name corresponding to each of Mac proteins. The prefixes represent the origins of the plant species, Os: *Oryza sativa*, At: *Arabidopsis thaliana*, Bd: *Brachypodium distachyon*, Zm: *Zea mays*, Vv: *Vitis vinifera*, and Pp: *Physcomitrella patens*, respectively. Phylogenetic analysis was conducted with Clustal X version 2.0 (Larkin et al. 2007) using the neighbor-joining algorithm. Scale represents the number of differences between sequences. Accession numbers of the amino acid sequence data are as follows: OsMac1 (NP_001058631.2), OsMac2 (NP_001047693.1), OsMac3 (NP_001056049.1), OsMac4 (AAU89247.1), AtMac1a (NP_974706.1), AtMac1b (NP_568039.1), AtMac3 (NP_565152.1), AtMac4 (NP_173720.3), BdMac1 (XP_003563248.1), BdMac2 (XP_003580331.1), BdMac3 (XP_003557936.1), BdMac4 (XP_003561557.1), ZmMac1a (AFW75721.1), ZmMac1b (AFW69580.1), ZmMac2a (DAA36442.1), ZmMac2b (DAA36440.1), ZmMac3 (AFW88420.1), ZmMac4a (NP_001132239.1), ZmMac4b (DAA45733.1), VvMac1 (XP_002282426.1), VvMac3 (CAN77212.1), VvMac4a (XP_002277706.1), VvMac4b (CBI27785.3), PpMacA (XP_001785207.1), PpMacB (XP_001754317.1), PpMacC (XP_001771788.1), PpMacD (XP_001777350.1), and PpMacE (XP_001763052.1), respectively.

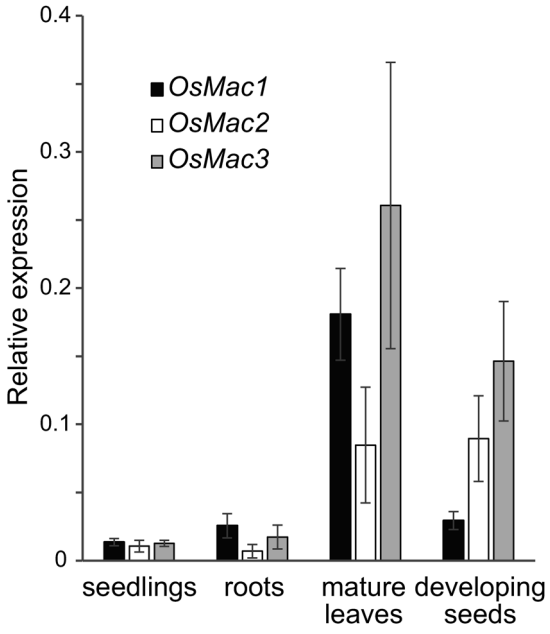

Figure 3. Expression of *OsMac1*, *OsMac2*, and *OsMac3* genes. Transcripts for each gene and the *Actin1* gene were detected by real-time RT-PCR using seedlings (3 days after germination), roots, mature leaves, and developing seeds (20 days after flowering). Expression levels of these genes are indicated by the relative values to *Actin1*. Error bars show *SD* (developing seed; $n=3$, and others; $n=5$).

35S::Mac1-GFP and 35S::Mac2-GFP genes in onion epidermal cells led to the detection of a fluorescent signal around the cell membrane region (Figure 4B), suggesting that these proteins are localized at the membrane. In the case of the cells expressing the 35S::Mac3-GFP gene, a fluorescent signal was detected in organelles, suggesting that the OsMac3 protein is localized at the membrane of an organelle (Figure 4B).

Genome structure and 5′UTR

The transcript of *OsMac1* had a long 5′UTR that was composed of the 526 nt that precede the main ORF. This region contained a CU-rich region at its 5′ region, and three short ORFs (uORFs) at the 3′ region (Figure 5A) (Teramura et al. 2012). The *OsMac2* and *OsMac3* genes contained nine exons (Figure 5B). In the *OsMac2* mRNA, the predicted main ORF started from the region in the second exon, showing that the 5′UTR encompassed two exons (Figure 5B). This 5′UTR consisted of 312 nt and contained three uORFs, but no apparent CU-rich region (Figure 5C). In the *OsMac3* mRNA, the predicted main ORF started from the region in the second exon. The 5′UTR of the *OsMac3* mRNA consisted of 318 nt and encompassed two exons (Figure 5B). This 5′UTR contained a CU-rich region in the first exon, and a uORF in the region in the second exon (Figure 5B and C). The nucleotide sequences of the 5′UTRs of *OsMac1*, *OsMac2*, and *OsMac3* showed no sequence homology with each other.

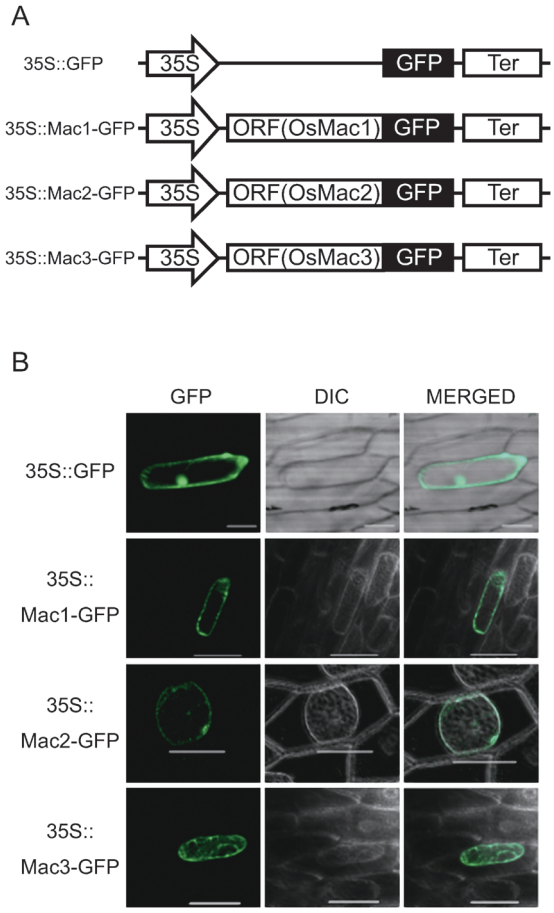

Figure 4. Translation of the main ORF of *OsMac1*, *OsMac2*, and *OsMac3* mRNA. (A) Schematic representation of the structure of the expression plasmids, which were constructed for transient expression in onion epidermal cells. 35S::Mac1-GFP, 35S::Mac2-GFP, and 35S::Mac3-GFP indicate the fusion genes consisting of the GFP gene following the ORFs encoded by *OsMac1*, *OsMac2*, *OsMac3* genes, respectively, which are driven by the CaMV 35S promoter (35S). 35S::GFP is the control plasmid consisting of the 35S promoter and the GFP gene. Ter: NOS terminator. (B) Detection and subcellular localization of the translated GFP, and OsMac1-GFP, OsMac2-GFP, and OsMac3-GFP fusion proteins in onion epidermal cells. Fluorescence images of GFP and differential interference contrast macroscopic images (DIC) are shown. Merged images are shown in the right sides of these figures. Genes were introduced using microprojectile methods. Scale bar = 100 μm.

Determination of the role of the 5′UTR in the translation of the downstream ORF

Next, we tested the effect of the *OsMac2* or *OsMac3* 5′UTRs on the translation of the downstream β-glucuronidase (*GUS*) reporter ORF. GUS ORF translation efficiency was monitored in transient-expression experiments in rice protoplasts transformed with the reporter construct harboring either the *OsMac2* or *OsMac3* 5′UTR upstream of the GUS ORF (OsMac2-UTR::GUS and OsMac3-UTR::GUS, respectively), a positive control containing OsMac1-UTRc (OsMac1-UTRc::GUS), and the control construct lacking a 5′UTR (35S::GUS) under control of the CaMV 35S-promoter (Figure 6A). GUS activity is used as a measure of

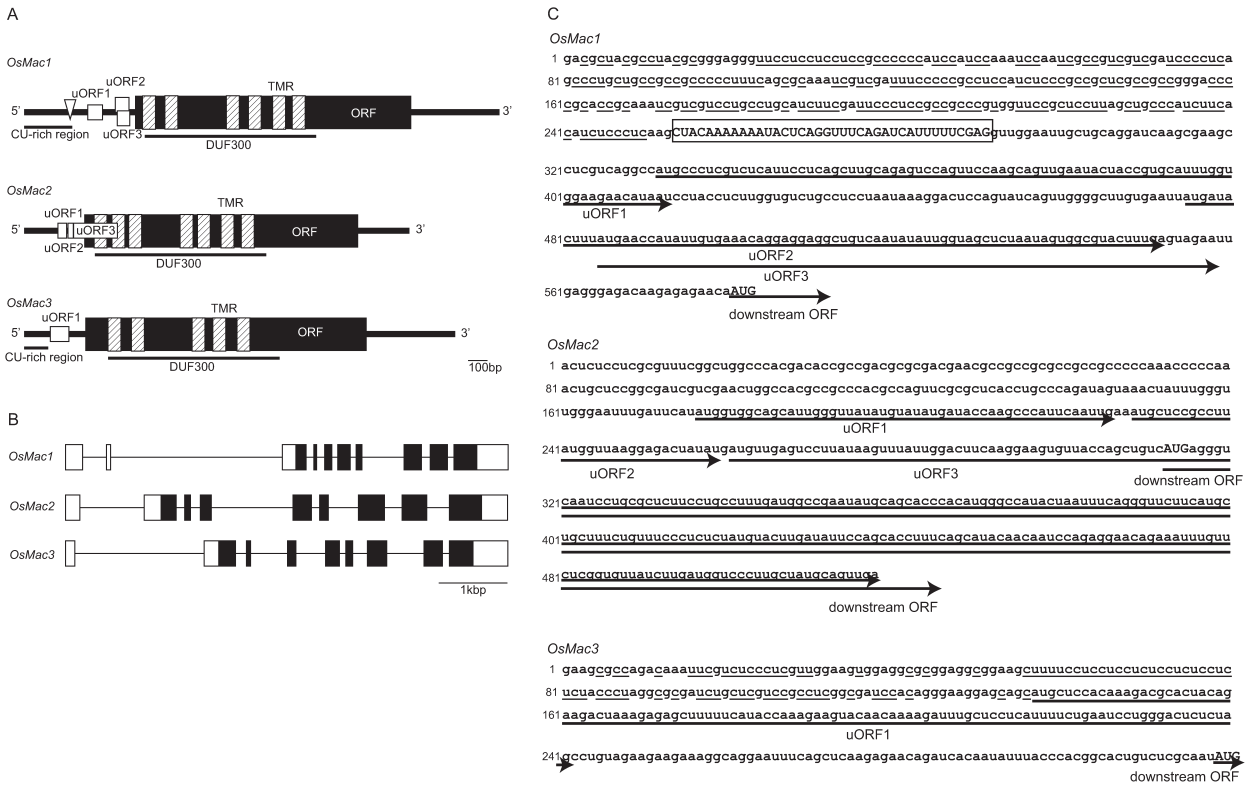

Figure 5. Comparison of *OsMac1*, *OsMac2*, and *OsMac3*. (A) Structure of the *OsMac1*, *OsMac2*, and *OsMac3* mRNAs. Open boxes show the uORFs. Filled boxes indicate the downstream ORFs. Hatched boxes in the downstream ORF show the location of the transmembrane regions (TMRs) in the DUF300 domains included in the predicted proteins. CU-rich regions and region corresponding to the DUF300 are shown by lines below. The position of the 38 nt sequence uniquely contained in UTRc of OsMac1 mRNA is indicated by an open triangle. (B) Genetic structure of the *OsMac1*, *OsMac2*, and *OsMac3* genes. Open and filled boxes indicate untranslated regions (5′UTR and 3′UTR) and a coding region in the exons. Introns are shown by lines. (C) Nucleotide sequences of the UTRs of the *OsMac1*, *OsMac2*, and *OsMac3* mRNAs. UTRc is shown as a representative UTR of *OsMac1* mRNA. The CU-rich sequences in *OsMac1* and *OsMac3* mRNAs are underlined. uORFs and the downstream ORFs are indicated by arrows placed below the nucleotide sequences. The specific 38-nt sequence found in the 5′UTR of *OsMac1* mRNA is shown by an open box.

translation efficiency. The transformation of the OsMac1-UTRc::GUS, OsMac2-UTR::GUS, or OsMac3-UTR::GUS plasmids resulted in the appearance of GUS activity, which was more than tenfold higher than that detected in protoplasts transformed with the control gene 35S::GUS (Figure 6B).

Significantly strong GUS activity was detected in protoplasts containing OsMac3-UTR::GUS, which was greater than that of OsMac1-UTRc::GUS. The protoplasts transfected with the OsMac2-UTR::GUS construct also exhibited a high level of GUS activity, which was similar to that of OsMac1-UTRc::GUS and significantly higher than that of 35S::GUS (Figure 6B). These results suggest that the 5′UTRs of *OsMac2* and *OsMac3* enhance the translation of the downstream ORF located after the long 5′UTR.

In a previous paper, we estimated the translational efficiency of the production of the desired protein in transgenic suspension-cultured rice cells. In this study, we determined the activity of the reporter protein in transformed protoplast cells, which were prepared from wild-type suspension cultured cells via the introduction of the desired gene. We obtained reproducible results using both experimental procedures in cells transformed with OsMac1-UTRc::GUS (Figure 6), which indicates that this assay provided reliable information for this study.

Discussion

The rice *OsMac2* and *OsMac3* mRNAs possess long 5′UTRs that consist of 312 and 318 nt, respectively, and precede the main ORFs, which allows the enhancement of the translational efficiency of the downstream ORF (Figure 6B). This observation coincided with the results obtained for the *OsMac1* mRNA. In the case of 5′UTR of the *OsMac1* mRNA, a splicing variant, UTRc, leads to significant translational enhancement of the downstream ORF, whereas no effect was shown by the shorter UTR that lacked the specific 38 nt region (Teramura et al. 2012). This fact suggests that this 38 nt sequence functions as an enhancer element for the translational efficiency that was uniquely contained in UTRc; however, no homologous sequence was found in the UTRs of *OsMac2* and *OsMac3*.

The 5′UTRs of the *OsMac1* and *OsMac3* mRNAs

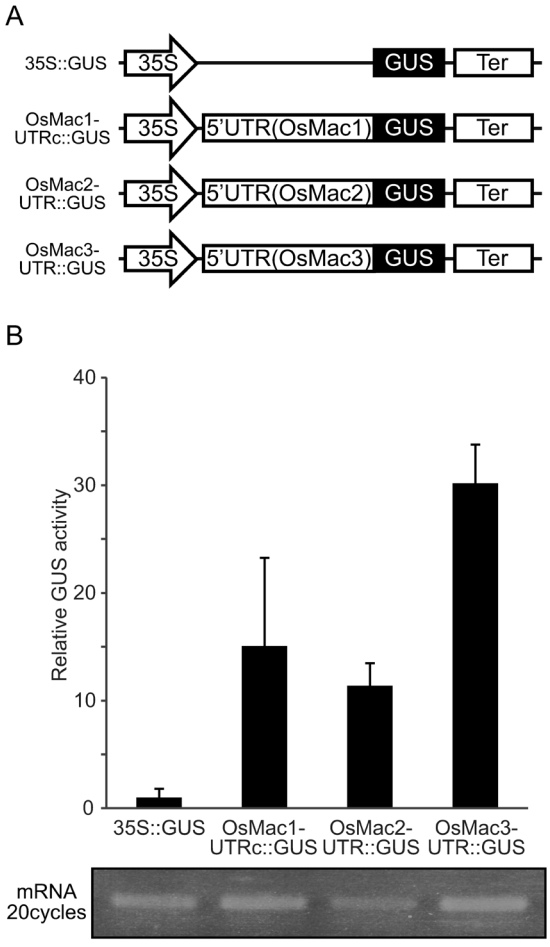

Figure 6. Translational efficiency of UTRs of the *OsMac1*, *OsMac2*, and *OsMac3* genes. (A) Structure of the reporter *GUS* genes placed downstream of the UTRs. 35S: CaMV 35S-promoter. Ter: NOS-terminator. (B) Translation efficiency of the GUS genes downstream 5′UTRs of these mRNAs. OsMac1-UTRc::GUS, OsMac2-UTR::GUS, and OsMac3-UTR::GUS indicate the fusion genes consisting of the GUS gene following the 5′UTR of the *OsMac1*, *OsMac2*, *OsMac3* mRNAs, respectively, which are driven by the CaMV 35S promoter (35S). 35S::GUS is the control plasmid consisting of the 35S promoter and the GUS gene. Translational efficiency is shown by the relative values of GUS activity of 35::GUS in the protoplast cells in which these plasmids were introduced along with the 35S::GFP. Error bars indicate the *SD* ($n=3$). Amount of mRNA in the protoplast cells are photometrically measured (150 ng in a reaction tube containing 10^6 cells). They are detected by RT-PCR and are indicated below the figure.

contained CU-rich regions, although no corresponding sequence occurred in the 5′UTR of the *OsMac2* mRNA (Figure 5C). Some mRNAs contain an oligopyrimidine tract at their transcriptional start site, such as 5′-TOP, which is composed of an uninterrupted stretch containing up to 13 pyrimidines and is involved in growth-dependent translational regulation (Avni et al. 1997; Crosio et al. 2000; Jefferies et al. 1997). It has been shown that the polypyrimidine-tract-binding protein promotes translation, and that it binds to RNA motifs that are predicted to form specific secondary structures (Amir-Ahmady et al. 2005; Galbán et al. 2008). The 5′UTRs of the *OsMac1* and *OsMac3* mRNAs yielded higher translational efficiency of the downstream ORF compared with that of *OsMac2* (Figure 6). The CU-rich region of the 5′UTRs of *OsMac1* and *OsMac3* contained sequences that were similar to the 5′TOP sequence. These sequences might be involved in a mechanism that leads to a high level of translational efficiency of the downstream ORFs.

The 5′UTRs of the *OsMac2* and *OsMac3* mRNAs also contained uORFs (Figure 5A). A uORF is a translational negative regulator (Rahmani et al. 2009). It has been reported that reinitiation after uORF translation is controlled by the TOR signaling pathway in Arabidopsis plants, and that TOR can trigger translation reinitiation via the phosphorylation of the plant reinitiation factor eIF3h, which promotes the translation of mRNAs harboring uORFs within their leaders (Schepetilnikov et al. 2013). However, it is presumed that the uORFs present in the 5′UTRs did not contribute significantly to the difference in the translational efficiency of the downstream ORF.

Models of Cap-independent mechanisms of translation initiation have been proposed. It is suggested that extensive protein–protein and protein–RNA interactions are required to bring together the translation factors, tRNAs, ribosomes, and mRNA (Gallie 2002; Thiébeauld et al. 2007). The 68 nt 5′ leader (termed Ω) sequence of the tobacco mosaic virus genomic RNA functions as a translational enhancer. The Ω sequence has a primary structure caused by two elements, a direct repeat of 8 nt and a CAA-rich region, which are responsible for translation enhancement (Gallie and Walbot 1992; Schmitz et al. 1996; Wells et al. 1998). The 5′UTRs of the *OsMac1*, *OsMac2*, and *OsMac3* mRNAs comprised more than 300 nt, and no homology to the Ω sequence was found. Therefore, these 5′UTRs may contain novel translational enhancer elements that enable the efficient translation of the downstream ORFs. There may be a common and conserved regulatory system for the translation of the downstream ORF in these genes.

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