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Abstract

Target of rapamycin (TOR) is a conserved eukaryotic serine/threonine kinase that functions as a central controller of cell growth. TOR protein is structurally defined by the presence several conserved domains such as the HEAT repeat, focal adhesion target (FAT), FKBP12/rapamycin binding (FRB), kinase, and FATC domains starting from the N-terminus. In most eukaryotes, TOR forms two distinct physical and functional complexes, which are termed as TOR complex 1 (TORC1) and TORC2. However, plants contain only TORC1 components, i.e., TOR, Raptor, and LST8. In this study, we analyzed the gene structure and functions of TORC components in rice to understand the properties of the TOR complex in plants. Comparison of the locations of introns in these genes among rice and other eukaryotes showed that they were well conserved among plants except for *Chlamydomonas*. Moreover, the intron positions in the coding sequence of human Raptor and LST8 were closer to those of plants than fly or nematode. Complementation tests of rice TOR (OsTOR) components in yeast showed that although OsTOR did not complement yeast *tor* mutants, chimeric TOR, which consisted of the HEAT repeat and FAT domain from yeast and other regions from rice, rescued the *tor* mutants, indicating that the HEAT repeat and FAT domains are important for species-specific signaling. OsRaptor perfectly complemented a *kog1* (yeast Raptor homolog) mutant, and OsLST8 partially complemented an *lst8* mutant. Together, these data suggest the importance of the N-terminal region of the TOR, HEAT and FAT domains for functional diversification of the TOR complex.

Keywords

Target of rapamycin, Raptor, LST8, rice, *Saccharomyces cerevisiae,* TORC1

Introduction

The target of rapamycin (TOR) signaling pathway plays a central role in regulating a wide range of growth-related cellular processes and is conserved in eukaryotic species. TOR is a large (280 kD) serine/threonine (Ser/Thr) protein kinase belonging to the family of phosphatidylinositol 3-kinase-related kinases. The activity of the TOR protein is inhibited by a complex of antifungal agents, rapamycin and FK506 binding protein 12 (FKBP12). TOR exists in two distinct complexes, rapamycin-sensitive TOR complex 1 (TORC1) and rapamycin insensitive TORC2, in most eukaryotic cells. TORC1 consists of TOR, Raptor (a yeast KOG1 homolog), and LST8 and is mainly regulated by nutrient and energy availability. It regulates translation and other processes necessary for growth. TORC2 consists of TOR, Rictor (a yeast AVO3 homolog), LST8, and SIN1 (a yeast AVO1 homolog) and is considered to be involved in the regulation of cytoskeleton organization, cell survival, and possibly in the regulation of TORC1 function (Dunlop and Tee, 2009; Jacinto, 2008; Loewith and Hall, 2011; Bai and Jiang, 2010; Robaglia et al., 2012). Among these factors, plants have homologs of TOR, Raptor, and LST8, but there are no homologs of factors unique to TORC2, i.e., Rictor and SIN1. Therefore, it is considered that TORC2 does not exist in plants. In this study, we set out to investigate the difference in gene structure of components of the TOR complex between plants and other eukaryotes and functional conservation of these components between plants and yeast.

TOR protein consists of highly conserved domains such as the HEAT repeat, focal adhesion target (FAT), FKBP12/rapamycin binding (FRB), kinase, and FATC domains starting from the N-terminus. The HEAT repeat domain interacts with Raptor or Rictor. The FAT domain, which is found in all the members of the phosphatidylinositol 3-kinase-related kinase family, is believed to be involved in interactions with other proteins. The FRB domain is the binding site for the FKBP-12-rapamycin complex. The kinase domain contains a conserved lipid Ser/Thr kinase motif. FATC is a putative scaffolding domain, which can be regulated by cytosolic redox potential (Watanabe et al., 2011; Inoki et al., 2005; Robaglia et al., 2012).

The first study on the function of *Arabidopsis* TOR (AtTOR) showed that AtTOR is insensitive to rapamycin, and disruption of *AtTOR* led to premature arrest of endosperm and embryo development (Menand Desnos et al., 2002). These properties of plant TOR have made it difficult to study the TOR pathway, and research on the function of TOR in plants has lagged behind that in other well-studied eukaryotes. However, recent studies using inducible knock-out plants and transgenic plants

overexpressing yeast FKBP12, which are sensitive to rapamycin, have clarified the important roles of TOR in plants, including autophagy regulation and cell wall modification as well as the regulation of growth and development (Deprost et al., 2007; Liu and Bassham, 2010; Caldana et al., 2013; Leiber et al., 2010). In addition, a recent study showed that the application of much higher concentrations of rapamycin than used in other eukaryotes was effective in *Arabidopsis* (Xiong et al., 2013; Xiong and Sheen, 2012).

In addition, the function of the components of TORC1 in plants has been studied. *Arabidopsis* contains two Raptor genes, *AtRaptor 1A* and *AtRaptor 1B*, and disruption of *AtRaptor 1B* showed a wide range of developmental defects, but such defects were not observed in *AtRaptor 1A*. The disruption of both *AtRaptor* genes showed normal embryonic development, but growth was arrested after germination. These results indicated that, in embryonic development, AtTOR does not require *AtRaptor* (Anderson et al., 2005). In addition, LST8 exists as two copies in *Arabidopsis*, *AtLST8-1* and *AtLST8-2*, but only *ATLST8-1* is functional. Mutation in *AtLST8-1* affected plant growth and development but had no lethal effects (Moreau et al., 2012).

In animals and yeasts, ribosome protein S6 protein kinase (S6K), a known target of TOR, participates in the TOR signaling pathway to regulate growth and life span in eukaryotes (Jacinto, 2008; Bai and Jiang, 2010). S6K was demonstrated to be a target of TOR in plants (Mahfouz et al., 2006; Xiong and Sheen, 2012; Xiong et al., 2013), indicating that the TOR signaling pathway in plants has common components downstream of TOR. In contrast, a recent study identified a novel target of AtTOR, E2Fa, which is not a known target of TOR in other eukaryotes (Xiong et al., 2013). E2Fa transcription factors are conserved key regulators of S-phase genes governing the cell cycle progression and DNA replication in plants and animals. Furthermore, the counterparts of signaling components upstream of the TOR complex identified in other eukaryotes have not been identified in plants. These findings indicated that there is a diversity of function and components in the TOR signaling pathway.

In this study, we isolated genes encoding components of TORC1, i.e., TOR, Raptor, and LST8 from rice plants and analyzed their gene structure. In addition, we examined whether TOR components from rice plants are functional in yeast *tor* mutants. Our results indicated that the HEAT repeat and FAT domain in TOR protein play an important role in species-specific signaling, and Raptor is the functionally most-conserved component in the TOR complex between plant and yeast.

Materials and Methods

Cloning of cDNA s of TOR components

The coding sequence (CDS) of OsTOR (RAP# Os5g0235300) was isolated by reverse transcription-polymerase chain reaction (RT-PCR) using the Transcriptor First Strand cDNA Synthesis Kit (Roche) and PrimeSTAR HS DNA Polymerase (TAKARA). Because the cDNA was very long (7.4 kbp), we amplified three overlapping fragments (fragments 1, 2, and 3; Fig. 1a). Then, we fused the fragments together using restriction enzymes *Eco*RI and *Bam*HI and cloned the full-length cDNA into the *Xba*I/*Hind*III site of a pCR-Blunt vector (Invitrogen). We used the primer sets OsTORXb-F/OsTORPSVG-R, OsTORNPSF-F/OsTORRMWH-R, and OsTOR3670-F/OsTORHd-R (Table 1a) for the amplification of fragments 1, 2, and 3, respectively. The obtained full-length cDNA was sequenced to confirm that it

encoded OsTOR.

The full-length cDNAs of the two *OsRaptor* sequences were amplified by RT-PCR. Because the sequences around the translation start site and stop codon of *OsRaptor 1* (LOC Os11g01872) and *OsRaptor 2* (LOC Os12g01922) were highly similar, we could not design specific primers. We amplified both *OsRaptor* cDNAs using the primer set of OsRaptor F/R (Table 1a). The cloned fragments was sequenced and all were found to be *OsRaptor 2*; therefore, we used only *OsRaptor 2* for further analyses.

OsLst8 and *AtLst8-1* were amplified by RT-PCR using primer sets of OsLST8F/R and AtLst8F/R (Table 1a). Amplified cDNAs were cloned into a pCR-Blunt vector.

Construction of vectors for yeast experiments

Yeast expression vectors for TOR and Raptor were constructed by PCR-directed recombination in *Saccharomyces cerevisiae.* The vector p426 GPD (Mumberg et al., 1995) was digested using *Eco*RI/*Hind*III and used for PCR-directed recombination (Oldenburg et al., 1997). *OsTOR* and *OsRaptor* were amplified using PrimeSTAR (TAKARA) and the primer set OsTOR TD F/R and OsRaptor TD F/R (Table 1a), respectively. *ScTOR1*, *ScTOR2*, and *ScKOG1* were amplified using the primer sets described in Table 1a and yeast genomic DNA as the template. For the cloning of *ScTOR1*, *ScTOR2*, and *ScKOG1* into a p426 GPD vector, these fragments were amplified by PCR using primer sets ScTOR1 TDF/R, ScTOR2 TDF/R, and ScKOG1 TDF/R, respectively. *OsLST8*, *AtLST8-1*, and *ScLST8* were ligated into the p426 GPD vector using restriction enzymes (*Bam*HI/*Sal*I).

Complementation test

Yeast strains used in the complementation test are listed in Table 2. SH121, SH229, and SH221 were obtained from M. Hall (University of Basel Biozentrum). YYK410 and RL99-3c were obtained from R. Loewith (University of Geneva) and Y. Kamada (National Institute for Basic Biology, Okazaki), respectively.

Yeast cells were grown and collected at a density of 0.4–0.6 OD/mL. Cells were then diluted to 6×10^7 cells/μL, and 10-fold serial dilutions were made. Ten μL of diluted cells were spotted on plates.

For the complementation test of TOR and Raptor, yeast were grown on Sabouraud's dextrose medium lacking uracil (SD-Ura) at 25°C or 37°C. For the complementation test of LST8, yeast were grown on SD glucose medium lacking uracil (SD Glc-Ura) or SD galactose medium lacking uracil (SD Gal-Ura) at 30°C.

Construction of vectors for chimeric TOR

A schematic representation of chimeric TOR construction is shown in Fig. 1b. Fragments described in Fig. 1b were amplified using the PCR using primer sets shown in Table 1b. These fragments were digested using the restriction enzymes shown in Fig. 1b and ligated to contiguous fragments. The full-length chimeric DNAs were inserted into a pCR-Blunt vector or pBluescript II SK+ vector (Stratagene).

Expression analysis

Rice plants (*Oryza sativa L.* cv. Nipponbare) were grown in soil in a greenhouse. Total RNA was extracted using TriPure isolation reagent (Roche), in accordance with the manufacturer's protocol. First-strand cDNA synthesis was performed using 1 μg of total RNA, oligo (dT), and ReverTra Ace (TOYOBO).

Real-time PCR amplification of cDNA was conducted using a LightCycler 480 (Roche Applied Science) in a 384-well PCR plate. The reaction was carried out in a 20 μL reaction volume containing 10 μL SYBR Premix Ex Taq II (TAKARA) with 0.3 μM each of the forward and reverse primers. Primers for real time-PCR were designed by the Universal ProbeLibrary Assay Design Center (Roche Applied Science). The primer sets used for real-time PCR are shown in Table 1c. All reactions were performed independently at least six times, and at least three sets of consistent data were used for analyses. Expression levels of actin (Os03g50890) were determined using specific primers (Table 1c) and were used for signal normalization of real-time PCR (Caldana et al. 2007). Relative gene expression level was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittegen, 2001). To validate the reliability of the data, amplification efficiencies

between the target genes and the house-keeping genes of all the real-time PCR reactions were compared, and dissociation curves of all PCR products were examined to ensure the quality of the reactions PCR. Because the sequences of *OsRaptor 1* and *OsRaptor 2* are almost identical and we could not make primer sets to distinguish *OsRaptor 1* and *OsRaptor 2,* we used a primer set to detect both *OsRaptor 1* and *OsRaptor 2.*

Results

Isolation of TOR cDNA from rice

The GenBank database showed that there is a single gene for TOR in the rice genome and cDNA was a partial fragment of the 3′ site (sequence ID: AK1204773, 4427bp, RAP# Os5g0235300). Therefore, we estimated the translation initiation site (TIS) using BLAST searches with the amino acid sequence of *Arabidopsis* TOR (AtTOR), and primers were designed to amplify the missing 5′ sequence of rice TOR (OsTOR) cDNA (primers OsTORXb F/OsTORPSVG R; Table 1a). We obtained a fragment of approximately 3.7 kb by PCR using rice cDNA (cv. Nipponbare), which was cloned into a pCR-Blunt vector, and its sequence was determined to confirm that the cloned cDNA fragment encoded the N-terminal part of TOR. To isolate the full-length OsTOR CDS, we amplified three overlapping fragments by RT-PCR and joined them together using unique restriction enzyme sites (Fig. 1a). The obtained cDNA (7,398 bp) (accession number: AB982929) was sequenced and the 3′ region (4,427 bp) was confirmed to be identical to the sequence in the GenBank database (NM_001061523 sequence ID:AK1204773). The 5′ region (2,971 bp) was similar to the sequence of *Oryza brachyantha TOR* (XM_006654108).

Analysis of the *OsTOR* genome structure revealed that the gene length (from TIS to stop codon) was 25,933 bp and there were 58 introns (Fig. 2a). Comparison of the genome structure of *OsTOR* with other *TOR* genes showed that plant *TOR* genes contained similar numbers of introns. In contrast, the intron number of *TOR* of *Drosophila melanogaster* (*DmTOR*) and *Caenorhabditis elegans* (*CeTOR*) was significantly different from that of *OsTOR*. Moreover, *Homo sapiens TOR (HsTOR)*, *Gallus gallus TOR* (*GgTOR*), and *Danio rerio TOR* (*DdTOR*) contain 56 introns, which is similar to that of plant *TORs* (gene lengths of *HsTOR*, *GgTOR*, and *DrTOR* are approximately 152, 62 and 244 kb, respectively).

Next, we analyzed the location of introns in the *TOR CDS* (Fig. 2b). Most intron positions were conserved among plants, although the positions of introns in *HsTOR*,

DmTOR, and *CeTOR* appeared to be different from those in *OsTOR*. It is notable that all of the intron positions were perfectly conserved among the vertebrates (Fig. 2b).

The TOR protein is composed of five domains, HEAT repeat, FAT, FRB, kinase, and FATC. The sequence alignment of OsTOR with TOR proteins from other organisms indicated that the kinase and FATC domains were relatively well conserved among the five domains (Fig. 2c). Each domain was relatively conserved among plants except for *Chlamydomonas* TOR (CrTOR). The amino acid sequences were highly conserved among the vertebrates described in Figure 2c (80–90% identical). Budding yeast (*Saccharomyces cerevisiae*) contains two TOR genes, *TOR1* and *TOR2*, which were designated as *ScTOR1* and *ScTOR2*, respectively, to distinguish them from other *TOR*s. ScTOR1 occurs only in TORC1, whereas ScTOR2 occurs in both TORC1 and TORC2. Neither ScTOR1 nor ScTOR2 contain introns.

Expression analysis showed that *OsTOR* was expressed in all tissues analyzed (Fig. 3a). Higher expression levels were detected in roots of 7-day-old seedlings and 21-day-old leaf blades.

Isolation of Raptor cDNA from rice

The GenBank database showed that there are two genes encoding Raptor in the rice genome (*OsRaptor 1*: LOC Os11g01872 and *OsRaptor 2*: LOC Os12g01922). Because these two genes are located in positions where genome duplication occurred (Jacquemin et al. 2011), the sequences of these genes showed high homology, including introns. Therefore, we used only one of them (*OsRaptor 2*), which was isolated first, for further analysis.

The gene lengths of *OsRaptor 1* and *OsRaptor 2* were 8,486 bp and 8,527 bp, respectively, and both contained 22 introns (Fig. 4a). The *Arabidopsis* genome also contains two *Raptor* genes (*AtRaptor 1A*: At5g01770 and *AtRaptor 1B*: At3g08850), and they contain 22 introns. Most intron positions are conserved among plants. Moreover, many intron positions of *OsRaptor* share homology with those of *HsRaptor* (46% identical and 54% similar) and *DrRaptor* (43% identical and 51% similar) (Fig. 4b), although the gene lengths of *HsRaptor* and *DrRaptor* are 420 kb and 273 kb, respectively, which is much longer than *OsRaptor*. The intron positions of *HsRaptor* and *DrRaptor* were mostly conserved, except for two additional introns in *DrRaptor*. In contrast to vertebrate *Raptor* genes, *DmRaptor* and *CeRaptor* did not contain shared intron positions.

Raptor proteins have three domains – Raptor N-terminal conserved (RCN) domain, HEAT repeat, and WD40 repeat. Each domain was compared with those of OsRaptor.

Amino acid sequences of plant Raptors showed relatively high homology, and the RCN domain showed the highest homology among the three domains.

Because the sequences of *OsRaptor 1* and *OsRaptor 2* were almost identical, including the 5'- and 3'-UTRs, we could not design primers to distinguish them. Therefore, we analyzed the expression of both *OsRaptor* genes without distinction between *OsRaptor 1* and *OsRaptor 2* (Fig. 3b). They were expressed in all tissues analyzed, and higher expression was observed in 7-day-old roots and shoots (Fig. 3b).

Isolation of LST8 cDNA from rice

LST8 is a single gene in rice, which is 3,728-bp long and contains nine introns (Fig. 5a). The *Arabidopsis* genome contains two *LST8* genes (*LST8-1*: At3g18140 and *LST8-2*: At2g22040) also containing nine introns. The location of introns was shared among rice, *Arabidopsis*, and *Populous*. However, *Chlamydomonas LST8* (*CrLST8*) did not show shared positions of introns. In contrast, *HsLST8* and *DrLST8* contain seven introns, and five of them share their positions with *OsLST8* (Fig. 5b). Although the human and zebrafish TOR and Raptor genes are much larger (longer than 100 kb) than those of rice (26 kb and 8.5 kb, respectively), *HsLST8* had a similar size to *OsLST8*. On the other hand, *DrLST8* (9,974bp) was much larger than *HsLST8* and *OsLST8.*

The amino acid sequence of AtLST8-1 showed high homology with OsLST8 (81%) but lower homology with AtLST8-2 (68%) (Fig. 5c).

OsLST8 was expressed at a similar level in all tissues analyzed (Fig. 3c). Relatively higher expression levels were detected in 3-day-old seedlings and 7-day-old roots and shoots.

Only the C-terminal region of OsTOR protein is compatible with that of yeast

After obtaining full-length OsTOR coding sequences, we tested whether OsTOR proteins complemented a *tor* mutant of *S. cerevisiae*. We used three temperature-sensitive mutants, SH121 (*tor2-21*^{ts}), SH229 (*tor1 tor2-29*^{ts}), and SH221 (*tor1 tor2-21*^{ts}) (Helliwell et al., 1998). SH121 was reported to be sensitive only for TORC2 function, and SH229 was sensitive only for TORC1 function. SH221 was sensitive for both TORC1 and TORC2 functions. The growth of these mutants was arrested at 37°C, although they can normally grow at 25°C.

The OsTOR cDNA was cloned into a p426 GPD vector (Mumberg et al., 1995) and was introduced into the three mutant strains. Yeast TOR genes (*ScTOR1* and *ScTOR2*) were also cloned into p426 GPD and were used to evaluate the extent of the complementation. All mutants expressing *OsTOR* failed to grow at 37°C (Fig. 6b). In

contrast, *ScTOR2* complemented all mutants. In our experiments, *ScTOR1* did not complement any mutant.

Next, we evaluated the part of TOR protein that has conserved functions between plant and yeast. Because the homology of the HEAT repeat domain between OsTOR and ScTOR was low, we constructed a chimeric OsTOR protein, in which the HEAT repeat was replaced with that from yeast (chimera TOR1, Fig. 6a) and introduced it into mutant yeasts. None of the mutants expressing chimeric TOR1 grew at 37°C (Fig. 6b). Therefore, we made another chimera consisting of the HEAT repeat and FAT domain of ScTOR and another region of OsTOR (chimera TOR2). This chimeric TOR2 complemented the SH121 and SH229 mutants very weakly but did not complement SH221 (Fig. 6b). When chimeric TOR containing the FRB domain of yeast TOR in addition to the HEAT repeat and FAT domain was tested (chimera TOR3), it weakly complemented SH221. Chimeras TOR4 and TOR5, in which only the kinase and FATC domains were derived from OsTOR, respectively, rescued all mutants similar to ScTOR2.

Next, we made chimeric yeast TOR, in which the FAT domain and HEAT repeat were replaced with those from OsTOR (chimeras TOR6 and TOR7, respectively). Chimeric TOR did not rescue any mutants (Fig. 6).

OsRaptor fully rescued a yeast *kog1* mutant

To test whether *OsRaptor 2* rescues a yeast *kog1* mutant, temperature-sensitive mutant YYK410 was used (Nakashima et al., 2008). We used *KOG1* as the positive control.

Although the homology of the amino acid sequences between OsRaptor and KOG1 was similar to that of TOR protein (approximately 30–40%), *OsRaptor 2* complemented the *kog1* mutant at a similar level as *KOG1* (Fig. 7).

OsLST8 weakly complemented the yeast *lst8* mutant

The homology of the amino acid sequence between OsLST8 and ScLST8 was higher (53%) than that of TOR and Raptor. In addition, *Chlamydomonas* LST8 (CrLST8) and *Arabidopsis* LST8 (AtLST8) were reported to complement a yeast *lst8* mutant (Diaz-Troya et al., 2008; Moreau et al., 2012). We tested whether OsLST8 also complemented the yeast *lst8* mutant. We used the RL99-3C mutant strain, in which the *ScLST8* gene was induced only in the presence of galactose, and this strain cannot grow on glucose medium (Loewith et al., 2002). We cloned *OsLST8* and *ScLST8* into a p426 GPD vector and introduced it into an *lst8* mutant.

OsLST rescued the *lst8* mutant very weakly compared with *ScLST8* (Fig. 8). In addition, to examine whether the weak complementation was attributed to the properties of *OsLST8*, we tested *AtLST8-1*, which encodes another plant LST8. The level of the complementation of the yeast *lst8* mutant by *AtLST8-1* was similar to that of *OsLST8* (Fig. 8).

Discussion

In this study, we cloned cDNAs of the TORC components, TOR, Raptor, and LST8, from rice and characterized their gene structure and function.

Our result showed that OsTOR did not complement *tor* mutants (Fig. 6). Complementation tests using chimeric forms of TOR showed that the functions of the kinase and FATC domains in OsTOR, which showed high homology with those of yeast, were compatible with those of yeast, whereas the functions of the HEAT repeat and FAT domain were not conserved. Because plants do not have a Rictor homolog, and the HEAT repeat of OsTOR may not interact with AVO3 (Rictor homolog in yeast), OsTOR could not rescue TORC2 function in yeast. Yang et al. (2013) recently reported the crystal structure of the part of mTOR that has enzymatic activity and encompasses many of its crucial domains (i.e., FAT, FRB, kinase, and FATC domains) in complex with mLST8. They showed that the FAT domain consisted mostly of α-helical repeats, which wind like a twisted telephone wire to form a C-shaped lobe around the kinase domain. The FAT domain wraps around the kinase domain, and the FRB site and mLST8 locate to opposite ends of the catalytic cleft to restrict substrate access to the active site. Although their model lacks HEAT repeats, these are considered to control the localization and recruitment of substrates to the TOR complex by binding with Raptor or Rictor. Our results indicated that the HEAT repeat and FAT domain may have evolved to localize species-specific substrates or components.

In contrast to OsTOR, OsRaptor complemented the *kog1* mutation perfectly (Fig. 7). These results indicated that OsRaptor interacts with the HEAT repeat in yeast TOR in spite of the low homology (Fig. 2c). In *Arabidopsis*, Raptor is reported to interact with the HEAT repeat of TOR and S6K, which is a major substrate of TOR. In addition, the phosphorylation site of S6K seems to be conserved at least between *Arabidopsis* and human (Mahfouz Kim et al., 2006; Xiong and Sheen, 2012). Taken together, the downstream components from Raptor may be relatively conserved among eukaryotes.

There are two copies of the Raptor gene in the rice genome, which are located in a duplicated genome region. Because of the high sequence homology between *OsRaptor 1*

and *OsRaptor 2*, including non-coding regions, we could not distinguish the expression of these two genes. Because all of the *Raptor* cDNA clones we isolated from rice were *OsRaptor 2,* it is not clear whether *OsRaptor 1* is functional or not. *Arabidopsis* also contains two genes for Raptor, *AtRaptor 1A* and *AtRaptor 1B*, and their predicted amino acid sequences shared 80% identity. The disruption of *AtRaptor 1B* showed growth arrest, whereas the *AtRaptor 1A* mutant showed no remarkable phenotypes. The difference in the mutant phenotype may be caused by a difference in expression level (Anderson et al., 2005). It was notable that the intron positions of *AtRaptor 1B* and *OsRaptor* were 100% conserved, whereas those in *AtRaptor 1A* were slightly different (Fig. 4a, b).

An analysis of intron positions provided another interesting finding. The intron positions of *Raptor* were well conserved between plants and vertebrates, particularly the region corresponding to the RCN domain and WD40 repeat (Fig. 4). Rogozin et al. (2003) reported that humans share many introns with the plant *Arabidopsis* than with fly or nematode. Our result supported this observation.

A complementation test of LST8 showed that OsLST8 and AtLST8 weakly complemented a yeast *lst8* mutant compared with ScLST8 (Fig. 8). Previous research showed that CrLST8 complemented a *lst8* mutant at a level similar to ScLST8 (Diaz-Troya et al., 2008). CrLST8 may be more similar to ScLST8 than to OsLST8 and AtLST8. The locations of introns were 100% conserved among rice, *Arabidopsis*, and *Physcomitrella patens*, whereas no intron positions in *CrLST8* were shared with those of other plants. In contrast, *HsLST8* and *DrLST8* shared the positions of five introns out of eight with plants. Similar to the case of *Raptor*, the structures of *HsLST8* and *DrLST8* were more similar to those of plants than to fly or nematode. These findings suggested that the intron positions of genes encoding these components were established before plants and animals diverged.

LST8 interacts with the kinase domain (Kim et al., 2003), which is well conserved among all eukaryotes. The kinase domain of OsTOR was fully functional in yeast, indicating that the kinase domain of OsTOR can interact well with ScLST8. In addition, overall homology of LST8 between plants and yeast is higher than that of Raptor (Fig. 4c and 5c). These findings led to a prediction that plant LST8 would fully complement the *lst8* mutant of yeast. However, OsLST8 and AtLST8 only complemented the yeast *lst8* mutant very weakly. Yeast LST8 may have additional functions that plant LST8s do not have. It was reported that human LST8 interacts with protein phosphatase 4 and functions as a negative regulator of NF-kB signaling (You et al., 2010).

Expression analysis showed that OsTOR was expressed at higher level in fully

differentiated leaf blades than in panicles, which contain many meristems. In *Arabidopsis*, AtTOR-GUS fusion protein expression occurred in proliferating cells and tissues, such as embryo and primary meristems, but not in differentiated organs such as fully expanded leaf and root tissues (Menand et al. 2002). However, AtTOR mRNA was detected at nearly equal levels in all plant tissues (Robaglia et al., 2004). These results indicated the post-transcriptional regulation of *AtTOR* gene expression. *OsRaptor* and *OsLST8* were also expressed in fully expanded leaf blades at similar levels to in panicles. Protein level analysis will be required to reveal whether there is post-transcriptional regulation of genes for TORC components in rice plants.

Our findings suggest that the N-terminal regions of TOR, the HEAT and FAT domains, are important for species-specific signaling among components of the TOR complex. Further investigation, including isolation of novel factors interacting with the N-terminal region of plant TOR, will be required to further elucidate the plant TOR signaling pathway.

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Figure legends

Fig. 1. Schematic representation of (a) strategy for the isolation of cDNAs of rice TOR and (b) strategy for the construction of chimeric TOR genes. a Fragments 1, 2, and 3 (blue bar) were amplified by PCR using the primer set shown on each line and fused together at the *Eco*RI and *Bam*HI sites. The full-length coding sequence was inserted into the *Xba*I/*Hind*III sites of a pCR-Blunt vector. **b** The fragments of ScTOR and OsTOR are shown as black and white bars, respectively. The fragments were amplified by PCR using the primer set described in Table 1b and fused together using the restriction sites shown at the junctions.

Fig. 2. Comparison of the gene and domain structures of TORs. a Schematic comparison of the gene structures of TOR from rice (*OsTOR*), *Arabidopsis* (*AtTOR*), *Physcomitrella patens* (*PpTOR*), *Drosophila melanogaster* (*DmTOR*), and *Caenorhabditis elegans* (*CeTOR*). Black boxes and bold lines show the exon and intron regions, respectively. Numbers on the left side of the panel indicate the size of the gene (from ATG to stop codon), and the numbers in parentheses indicate the number of introns. **b** Schematic comparison of the location of introns in cDNAs of *OsTOR*, *AtTOR*, *PpTOR*, human TOR (*HsTOR*), chicken TOR (*GgTOR*), zebrafish TOR (*DrTOR*), *DmTOR*, *CeTOR*, and yeast TOR (*ScTOR*). cDNAs are indicated by white boxes. Introns occurring in the exact same position in the aligned amino acid sequences compared with that of rice are shown with red hatching. When the location of an intron was shifted by five amino acids or fewer from that of rice, the intron is shown with green hatching, whereas others are shown with blue hatching. Numbers on the left side of the panel indicate the size of the cDNA. Numbers in parentheses indicate the number of introns. Colored bars placed above the cDNA panels show the TOR domains corresponding to the region (the colors of boxes are congruent with those in Fig. 1c). **c** Schematic comparison of the domain structure of OsTOR, AtTOR, PpTOR, *Chlamydomonas reinhardtii* TOR (CrTOR), HsTOR, GgTOR, DrTOR, DmTOR, CeTOR, and ScTOR. TOR protein contains the HEAT repeat (orange), focal adhesion target (FAT; green), FKBP12/rapamycin binding (FRB; blue), kinase (red) and FATC (purple) domains from the N-terminus. Numbers on the left side of the panel indicate the number of amino acids. Numbers in parentheses indicate the overall homology compared with OsTOR. The homology of each

domain compared with that of OsTOR is described below each domain.

Fig. 3. Expression of genes encoding the components of rice target of rapamycin complex (TORC) in various organs.
The expression levels of rice target of rapamycin (*OsTOR*) (**a**), *OsRaptor* (**b**), and *OsLST8* (**c**) in the indicated tissues were determined by real-time PCR and presented as relative values to the level in 3-day-old seedlings. The gene expression levels were normalized to *OsActin*. Data shown represent the mean ± SD of three replicates.

Fig. 4. Comparison of the gene and domain structures of Raptor. a Schematic comparison of the gene structures of Raptor from rice (*OsRaptor*) *1* and *2*, *Arabidopsis* (*AtRaptor*) *1A* and *1B*, *Physcomitrella patens* (*PpRaptor*), *Drosophila melanogaster* (*DmRaptor*), and *Caenorhabditis elegans* (*CeRaptor*). Black boxes and bold lines show the exon and intron regions, respectively. Numbers on the left side of the panel indicate the size of the genes (from ATG to stop codon), and numbers in parentheses indicate the number of introns. **b** Schematic comparison of the location of introns in cDNAs of *OsRaptor 1* and *2*, *AtRaptor 1A* and *1B*, *PpRaptor*, human Raptor (*HsRaptor*), zebrafish Raptor (*DrRaptor*), *DmRaptor*, *CeRaptor*, and yeast KOG1 (*ScKOG1*). cDNAs are indicated by white boxes, and introns occurring in the exact same position in the aligned amino acid sequence compared with that of rice are shown with red hatching. When the location of an intron was shifted by five amino acids or fewer from that of rice, the intron is shown with green hatching, whereas others are shown with blue hatching. Numbers on the left side of the panel indicate the size of the cDNA. Numbers in parentheses indicate the number of introns. Colored bars above the cDNA panels show the Raptor domains corresponding to the regions of the gene (the colors of the boxes are congruent with those in Fig. 4c). **c** Schematic comparison of the domain structure of OsRaptor 1 and 2, AtRaptor, PpRaptor 1A and 1B, HsRaptor, DrRaptor, DmRaptor, CeRaptor, and ScKOG1. The Raptor protein contains the RCN domain (green), HEAT repeat (gray), and WD40 repeat (orange), from the N-terminus. Numbers on the left side of the panel indicate the number of amino acids. Numbers in parentheses indicate the overall homology compared with OsRaptor 1. The homology of each domain compared with that of OsRaptor 1 is described below each domain.

Fig. 5. Comparison of the gene and domain structure of LST8. a Schematic comparison of the gene structures of LST8 from rice (*Os LST8*), *Arabidopsis* (*AtLST8*) 1 and 2, *Physcomitrella patens* (*PpLST8*), *Chlamydomonas reinhardtii* (*CrLST8*), human

(*HsLST8*), *Drosophila melanogaster* (*DmLST8*), and *Caenorhabditis elegans* (*CeLST8*). Black boxes and bold lines show the exon and intron regions, respectively. Numbers on the left side of the panel indicate the size of genes (from ATG to stop codon) and numbers in parentheses indicate the number of introns. **b** Schematic comparison of the location of introns in the cDNAs of *OsLST8*, *AtLST8* 1 and 2, *PpLST8*, *CrLST8*, *HsLST8*, zebrafish LST8 (*DrLST8*), *DmLST8*, *CeLST8*, and yeast LST8 (*ScLST8*). cDNAs are shown as white boxes, and introns occurring in the exact same position in the aligned amino acid sequence compared with that of rice are shown with red hatching. When the location of an intron was shifted by five amino acids or fewer from that of rice, the intron is shown with green hatching, whereas others are shown with blue hatching. Numbers on the left side of the panel indicate the size of the cDNA. Numbers in parentheses indicate the number of introns. **c** Schematic comparison of proteins OsLST8, AtLST8 1 and 2, PpLST8, CrLST8, HsLST8, DrLST8, DmLST8, CeLST8, and ScLST8. Numbers in parentheses indicate the overall homology compared with OsLST8.

Fig. 6. Complementation test of rice target of rapamycin (OsTOR) and chimeric TORs. **a** Schematic representation of the chimeric TOR structure. Black and white bars indicate the fragments of yeast TOR (ScTOR) and OsTOR, respectively. **b** Serial dilutions of temperature-sensitive *torc* mutants, i.e., *torc1*, *torc2*, and *torc1/2* expressing *OsTOR*, chimeric *TORs*, and *ScTOR1* and *2* were spotted on Sabouraud's dextrose medium lacking uracil (SD-Ura). Plates were placed at 25°C or 30°C.

Fig. 7. Complementation test of OsRaptor. Serial dilutions of temperature-sensitive *kog1* mutants, YYK410 expressing *OsRaptor*, or yeast KOG1 (*ScKOG1*) were spotted on Sabouraud's dextrose medium lacking uracil (SD-Ura). Plates were placed at 25°C or 30°C.

Fig. 8. Complementation test of OsLST8. Yeast cells expressing LST8 from the glucose-repressible and galactose-inducible GAL1 promoter (RL99-3c) were transformed with an empty vector or expression vector of rice LST8 (*OsLST8*), Arabidopsis LST8-1 (*AtLST8-1*), and yeast LST8 (*ScLST8*). Serial dilutions of cells were spotted on SD glucose medium lacking uracil (SD Glc-Ura) or SD galactose medium lacking uracil (SD Gal-Ura) and grown at 30°C.

Table 1a. Primer set used for cloning and vector construction

primers for cloning of TORC components cDNA	
OsTORXb F	GCTCTAGAATGAAGCCCTCGCCGCACTTC
OsTORPSVG R	CTCACGACCAACAGAAGGCTG
OsTORNPSF F	GCTCTATATCGGAATCCATC
OsTORMWH R	CTCAAGAGCCTCATGCCACAT
OsTOR3670 F	CAGACGCCGTGCCATTGTCACTTTG
OsTORHd R	GCAAGCTTTCACCAAAACGGGCACCACCC
OsRAPTOR F	GAGCTCATGGCATTGGGGGATCTC
OsRAPTOR R	GTCGACTCATCGTACTTGGTAATTG
OsLST8 F	GGATCCATGGCTCAACCTTCTGTTATC
OsLST8 R	GTCGACTTATGATGGTGCTGATTC
primers for yeast vector construction	
OsTOR TDF	ACTAGTGGATCCCCCGGGCTGCAGGAATTCATGAAGCCCTCGCCGCACTTC
OsTOR TDR	TGACTCGAGGTCGACGGTATCGATAAGCTTTCACCAAAACGGGCACCACC
ScTOR1 TDF	ACTAGTGGATCCCCCGGGCTGCAGGAATTCATGGAACCGCATGAGGAGCAG
ScTOR1 TDR	TGACTCGAGGTCGACGGTATCGATAAGCTTTCACCAGAATGGGCACCATC
ScTOR2 TDF	ACTAGTGGATCCCCCGGGCTGCAGGAATTGATGAATAAATACATTAACAAATAC
ScTOR2 TDR	TGACTCGAGGTCAGCGGTATCGAGAAGCTTCTACCAGAATGGACACCAAC
OsRAPTOR TDF	ACTAGTGGATCCCCCGGGCTGCAGGAATTCATGGCATTGGGGGATCTCATG
OsRAPTOR TDR	TGACTCGAGGTCGACGGTATCGATAAGCTTTCATCGTACTTGGTAATTGTC
ScKOG1 TDF	ACTAGTGGATCCCCCGGGCTGCAGGAATTCATGCCGGAGATTTATGGACC
ScKOG1 TDR	TGACTCGAGGTCGACGGTATCGATAAGCTTTCAAAAATAATCAATTCTCTC
OsLST8 F	GGATCCATGGCTCAACCTTCTGTTATC
OsLST8 R	GTCGACTTATGATGGTGCTGATTC
AtLST8-1 F	GGAATTCATGAGTCAGCCTTCTGT
AtLST8-1 R	GAAGCTTTTAATCGTGAAGTGCACAG
ScLST8 F	GGATCCATGTCTGTTATCTTAGTATC
ScLST8 R	CTGCAGCTATACATCGTTTAATGCG

Table1b. Primer set used for chimera TOR construction

fragments	Forward	Reverse
ScTOR2-1 (Sc1)	GTCGACATGAATAAATACATTAACAAATAC	CCGCGGTCTTAATTTTCCCATCATCAC
Sc2	CTGCAGATGAATAAATACATTAAC	GGTACCTAGTTGTAAAAGGAGC
Sc3	GGTACCGACTTTGTGGTCTTTGTG	CTCGAGACGTATCAATTCGTGGCTG
Sc4	CTGCAGATGAATAAATACATTAAC	CGCTCGAGTTTAACAATTGGTTTTCC
Sc5	CGGGATCCGGTTTTGACTTACCAACAAAG	CGGAATTCCTACCAGAATGGACACCAAC
Sc6	CTGCAGATGAATAAATACATTAAC	CGCTCGAGATTAAACCTTCTTATATC
Sc7	CTGCAGATGAATAAATACATTAAC	GCAAGCTTATGGATTGGGATAGGCAATG
Sc8	GCGCGGCCGCGGAATCTCTCTCACGACAG	GCGGGCCCCTACCAGAATGGACACCAAC
Sc9	GCCTCGAGACATTAGGTAAGTATGCC	CGCTGCAGCTACCAGAATGGACACCAACC
OsTOR-1 (Os1)	TCTAGAATGCCGCGGTGTCTAGCAGCCTTGGCCCGTTG	GCAAGCTTTCACCAAAACGGGCACCACCC
Os2	GTCGACGAACTGATACGAGTTGCC	GCAAGCTTTCACCAAAACGGGCACCACCC
Os3	CGGTCGACGCATTCTTGCTGAAAGGC	GGATCCGAATTCCCAAAACGGGCACCACCC
Os4	CGGTCGACGCATTCTTGCTGAAAGGC	CGGGATCCCAGACGCCAATTGATTAATG
Os5	CGGTCGACTTATCCGTGAAGGTTCAG	CGGCGGCCGCTCACCAAAACGGGCACCACC
Os6	GCAAGCTTCTGCTCGGTGCACTTGCTG	GGGCGGCCGCTGATTTGCAAGCAACCAAC
Os7	GCTCTAGAATGAAGCCCTCGCCGCACTTC	GCGTCGACTCTTGTATCAATTGGAAG

Table1c. Primer set used for expression analysis

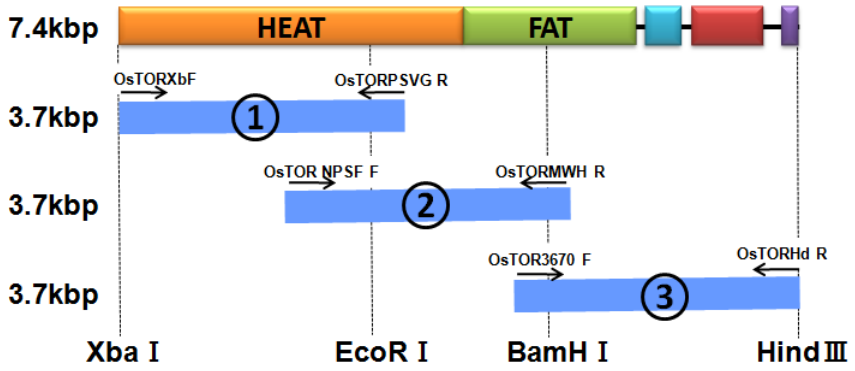
	Forward	**Reverse**
OsTOR	CAAATCGTATGGGAGGAGCTA	GCAGCCATAAGAAGTTTCTCCA
OsRaptor	AGATCTGAGGTTGCCGTAGC	TGAATTGGTTTGAGGTTTCCA
OsLST8	AGTTCTGTGACCCCAACAGG	CCCAACGCTGGTGACCTA
OsActin	CTCCCCCATGCTATCCTTCG	TGAATGAGTAACCACGCTCCG

Table 2. yeast strains used for complementation test

Strain	Genotype	Phenotype	Resource
SH121	JK9-3da tor2::ADE2-3 YCplac111[tor2-21]	*torc2(TOR1 ror2-21^{ts})*	M N.Hall, 1998
SH229	JK9-3da ade2 his3 HIS4 tor1::HIS3 tor2::ADE2/YCplac111::tor2-29	*torc1(tor1 tors-29^{ts})*	M N.Hall, 1998
SH221	JK9-3da ade2 his3 HIS4 tor1::HIS3 tor2::ADE2/YCplac111::tor2-21	*torc1/2 (tor1 tor2-21^{ts})*	M N.Hall, 1998
YYK410	W303-1B kog1 ::LEU2 pRS313[kog1-105]	*kog1*	Y. Kamada, 2008
RL99-3c	TB50a tor2::kanMX4 (kanMX4)-GAL1p-LST8/pRS314TOR2(HA)3	*lst8*	R. Loewith, 2005

Fig.1

a


7.4kbp
HEAT
FAT
OsTORXbF
OsTORPSVG R
3.7kbp
1
OsTOR NPSF F
OsTORMWH R
3.7kbp
2
OsTOR3670 F
OsTORHd R
3.7kbp
3
Xba I
EcoR I
BamH I
Hind Ⅲ

b

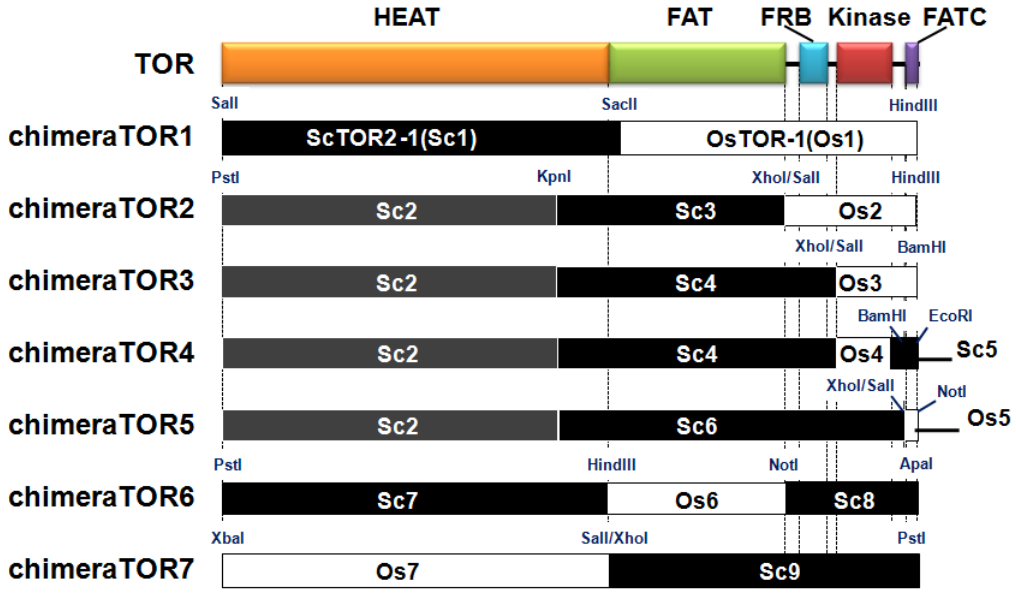
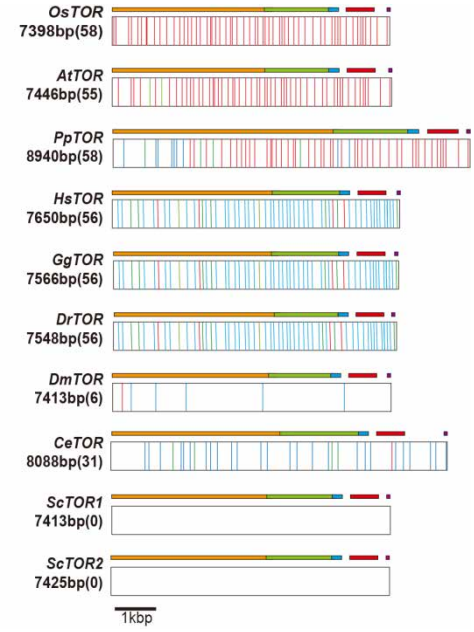

HEAT
FAT
FRB
Kinase
FATC
TOR
SalI
SacII
HindIII
chimeraTOR1
ScTOR2-1(Sc1)
OsTOR-1(Os1)
PstI
KpnI
XhoI/SalI
HindIII
chimeraTOR2
Sc2
Sc3
Os2
XhoI/SalI
BamHI
chimeraTOR3
Sc2
Sc4
Os3
BamHI
EcoRI
chimeraTOR4
Sc2
Sc4
Os4
Sc5
XhoI/SalI
NotI
chimeraTOR5
Sc2
Sc6
Os5
PstI
HindIII
NotI
ApaI
chimeraTOR6
Sc7
Os6
Sc8
XbaI
SalI/XhoI
PstI
chimeraTOR7
Os7
Sc9

Fig.2

a


OsTOR
25993bp(58)
AtTOR
16994bp(55)
PpTOR
21460bp(58)
DmTOR
8416bp(6)
CeTOR
17210bp(31)
1kbp

b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7398bp(58)
AtTOR
7446bp(55)
PpTOR
8940bp(58)
HsTOR
7650bp(56)
GgTOR
7566bp(56)
DrTOR
7548bp(56)
DmTOR
7413bp(6)
CeTOR
8088bp(31)
ScTOR1
7413bp(0)
ScTOR2
7425bp(0)
1kbp

c

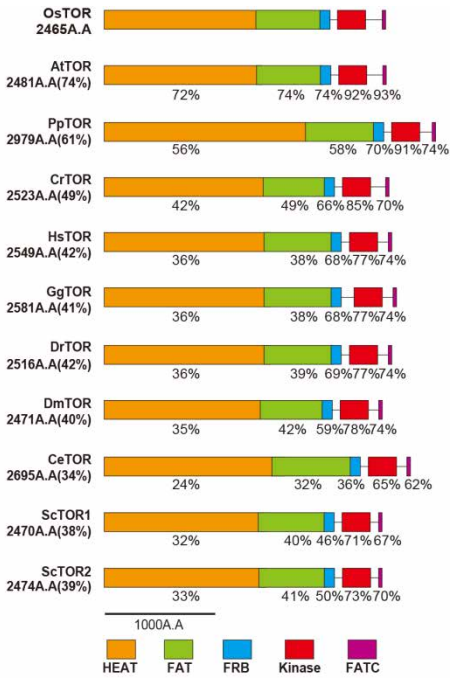
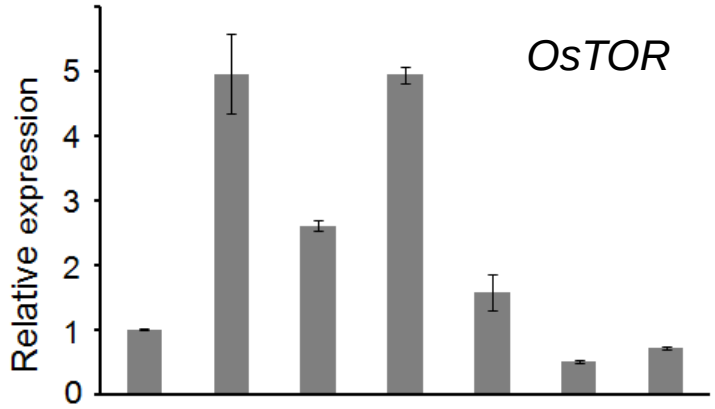
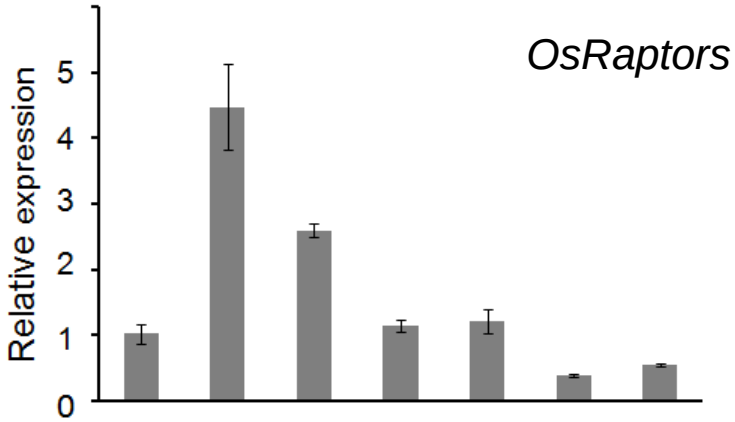

OsTOR
2465A.A
AtTOR
2481A.A(74%)
72% 74% 74%92%93%
PpTOR
2979A.A(61%)
56% 58% 70%91%74%
CrTOR
2523A.A(49%)
42% 49% 66%85% 70%
HsTOR
2549A.A(42%)
36% 38% 68%77%74%
GgTOR
2581A.A(41%)
36% 38% 68%77%74%
DrTOR
2516A.A(42%)
36% 39% 69%77%74%
DmTOR
2471A.A(40%)
35% 42% 59%78%74%
CeTOR
2695A.A(34%)
24% 32% 36% 65% 62%
ScTOR1
2470A.A(38%)
32% 40% 46%71%67%
ScTOR2
2474A.A(39%)
33% 41% 50%73%70%
1000A.A
HEAT
FAT
FRB
Kinase
FATC

Fig.3

a


OsTOR
Relative expression
0
1
2
3
4
5

b


OsRaptors
Relative expression
0
1
2
3
4
5

c

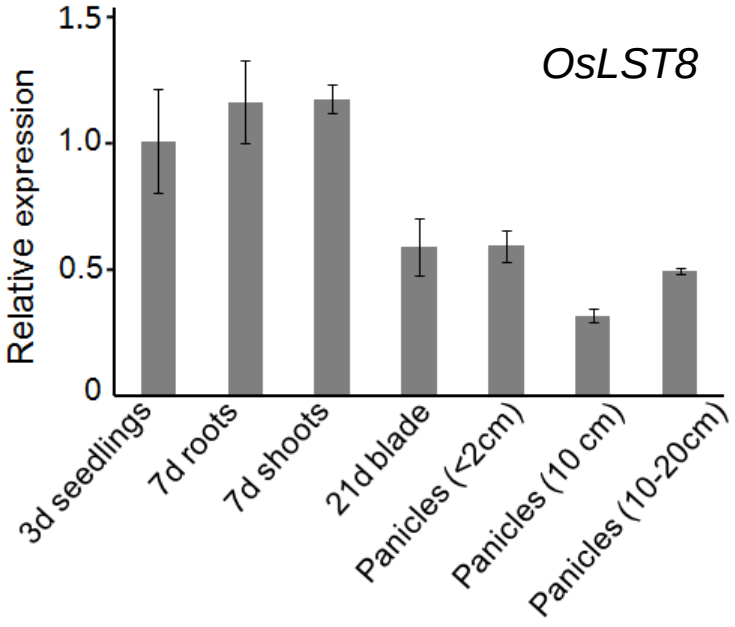

OsLST8
Relative expression
0
0.5
1.0
1.5
3d seedlings
7d roots
7d shoots
21d blade
Panicles (<2cm)
Panicles (10 cm)
Panicles (10-20cm)

Fig.4

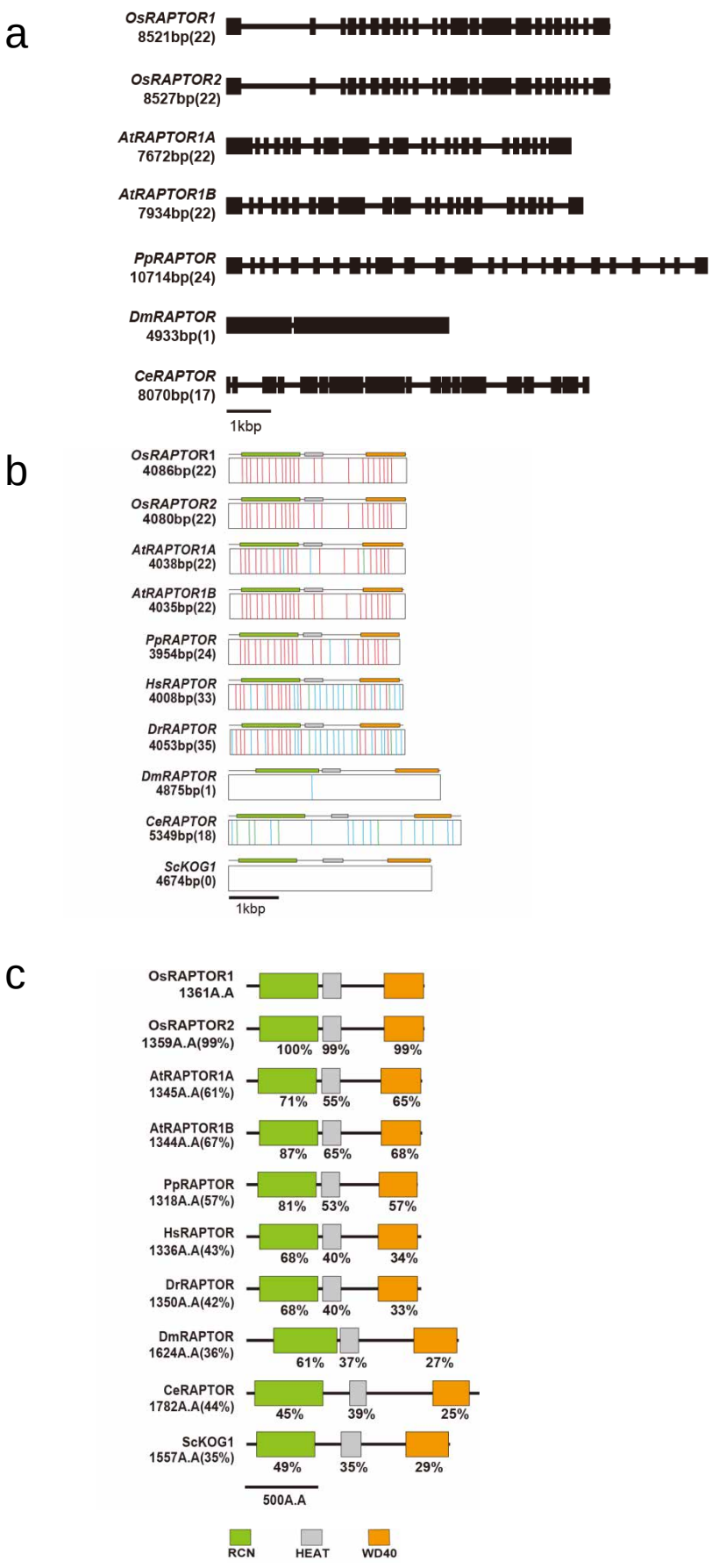
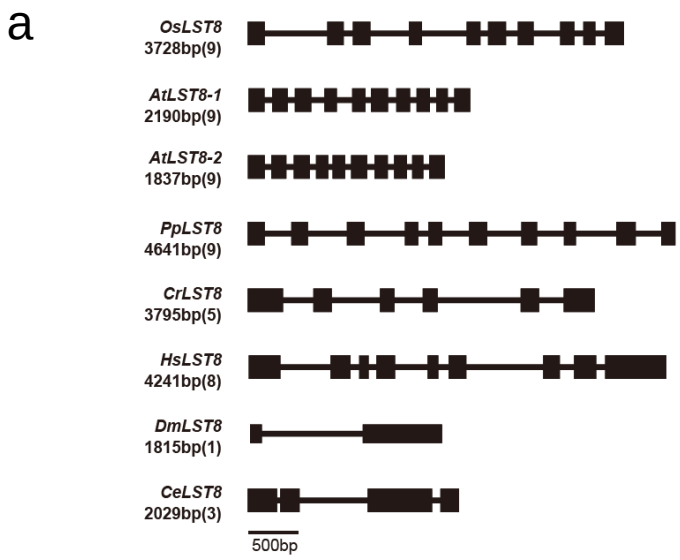

a
OsRAPTOR1
8521bp(22)
OsRAPTOR2
8527bp(22)
AtRAPTOR1A
7672bp(22)
AtRAPTOR1B
7934bp(22)
PpRAPTOR
10714bp(24)
DmRAPTOR
4933bp(1)
CeRAPTOR
8070bp(17)
1kbp
b
OsRAPTOR1
4086bp(22)
OsRAPTOR2
4080bp(22)
AtRAPTOR1A
4038bp(22)
AtRAPTOR1B
4035bp(22)
PpRAPTOR
3954bp(24)
HsRAPTOR
4008bp(33)
DrRAPTOR
4053bp(35)
DmRAPTOR
4875bp(1)
CeRAPTOR
5349bp(18)
ScKOG1
4674bp(0)
1kbp
c
OsRAPTOR1
1361A.A
OsRAPTOR2
1359A.A(99%)
100% 99% 99%
AtRAPTOR1A
1345A.A(61%)
71% 55% 65%
AtRAPTOR1B
1344A.A(67%)
87% 65% 68%
PpRAPTOR
1318A.A(57%)
81% 53% 57%
HsRAPTOR
1336A.A(43%)
68% 40% 34%
DrRAPTOR
1350A.A(42%)
68% 40% 33%
DmRAPTOR
1624A.A(36%)
61% 37% 27%
CeRAPTOR
1782A.A(44%)
45% 39% 25%
ScKOG1
1557A.A(35%)
49% 35% 29%
500A.A
RCN
HEAT
WD40

Fig.5


a
OsLST8
3728bp(9)
AtLST8-1
2190bp(9)
AtLST8-2
1837bp(9)
PpLST8
4641bp(9)
CrLST8
3795bp(5)
HsLST8
4241bp(8)
DmLST8
1815bp(1)
CeLST8
2029bp(3)
500bp


b
OsLST8
951bp(9)
AtLST8-1
918bp(9)
AtLST8-2
942bp(9)
PpLST8
951bp(9)
CrLST8
948bp(5)
HsLST8
981bp(8)
DrLST8
981bp(8)
DmLST8
933bp(1)
CeLST8
1149bp(3)
ScLST8
912bp(0)
300bp

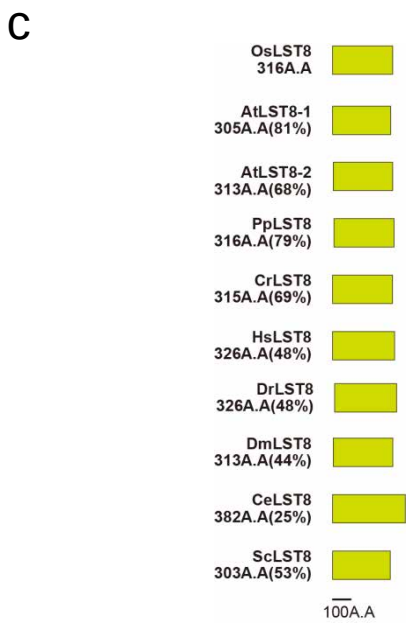
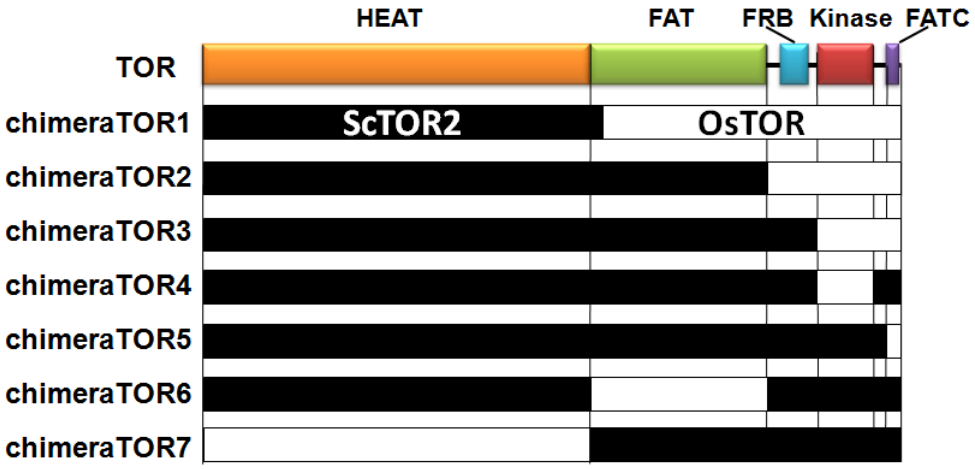

c
OsLST8
316A.A
AtLST8-1
305A.A(81%)
AtLST8-2
313A.A(68%)
PpLST8
316A.A(79%)
CrLST8
315A.A(69%)
HsLST8
326A.A(48%)
DrLST8
326A.A(48%)
DmLST8
313A.A(44%)
CeLST8
382A.A(25%)
ScLST8
303A.A(53%)
100A.A

Fig.6

a


HEAT
FAT
FRB
Kinase
FATC
TOR
chimeraTOR1
ScTOR2
OsTOR
chimeraTOR2
chimeraTOR3
chimeraTOR4
chimeraTOR5
chimeraTOR6
chimeraTOR7

b

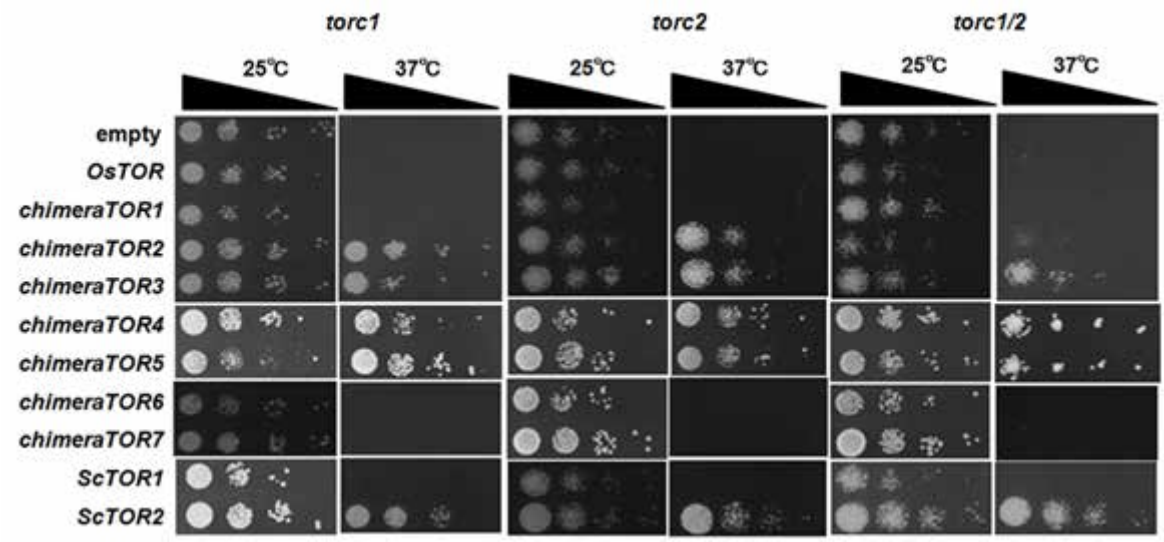

torc1
torc2
torc1/2
25℃
37℃
25℃
37℃
25℃
37℃
empty
OsTOR
chimeraTOR1
chimeraTOR2
chimeraTOR3
chimeraTOR4
chimeraTOR5
chimeraTOR6
chimeraTOR7
ScTOR1
ScTOR2

Fig.7

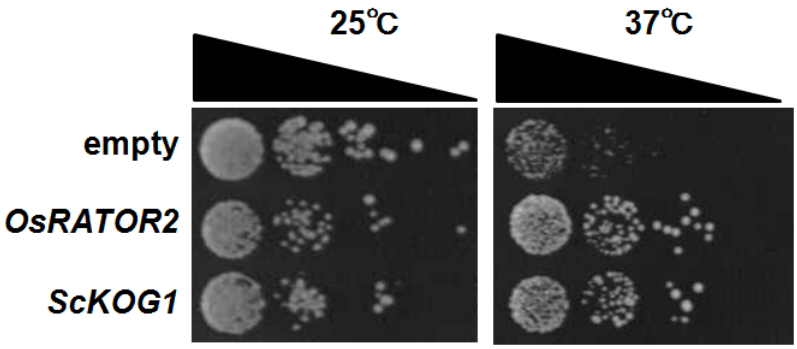


Fig.8