Target of Rapamycin Regulates Development and Ribosomal RNA Expression through Kinase Domain in Arabidopsis¹[W][OA]

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Target of rapamycin (TOR) is a central regulator of cell growth, cell death, nutrition, starvation, hormone, and stress responses in diverse eukaryotes. However, very little is known about TOR signaling and the associated functional domains in plants. We have taken a genetic approach to dissect TOR functions in Arabidopsis (Arabidopsis thaliana) and report here that the kinase domain is essential for the role of TOR in embryogenesis and 45S rRNA expression. Twelve new T-DNA insertion mutants, spanning 14.2 kb of TOR-encoding genomic region, have been characterized. Nine of these share expression of defective kinase domain and embryo arrest at 16 to 32 cell stage. However, three T-DNA insertion lines affecting FATC domain displayed normal embryo development, indicating that FATC domain was dispensable in Arabidopsis. Genetic complementation showed that the TOR kinase domain alone in tor-10/tor-10 mutant background can rescue early embryo lethality and restore normal development. Overexpression of full-length TOR or kinase domain in Arabidopsis displayed developmental abnormalities in meristem, leaf, root, stem, flowering time, and senescence. We further show that TOR, especially the kinase domain, plays a role in ribosome biogenesis by activating 45S rRNA production. Of the six putative nuclear localization sequences in the kinase domain, nuclear localization sequence 6 was identified to confer TOR nuclear targeting in transient expression assays. Chromatin immunoprecipitation studies revealed that the HEAT repeat domain binds to sequences in the kinase domain, nuclear localization sequence 6 was identified to confer TOR nuclear targeting in transient expression assays. Together, these results show that TOR controls the embryogenesis, postembryonic development, and 45S rRNA production through its kinase domain in Arabidopsis.

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TORC2 is rapamycin insensitive and is involved in the regulation of cytoskeleton structure, cell cycle, apoptosis, and transcription (Guertin et al., 2006; Jones et al., 2009). AKT and SGK1 have been shown to be substrates for TORC2 in animals (Guertin et al., 2006; Jones et al., 2009).

TOR proteins in diverse eukaryotes consist of highly conserved domains (Fig. 1A). The N-terminal 1,200 residues consist of 20 HEAT repeats. These HEAT repeats have been implicated in protein-protein interactions (Andrade and Bork, 1995; Kunz et al., 2000). The focal adhesion target (FAT) domain, located at the C-terminal end of HEAT repeats has been also implicated in protein-protein interactions (Bosotti et al., 2000). The FRB (FKBP12/rapamycin binding) domain is the binding site for FKBP12 (FK506 binding protein 12) protein, which in turn is the target for rapamycin-mediated inhibition (Schmelzle and Hall, 2000; Kim et al., 2002; Loewith et al., 2002). The kinase domain contains a conserved lipid Ser/Thr kinase motif. A putative scaffolding domain, FATC, is located at the carboxy terminus of TOR protein (Takahashi et al., 2000; Dames, 2010).

One of the key functions of TOR is its involvement in ribosome biogenesis (Li et al., 2006; Wei et al., 2009; Tsang et al., 2010). The rate of cell proliferation and growth directly depends on ribosome biogenesis. This requires coordination of the production of several ribosomal components, including four different rRNAs and about 130 ribosomal proteins (Michels and Hernandez, 2006). TOR has been implicated to play an important regulatory role in the expression of several of these components and also in the biogenesis of ribosomes (Li et al., 2006; Michels and Hernandez, 2006; Wei et al., 2009; Tsang et al., 2010). In yeast and animals, inhibition of the TOR signaling pathway by nutrient starvation or rapamycin treatment results in a rapid down regulation of 18S, 5.8S, 25S, and 5S rRNAs (Li et al., 2006; Michels and Hernandez, 2006).

Loss of functions associated with TOR mutations in yeast is evidenced by small cell size, slow growth rate, hypersensitivity to temperature and osmotic stress, and defects in cell cycle and translation initiation (Barbet et al., 1996; Weisman and Choder, 2001). Knockouts of Drosophila TOR show reduced nuclear size and lipid vesicle aggregation in the larval fat body (Zhang et al., 2000). TOR deficiency in Caenorhabditis elegans results in arrest at mid-to-late developmental stages of larvae (Long et al., 2002). In mice, the disruption of TOR causes early embryo lethality and also arrests the proliferation of embryonic stem cells (Murakami et al., 2004). Together, these findings show that TOR plays a critical role in the growth and development of diverse eukaryotic species.

In contrast to the knowledge on TOR in other eukaryotes, very little is known about TOR signaling in plants (Menand et al., 2002; Anderson et al., 2005; Mahfouz et al., 2006; Deprost et al., 2007; Sormani et al., 2007). Unlike in yeast and animals, Arabidopsis (Arabidopsis thaliana) TOR is insensitive to rapamycin (Sormani et al., 2007). The putative FKBP12 homologs in Arabidopsis do not bind or interact with rapamycin, and this failure to form the functional in vivo rapamycin-FKBP12 complex likely contributes to rapamycin insensitivity in plants (Sormani et al., 2007). Insensitivity to rapamycin has been a major impediment to making progress with determination of the biological effects of TOR activity in plants. Therefore, much less is known about the function of TOR in plants than in yeast and animal systems. The Arabidopsis genome contains a single copy of TOR and the loss-of-function mutations cause embryo lethality in Arabidopsis (Menand et al., 2002). The TOR protein contains all the conserved regions found in other species: HEAT repeats, FAT domain, FRB domain, kinase domain, and the FATC domain (Menand et al., 2002; Mahfouz et al., 2006). However, it is not known which of the five domains are critical for embryonic development or other regulatory functions of the TOR complexes. In this study, we characterize TOR...
functional domains required for plant growth and development and show that the kinase domain plays critical roles in development, nuclear localization, and rRNA expression in Arabidopsis.

RESULTS

Characterization of 12 New T-DNA Insertion Lines Spanning 14.2 kb of the TOR-Encoding Genomic Region

TOR is a multidomain protein and these domains are implicated in different functions (De Virgilio and Loewith, 2006b). The Arabidopsis TOR gene spans 17.6-kb genomic region with 56 exons and 55 introns (Menand et al., 2002). An earlier study had shown embryo lethality in two T-DNA insertional mutants in the region preceding the FRB domain (Menand et al., 2002). It is possible to identify mutations in different regions that produce truncated proteins for further functional analysis. Toward this, we screened SALK lines for additional insertional mutations and identified 12 new T-DNA insertion lines spanning 14.2 kb of TOR-encoding genomic region (Table I; Fig. 1; http://signal.salk.edu/cgi-bin/tdnaexpress). The flanking sequences of these T-DNA lines were further confirmed by PCR and sequencing (Table I; Supplemental Materials and Methods S1). The sequencing results showed that all the left border flanking sequences of the 12 mutants are consistent with the information provided in the T-DNA Express database (see http://signal.salk.edu/cgi-bin/tdnaexpress; Table I). As shown in Table I, the right border of T-DNAs was not detectable in tor-6 and tor-12, possibly due to some DNA deletions or rearrangements. The other 10 lines showed the correct T-DNA right border flanking sequence (Table I).

To further examine whether TOR insertion lines express truncated versions, we performed quantitative reverse transcription (RT)-PCR using the embryo samples to test TOR transcripts corresponding to upstream, downstream, and also the sequences that span the T-DNA insertion sites (Table II; Supplemental Table S2; Supplemental Materials and Methods S1). TOR transcription downstream of T-DNA insertion sites was not detectable in tor-4, tor-5, tor-6, tor-7, tor-12, tor-13, and tor-14 but low expression was detectable in tor-3, tor-8, tor-9, tor-10, and tor-11 (Table II). These results are explainable on the basis of the orientation of the T-DNA insertion (Table I) because some transcripts can be produced in the forward T-DNA insertion orientation lines in which 35S promoter adjacent to T-DNA left border can potentially express the downstream coding sequence (Deprost et al., 2007). No expression was detected corresponding to TOR in the reverse T-DNA insertion orientation mutants (Table II). It should be noted in tor-3, tor-4, and tor-5, transcription of the sequence upstream to the insertion site was not evident (Table II), indicating that truncated transcripts were not stable enough for detection in the three lines. However, the truncated versions of TOR transcripts, expressed under the control of native promoter, corresponding to upstream region of the T-DNA insertion sites, were detected in tor-6, tor-7, tor-8, tor-9, tor-10, tor-11, tor-12, tor-13, and tor-14 lines (Table II). The T-DNA insertions occurred in the introns of TOR genomic region in tor-4, tor-5, tor-6, tor-7, tor-8, tor-11, tor-13, and tor-14 lines. The RT-PCR analysis of these lines did not detect TOR transcription spanning the introns, suggesting no restoration of full-length transcript via splicing of T-DNA inserts from the respective introns (Tables I and II).

The FATC Domain Is Dispensable in Arabidopsis

The FATC domain is essential for TOR functions in yeast and mammalian system (Takahashi et al., 2000; Dames, 2010). However, T-DNA insertions that are predicted to affect the FATC domain in tor-12, tor-13, and tor-14 showed normal embryo development (Ta-

<table>
<thead>
<tr>
<th>TOR Mutant</th>
<th>SALK Lines</th>
<th>Embryo Phenotypes</th>
<th>Insertion Position/Domain/Orientation</th>
<th>Distance to Transcription Initiation Site bp</th>
<th>Right Border Sequence of T-DNA</th>
<th>Left Border Sequence of T-DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>tor-3</td>
<td>SALK_036379</td>
<td>Lethal</td>
<td>10th exon/HEAT/F</td>
<td>3,604</td>
<td>No deletion</td>
<td>Correct position</td>
</tr>
<tr>
<td>tor-4</td>
<td>SALK_007654</td>
<td>Lethal</td>
<td>11th intron/HEAT/R</td>
<td>4,016</td>
<td>No deletion</td>
<td>Correct position</td>
</tr>
<tr>
<td>tor-5</td>
<td>SALK_147473</td>
<td>Lethal</td>
<td>15th intron/HEAT/R</td>
<td>5,766</td>
<td>No deletion</td>
<td>Correct position</td>
</tr>
<tr>
<td>tor-6</td>
<td>SALK_017177</td>
<td>Lethal</td>
<td>19th intron/HEAT/R</td>
<td>6,795</td>
<td>Not detectable</td>
<td>Correct position</td>
</tr>
<tr>
<td>tor-7</td>
<td>SALK_028697</td>
<td>Lethal</td>
<td>19th intron/HEAT/R</td>
<td>6,797</td>
<td>No deletion</td>
<td>Correct position</td>
</tr>
<tr>
<td>tor-8</td>
<td>SALK_016286</td>
<td>Lethal</td>
<td>28th intron/FAT/F</td>
<td>9,870</td>
<td>No deletion</td>
<td>Correct position</td>
</tr>
<tr>
<td>tor-9</td>
<td>SALK_013925</td>
<td>Lethal</td>
<td>46th exon/FRB/F</td>
<td>15,034</td>
<td>No deletion</td>
<td>Correct position</td>
</tr>
<tr>
<td>tor-10</td>
<td>SALK_138622</td>
<td>Lethal</td>
<td>47th exon/kinase/F</td>
<td>15,159</td>
<td>No deletion</td>
<td>Correct position</td>
</tr>
<tr>
<td>tor-11</td>
<td>SALK_043130</td>
<td>Lethal</td>
<td>53 intron/Kinase/F</td>
<td>16,572</td>
<td>No deletion</td>
<td>Correct position</td>
</tr>
<tr>
<td>tor-12</td>
<td>SALK_056253</td>
<td>Normal</td>
<td>55 exon/FATC/R</td>
<td>17,093</td>
<td>Not detectable</td>
<td>Correct position</td>
</tr>
<tr>
<td>tor-13</td>
<td>SALK_108347</td>
<td>Normal</td>
<td>55th intron/FATC/R</td>
<td>17,267</td>
<td>No deletion</td>
<td>Correct position</td>
</tr>
<tr>
<td>tor-14</td>
<td>SALK_056209</td>
<td>Normal</td>
<td>55 intron/FATC/R</td>
<td>17,283</td>
<td>No deletion</td>
<td>Correct position</td>
</tr>
</tbody>
</table>
ble I), indicating that the truncated versions of TOR without FATC domain is functional and FATC domain is likely not essential for embryo development in Arabidopsis. In quantitative RT-PCR assays, we found no evidence of FATC domain transcript sequences downstream of the T-DNA insertions in tor-12, tor-13, and tor-14 (Table II). However, nearly normal levels of transcript from the upstream region were found (Table II). Embryogenesis, vegetative growth, and reproductive development were also normal in these mutants (Table I; Supplemental Table S3). These observations suggest that the FATC domain did not negatively impact the life cycle, indicating that this domain was not required for plant development under laboratory conditions. Multiple sequence alignment identified that the TOR FATC domain (80 amino acids at C terminal of TOR proteins) shows high conservation in yeast, mouse, and human (Supplemental Fig. S1), while 22 additional residues (SSSAIPSNPIADHHN-LLGGDHS) were found in the corresponding Arabidopsis FATC domain (see Supplemental Fig. S1). Further, these additional residues are also found in the FATC domain of TOR proteins in diverse plant species, suggesting plant lineage diverged from the corresponding domain in yeast and animal species (Supplemental Fig. S1). Taken together, these observations likely reflect the functional diversities between plant and nonplant species of TOR proteins.

### The Kinase Domain Is Required for Embryo Development

The insertions in tor-3, tor-4, tor-5, tor-6, and tor-7 occurred in the HEAT repeats domain (Fig. 1A; Table I). A low level of transcript (approximately 7% of the wild-type level) downstream of T-DNA insertion site was evident in tor-3 (Table II) due to the forward orientation of T-DNA insertion, but none was observed in the other four mutants. However, all these mutants showed embryo lethality (Tables I and II; Fig. 1). The T-DNA insertions occurred in the FAT and FRB domain in the lines tor-8 and tor-9, respectively (Table I; Fig. 1), permitting low-level expression (13% and 9%, respectively; Table II) because of the permissive orientation as in tor-3. However, tor-8 and tor-9 also cause embryo lethality (Table I; Fig. 1). As for tor-10 and tor-11, despite their permissive orientation, the T-DNA insertion in kinase domain abolishes embryo development (Table I; Fig. 1). Although low level of TOR transcription downstream of T-DNA insertion site were detected in tor-3, tor-8, tor-9, tor-10, and tor-11 due to the forward T-DNA insertion orientation, they still exhibited the same embryo lethality as tor-4, tor-5, tor-6, and tor-7 with the reverse T-DNA insertion orientation (Table II). Therefore, it is likely that low level of transcription downstream of T-DNA insertion site in these mutants is inadequate to support embryo development or these transcripts failed to translate to functional TOR protein. Nearly normal levels of transcription upstream of the T-DNA insertion site were detected in lines tor-6 to tor-14 but not in tor-3, tor-4, and tor-5 (Table II), indicating that production of TOR transcripts encompassing the HEAT, FAT, and FRB domains is insufficient for embryo development. However, tor-3 to tor-11 lines share defective kinase domain and mutant embryo phenotypes (Fig. 1B; Table I), indicating that the kinase domain is a critical part of the TOR with respect to embryo development.

To further confirm that embryo-lethal phenotypes observed in tor-3 to tor-11 lines were the result of the respective T-DNA insertions, we crossed these lines independently with wild-type Arabidopsis (Columbia-0 [Col-0]). In the F2 population, the T-DNA co-segregated with embryo-lethal phenotypes, indicating that T-DNA insertions caused embryo-lethal phenotypes in tor-3 to tor-11 lines.

### Table II. Analysis of the transcription level and embryo phenotypes of ATOR mutant

<table>
<thead>
<tr>
<th>TOR Mutant</th>
<th>Transcripts with Sequences from Upstream of the Insertion Sitea</th>
<th>Transcripts with Sequences from Downstream of the Insertion Sitea</th>
<th>Transcripts Spanning the Insertion Site</th>
<th>Percentage of Aborted Seedsb</th>
<th>Mutant Embryo Arrested Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>tor-3</td>
<td>Not detected</td>
<td>0.07 (±0)</td>
<td>Not detected</td>
<td>25.9 (n = 2,537)</td>
<td>16–32 cells</td>
</tr>
<tr>
<td>tor-4</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>25.2 (n = 2,511)</td>
<td>16–32 cells</td>
</tr>
<tr>
<td>tor-5</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>25.1 (n = 2,493)</td>
<td>16–32 cells</td>
</tr>
<tr>
<td>tor-6</td>
<td>0.76 (±0.05)</td>
<td>Not detected</td>
<td>Not detected</td>
<td>24.9 (n = 2,498)</td>
<td>16–32 cells</td>
</tr>
<tr>
<td>tor-7</td>
<td>0.74 (±0)</td>
<td>Not detected</td>
<td>Not detected</td>
<td>25.3 (n = 2,527)</td>
<td>16–32 cells</td>
</tr>
<tr>
<td>tor-8</td>
<td>0.82 (±0.03)</td>
<td>0.13 (±0.01)</td>
<td>Not detected</td>
<td>24.8 (n = 2,522)</td>
<td>16–32 cells</td>
</tr>
<tr>
<td>tor-9</td>
<td>0.96 (±0.01)</td>
<td>0.09 (±0)</td>
<td>Not detected</td>
<td>25.4 (n = 2,505)</td>
<td>16–32 cells</td>
</tr>
<tr>
<td>tor-10</td>
<td>1.05 (±0)</td>
<td>0.11 (±0.02)</td>
<td>Not detected</td>
<td>25.1 (n = 2,517)</td>
<td>16–32 cells</td>
</tr>
<tr>
<td>tor-11</td>
<td>1.09 (±0)</td>
<td>0.14 (±0)</td>
<td>Not detected</td>
<td>25.0 (n = 2,523)</td>
<td>16–32 cells</td>
</tr>
<tr>
<td>tor-12</td>
<td>1.07 (±0.02)</td>
<td>Not detected</td>
<td>0 (n = 2,579)</td>
<td>Normal embryos</td>
<td></td>
</tr>
<tr>
<td>tor-13</td>
<td>0.97 (±0.01)</td>
<td>Not detected</td>
<td>0 (n = 2,508)</td>
<td>Normal embryos</td>
<td></td>
</tr>
<tr>
<td>tor-14</td>
<td>1.01 (±0)</td>
<td>Not detected</td>
<td>0 (n = 2,583)</td>
<td>Normal embryos</td>
<td></td>
</tr>
</tbody>
</table>

Values shown are mean number ± so of TOR relative expression levels in 6 d after flower embryos against that of wild-type control. RNA was extracted from 6 DAF embryos and qualitatively analyzed by RT-PCR and then quantitatively analyzed by real-time PCR, and normalized against Actin2 expression. b Fifty siliques were used for each mutant line.
Kinase Domain Is Critical for TOR Functions in Vivo

To further assess the functional domains of TOR in vivo, we first assembled the full-length wild-type cDNA clone of TOR (7,446 bp) and 3 kb of the putative upstream promoter region. Expression constructs were generated with full-length cDNA and a series of truncated versions under the control of its native promoter (Fig. 2; Supplemental Table S4; Supplemental Materials and Methods S1). The tor-3 line, with the insertion closest to the 5’ end of the open reading frame among all other insertion mutations discussed in this study (Table I), represents a mutant with near obliteration of TOR transcription. This line shows a strong embryo-defective phenotype with 88% (n = 2,537) of the embryos arrested at the 16-cell stage and all of the remainder at the 32-cell stage (Supplemental Table S5). The segregating mutant embryos displayed slower growth than the wild-type embryos (Supplemental Table S5). While the wild-type embryos were at the heart stage, the mutant embryos were only at dermatogen stage, suggesting that loss of TOR functions in these embryos affected the cell division and growth rates. The tor-10 allele, the earliest insertion in kinase domain, was selected to represent the kinase domain deletion. Examination of the tor-10 also identified similar growth defects in the segregating mutant embryos (Supplemental Table S5). However, more embryos (55%; n = 2,517) progressed up to 32 cells and none beyond this stage (Supplemental Table S5).

In the tor-3 mutant background, the full-length TOR cDNA (PTOR:TOR) completely rescued the embryo lethality to wild type and produced normal plants in T2 generation plants carrying both PTOR:TOR construct (Basta-selective marker for plant) and T-DNA insertion of tor-3 (Fig. 2A; Supplemental Table S6), whereas the kinase domain construct (PTOR:TOR2031-2350) was able to rescue the embryo lethality from the early globular stage block to the late heart stage in T2 generation (Fig. 2, A and B; Supplemental Table S6). The other deletion constructs lacking the kinase domain alone did not rescue the tor-3 embryos from globular stage arrest (Supplemental Table S6; Fig. 2A), suggesting that the kinase domain though critical by itself is not sufficient to restore wild-type TOR functions. In the tor-10 mutant line, full-length TOR (PTOR:TOR) completely rescued the embryo lethality and produced normal plants in T2 generation (Fig. 2C; Supplemental Table S7). Interestingly, transgenic plants containing the constructs that expressed: the kinase domain alone (PTOR:TOR2031-2350), deletion of N-terminal HEAT repeats (PTOR:TOR1433-2482), and deletion of both the HEAT repeats and FAT domain (PTOR:TOR1832-2482), fully rescued the tor-10 mutant and produced normal plants (Fig. 2, C and D; Supplemental Table S7). These results suggest that kinase domain alone or in combination with HEAT and/or FAT domain deletions is sufficient to restore wild-type TOR functions in tor-10 mutant background. The complementation of two partial TOR proteins to restore native functions is likely mediated through interaction and in vivo assembly of these peptides. To test potential interactions between different Arabidopsis TOR protein domains, we performed yeast two-hybrid assays with full-length protein, TOR kinase domain (TOR2031-2350), and TOR protein without kinase domain (TOR1-2031). These analyses showed that: TOR protein interacts with itself, TOR protein interacts with TOR2031-2350 (kinase domain), and TOR protein can also bind to TOR1-2031 (kinase and FATC domain deletion; Supplemental Fig. S2). Similar intragenic complementation phenotypes of TOR have also been observed in Drosophila (Zhang et al., 2006b). Consistent with these observations, PTOR:TOR1-2031 (kinase and FATC domain deletion) and pTOR:TOR2031-2350 (kinase domain deletion) constructs failed to rescue embryo-defective phenotypes of tor-10 (Fig. 2C; Supplemental Table S7). Taken together, these results further demonstrate that the TOR kinase domain is essential for embryo development.

Perturbation of the TOR and Kinase Domain Functions Impairs Plant Development

To investigate the functions of TOR in other phases of Arabidopsis development, we generated transgenic lines with P35S:TOR construct using Basta resistance selection marker. These transgenic lines displayed a range of developmental phenotypes involving leaf, stem, root, and meristem (Fig. 3, A and C). Based on TOR expression levels examined by real-time PCR (Supplemental Tables S8 and S9) and the severity of phenotypes, these lines were classified into strong, medium, weak, and normal groups.

The strong phenotype was defined as one with severe primary and lateral inflorescence meristem defects and shorter hypocotyls. Twenty nine of the 300 transgenic lines showed this strong phenotype (Fig. 3A). Development of primary shoots and root meristems was arrested in these plants at the early vegetative stage (Fig. 3A, plant number 1). The medium group (58/300) was defined as one where the shoot meristem had collapsed around 35 d after germination (DAG; Fig. 3A, plant number 2). Additionally, the following changes in leaf patterning were observed: Leaf lamina was significantly larger, rounder, and thicker than the wild type and was noticeably wrinkled with severe changes in proximodistal and mediolateral axis. Short hypocotyls were also observed (Supplemental Fig. S5). The lines defined as the weak group (96/300; Fig. 3A, plant number 3) displayed compact architecture with short petioles and early senescence of rosette leaves, reduced stem elongation, compressed internodes, and defects in lateral root initiation (Fig. 3, B and C). The remaining lines (146/300) were phenotypically normal. To further examine whether P35S:TOR produced the functional protein in transgenic Arabidopsis, we transformed P35S:TOR construct into TOR/tor-3 and TOR/tor-10 backgrounds. Except displaying similar overexpres-
sion phenotypes, both tor-3/tor-3 and tor-10/tor-10 mutant embryos can be rescued by P35S:TOR in T2 generation (Supplemental Tables S6 and S7), suggesting that the P35S:TOR construct produced full-length functional TOR protein.

Since the kinase domain is critical for TOR in vivo function, we focused on whether overexpression of the kinase domain would show similar or different phenotypes as compared to transformants carrying full-length TOR (P35S:TOR). We constructed P35S:TOR2031-2350 construct (Supplemental Table S4) using Basta resistance selection marker and produced independent transgenic lines. The overexpression lines with this construct (Supplemental Table S10) showed similar phenotypes of short hypocotyls and leaf petioles as observed in P35S:TOR lines (Supplemental Fig. S3), but the P35S:TOR2031-2350 lines also showed some distinct developmental phenotypes (Fig. 3, D and F). There were significant alterations in root morphogenesis and abnormal cell expansion in specialization zones (Fig. 3H; Supplemental Fig. S4). The pith cells in the root had an increased number of columns and this phenotype extended to the top part of the root (bracketed area) producing a tuberous root phenotype (Supplemental Fig. S4). There were changes in proximal-distal axis; dark-green and asym-

Figure 2. TOR kinase domain is critical for TOR functions in vivo. A, Functional complementation assay in TOR/tor-3 background that shows partial rescue of early embryo lethality with kinase domain in tor-3/tor-3 mutant embryos, while constructs without the kinase domain fail to rescue the early embryo lethality. ++, Complete rescue of early embryo lethality; +, partial rescue of early embryo lethality; −, no rescue of embryo lethality. All the complementation constructs are expressed under the control of 3-kb TOR native promoter. B, Representative Nomarski images of kinase domain alone (PTOR:TOR2031-2350) that shows partial complementation of tor-3/tor-3 mutant embryos. Bars = 0.01 mm unless indicated in figures. C, Functional complementation assay in TOR/tor-10 background shows that the kinase domain was able to rescue the tor-10/tor-10 mutant embryo phenotype, while deletion constructs without the kinase domain failed to rescue embryo lethality. ++, Complete rescue of early embryo lethality; −, constructs did not restore the mutant embryos. D, Representative Nomarski images of functional complementation of tor-10/tor-10 defective embryos. PTOR:TOR2031-2350 constructs completely complement the tor-10/tor-10 mutant embryos. Bars = 0.01 mm unless indicated in figures.
metric rosette leaves (Fig. 3, E–G); delayed senescence and flowering (Fig. 3, E–G); altered phyllotactic pattern in the inflorescence stem; bent and longer siliques (Fig. 3I); defective primary meristem resulting in loss of apical dominance; and the simultaneous formation of axillary inflorescence shoots (Fig. 3G). These phenotypes were observed in 96/300 lines. In comparison, 10/300 lines showed enlarged shoot apical meristem, often becoming fasciated, increased number of floral organs, and enlarged floral meristem, suggesting that TOR kinase domain likely mediates functions in meristem. We further introduced \( P35S:TOR^{2031-2350} \) construct into \( TOR/tor-3 \) and \( TOR/tor-10 \) lines. In T2 generation, except displaying similar overexpression phenotypes, \( P35S:TOR^{2031-2350} \) is able to restore the \( tor-10/tor-10 \) mutant embryos to normal growth and partially rescue \( tor-3/tor-3 \) to late heart stage (Supplemental Tables S6 and S7), indicating \( P35S:TOR^{2031-2350} \) produced the same functional kinase domain protein as \( PTOR:TOR^{2031-2350} \).

To test the contributions of other TOR domains, a deletion derivative without the kinase domain was used to construct \( P35S:TOR^{2031-2350} \) (Supplemental Table S4). Independent transgenic lines were made using this construct, again in the wild-type Arabidopsis background. These lines also displayed some phenotypes similar to full-length TOR overexpression (Fig. 3I): short leaf lamina petioles, compact and yellowing rosette leaves (Fig. 3J); but some distinct phenotypes were also observed: dwarf plants, rapid ageing in rosette leaves and primary stem, and shorter overall lifespan (Fig. 3K).

To test any influence of TOR transgene on the expression of native genomic TOR gene, RT-PCR analysis was performed using specific 5\# and 3\# primers (Supplemental Table S8) that distinguish native and transgene expression in transgenic lines produced with \( P35S:TOR \), \( P35S:TOR^{2031-2350} \), and \( P35S:TOR^{D2031-2350} \) constructs. This analysis showed that the expression of endogenous TOR transcript levels (Supplemental Table S10) were very similar to wild-type controls, indicating that the overexpression phenotypes with these constructs are the outcome of the observed high expression levels of respective TOR transgenes.

The Kinase Domain Regulates rRNA Expression

TOR has been implicated in the regulation of rRNA transcription in yeast and animals (Li et al., 2006; Wei et al., 2009; Tsang et al., 2010). To determine if plant
TOR is also involved in this process, we investigated whether transcription of pre-45S rRNA, consisting of 18S, 5.8S, and 25S rRNAs, was affected in the TOR mutants and in overexpression lines. Total RNA was isolated independently from embryos in tor-3/tor-3 and tor-10/tor-10 mutants and P35S:TOR, P35S:TOR2031-2350, and P35S:TOR2031-2350 transgenic lines (Supplemental Fig. S5). Using specific primers designed for 18S, 5.8S, and 25S RNA regions (Supplemental Table S8), quantitative analysis of expression levels was performed using real-time PCR-based assays. The expression levels of 18S, 5.8S, and 25S rRNA were significantly lower (less than 40%) in tor-3/tor-3 and tor-10/tor-10 mutant embryos relative to the wild type (Fig. 4A). The rRNA levels were 8 times higher in P35S:TOR transgenic lines. The kinase domain overexpression lines (P35S:TOR2031-2350) showed 6-fold greater level of rRNA expression relative to wild-type control (Fig. 4B). Notably, the positive influence of higher rRNA expression was abolished, if the kinase domain was deleted (P35S:TOR2031-2350, Fig. 4B). These results suggest that TOR regulates rRNAs transcript levels through the activity of its kinase domain.

The Kinase Domain of TOR Is Involved in Nuclear Localization

To further investigate TOR-mediated regulation of rRNA expression in Arabidopsis, we have examined TOR subcellular localization, using GFP translational fusion constructs (P35S:TOR-GFP) and onion (Allium cepa) epidermal cell-based transient expression system. The results revealed that TOR protein is present in both the cytoplasm and the nucleus (Fig. 5). To map the nuclear localization sequences (NLSs) of TOR, we identified six putative NLSs using PSORT software: 380PRKGRPL (NLS1); 560RRRR (NLS2); 1334PRSKRDPM (NLS3); 1673KRK (NLS4); 2077RPRK (NLS6); and a bipartite nuclear localization signal 1506RRAKYDERAYEYERARK (NLS5; Fig. 5A). To test which of these putative NLSs are involved in the nuclear targeting, we constructed the corresponding deletion mutants for expression in the onion epidermal cell-based transient expression system. Since these putative NLSs are distributed in different domains of TOR protein, the following independent constructs (Supplemental Table S4) were made: TOR1433-2482 (NLS1, NLS2, and NLS3 deletions), TOR1832-2482 (NLS1, NLS2, NLS3, NLS4, and NLS5 deletions), TOR2031-2350 (containing NLS6 motif), TOR1-2031 (NLS6 deletion), and TOR2031-2350 (kinase domain deletion; Fig. 5A). These derivatives were fused with GFP at C termini to determine nuclear targeting. Deletions involving the putative NLS1-5 motifs did not affect the nuclear localization. However, the loss of NLS6 region resulted in most of the GFP signal being retained in the cytoplasm (Fig. 5C). This analysis suggests that the NLS6 motif (located in the kinase domain) of TOR is critical for nuclear localization. To further confirm that the GFP signal is not caused by the cleaved TOR protein, we fused GFP reporter at N terminal of TOR, TOR2031-2350, and TOR2031-2350/S4 (containing NLS6 motif), and tested in onion epidermal cell assay. These constructs displayed consistent localization pattern as their C-terminal GFP fusions (Supplemental Fig. S6), indicating that TOR protein wasn’t cleaved in the onion epidermal cells.

To further define the regions of NLS6 required for nuclear localization, additional deletion mutants of this region as C-terminal GFP fusions were generated (Fig. 5B) using specific PCR primers (Supplemental Materials and Methods S1; Supplemental Table S4). Transient onion cell expression studies showed deletions targeted in the RPRK motif, located in the kinase domain from amino acids 2,077 to 2,080, resulted in loss of nuclear localization (Fig. 5C). Furthermore, constructs involving fusion of the peptide RPRK to GFP at the N terminus or C terminus produced predominantly nuclear localization, suggesting that these four residues are sufficient for nuclear targeting (Supplemental Fig. S6). These results provide evidence that the NLS6 within the kinase domain is required for targeting TOR to the nucleus.

We further asked whether N-terminal and C-terminal GFP fusion with TOR would affect TOR protein functions in plants. Both P35S:GFP-TOR and P35S:TOR-GFP constructs were introduced into TOR/tor-3 line. In the T2 generation, except displaying similar phenotypes as P35S:TOR (Supplemental Fig. S6), P35S:GFP-TOR and P35S:TOR-GFP were able to comple-
ment the tor-3/tor-3 mutant embryos to normal growth (Supplemental Table S6), indicating that GFP fusion at the carboxy or amino end did not affect TOR functions in transgenic Arabidopsis lines.

**TOR Directly Interacts with 45S Ribosome RNA Promoter in Vivo**

Chromatin immunoprecipitation (ChIP) experiments (Tsang et al., 2003; Xie et al., 2004) were performed to determine whether TOR directly binds to sequences within the 45S ribosome promoter region. Since P35S:GFP-TOR and P35S:TOR-GFP showed similar localization in transient assays in onion epidermal system and same phenotypes as P35S:TOR in transgenic Arabidopsis lines, we selected P35S:GFP-TOR transgenic Arabidopsis plants (10 DAG) to perform ChIP experiments. In this assay, P35S:GFP transgenic and wild-type lines were used as controls. Chromatin-associated proteins were cross-linked to the DNA with formaldehyde. The cross-linked chromatin was fragmented by sonication and then mixed with anti-GFP antibody to select and isolate DNA associated with the GFP-TOR fusion protein. PCR primer pairs (Supplemental Table S11) were designed for amplifying 18 overlapping DNA fragments spanning the 45S rRNA promoter and 5'-external transcribed spacer elements (ETS) regions (Fig. 6A). After immunoprecipitation (IP) with GFP antibody, the associated chromatin fragments extracted from P35S:GFP-TOR, P35S:GFP, and wild-type control were analyzed by quantitative PCR amplification with the 18 primer sets (Fig. 6B). Specific PCR bands were displayed corresponding to primer pairs R6, R7, R12, R13, R14, R15, R17, and R18 only in IP chromatin samples of P35S:GFP-TOR transgenic line and no detectable amplified DNA bands in P35S:GFP or wild-type control lines (Fig. 6B), whereas the control experiment without antibody resulted in little or no detectable amplified DNA bands.
Figure 6. TOR interacts with the promoter and 5′-ETS of the ribosome RNA gene and regulates ribosome rRNA expression in Arabidopsis. A, The organization of the 45S rRNA gene in Arabidopsis and the location of 18 overlapping PCR fragments spanning 45S rRNA genomic sequences designed for ChIP assay. Eighteen PCR primer sets are listed in Supplemental Table S11. B, Mapping TOR binding regions in 45S rRNA promoter and 5′-ETS by ChIP assay. The ChIP assay was performed with GFP antibody using the samples derived from: 1, Wild type; 2, transgenic line produced with P35S:GFP; and 3, transgenic line produced with P35S:GFP-TOR line. INPUT represents genomic DNA used as template to show the amplification of predicted size fragments with R1 to R18 primer set corresponding to 18 target genomic sequences (top section). In the IP chromatin samples recovered with GFP antibody, PCR amplification of genomic sequences was observed only with R6, R7, R12, R13, R14, R15, R17, and R18 primer sets in P35S:GFP-TOR transgenic line (3), and no detectable amplified DNA bands in P35S:GFP (2) or wild-type control (1) lines (middle section). As negative control MOCK assay was performed after processing the same samples in ChIP assay but without antibody and this analysis show no amplification of target sequences (bottom section). The agarose gel was stained using GelRed dye solution. C, Quantification of TOR binding to the 45S rRNA promoter and 5′-ETS region. The R7 genomic region was set as the control region and the quantity of relative binding by TOR in other regions are shown in the bar graph as fold of enrichment compared with the control sample. Error bars indicate ± so for triplicates. D, DNA-binding domain is critical for the ability of TOR protein to bind the 45S rRNA promoter and 5′-ETS region. The R7 genomic region was set as the control region and the quantity of relative binding by TOR in other regions are shown in the bar graph as fold of enrichment compared with the control sample. Error bars indicate ± so for triplicates. D, DNA-binding domain is critical for the ability of TOR protein to bind the 45S rRNA promoter and 5′-ETS region. The R7 genomic region was set as the control region and the quantity of relative binding by TOR in other regions are shown in the bar graph as fold of enrichment compared with the control sample. Error bars indicate ± so for triplicates. D, DNA-binding domain is critical for the ability of TOR protein to bind the 45S rRNA promoter and 5′-ETS region. The R7 genomic region was set as the control region and the quantity of relative binding by TOR in other regions are shown in the bar graph as fold of enrichment compared with the control sample. Error bars indicate ± so for triplicates. 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DNA for any of the primer pairs (Fig. 6B). Furthermore, we selected five genes on different chromosomes of Arabidopsis to further validate the ChiP results. As shown in Supplemental Figure S8, no detectable amplified DNA bands for any of these primer pairs in GFP-antibody-based IP chromatin samples of P35S:GFP-TOR, P35S:GFP, and wild-type Arabidopsis, indicated that the observed interactions of AtTOR with 45S rRNA promoter and 5′-ETS regions are not due to random nonspecific binding to genomic sequences.

Mapping the amplicons to the 45S rRNA promoter region showed that TOR binding peaks occurred in the 5′-ETS and the transcription initiation site for RNA polymerase I located within the 113-bp repeat region in Arabidopsis. However, transgenic plants containing the 113-bp repeat construct were 5 times higher in the P35S:TOR background compared to wild-type background (Fig. 7B). These results show that TOR activates 45S rRNA promoter and that the activation is significantly enhanced if 5′-ETS is also present.

**DISCUSSION**

In this study, we have dissected functional domains of Arabidopsis TOR protein: the kinase domain that controls embryogenesis and the expression of rRNAs; a NLS, residing in the kinase domain that directs nuclear import of TOR protein; and the DNA-binding motif, residing in the HEAT repeat, which mediates the interaction between TOR protein and the promoter of 45S rRNA through binding to its promoter and 5′-ETS region in Arabidopsis.

**The Leu Zipper within the HEAT Domain Is Responsible for DNA Binding of TOR**

We further considered whether TOR protein binds the 45S rRNA promoter through DNA-binding motifs. Using PSORT software, we identified a putative DNA-binding (Leu zipper) sequence, 1028-1050 (Leu zipper 45S rRNA promoter), within the TOR HEAT repeat domain. Transgenic plants with P35S:GFP-TOR (Leu zipper deletion) construct (Supplemental Table S4) were generated and ChiP assay was performed as noted above. Primer pairs R6, R7, R12, R13, R14, R15, R17, and R18 were employed for quantitative PCR analysis. Wild-type and P35S:GFP-TOR transgenic Arabidopsis were used for negative and positive control, respectively. TOR protein without the DNA-binding motif (GFP-TOR 1028-1050) did not produce amplified DNA fragments from the target regions while the samples from GFP-TOR transgenic plants showed specific PCR fragments (Fig. 6D). These results suggested that TOR directly bound to 45S rRNA promoter and 5′-ETS region by its Leu zipper motif.

**TOR Activates 45S rRNA Promoter in a 5′-ETS-Dependent Manner**

Having established that TOR binds to the 45S rRNA promoter and 5′-ETS via the Leu zipper sequence within the HEAT repeat domain, we considered whether the promoter and/or the 5′-ETS regions played more important roles in modulating transcription of 45S rRNA. We therefore cloned two PCR fragments, a 2,885-bp fragment containing the 45S rRNA promoter region and a larger 4,162-bp fragment spanning both the promoter and 5′-ETS (Supplemental Table S4), and fused them with GFP to generate two constructs P45S rRNA:GFP and P45S rRNA:5′-ETS-GFP (Fig. 7A). Transgenic plants harboring these GFP constructs were crossed with P35S:TOR transgenic plants (Fig. 7A). For each of the four lines, GFP and TOR transcript levels were examined with real-time PCR in 20 F2 generation plants. GFP RNA levels of the P45S rRNA:GFP construct were 5 times higher in the P35S:TOR background compared to wild-type background (Fig. 7B). However, transgenic plants containing the P45S rRNA:5′-ETS-GFP construct had 25 times higher GFP RNA levels in the P35S:TOR background, compared with wild-type background (Fig. 7B). These results show that TOR activates 45S rRNA promoter and that the activation is significantly enhanced if 5′-ETS is also present.

**Figure 7.** Quantification of 45S rRNA promoter activity in wild-type and TOR overexpression backgrounds. A, Diagram of GFP expression constructs produced by 45S rRNA promoter with or without 5′-ETS. These expression constructs were transformed into wild-type and TOR overexpression Arabidopsis backgrounds. 1, P45S rRNA:GFP construct in wild-type Arabidopsis background; 2, P45S rRNA:5′-ETS-GFP construct in wild-type Arabidopsis background; 3, P45S rRNA:GFP construct in TOR overexpression background; 4, P45S rRNA:5′-ETS-GFP in TOR overexpression background. B, Bar graph showing relative amounts of expressed GFP as determined by quantitative real-time PCR. The GFP expression with P45S rRNA:GFP construct in wild-type Arabidopsis was used as control (value set as 1) and the relative GFP expression levels in other lines are shown in the bar graph as fold change compared to the wild-type control. The numbers 1, 2, 3, and 4 correspond to the constructs and background Arabidopsis lines as indicated in A. Quantities of GFP expression levels are relative to the amount of expression observed for construct 1. Error bars indicated ± so for triplicates.
45S rRNA. Plant TOR is insensitive to rapamycin and therefore genetic perturbations that alter the activity are required in delineating the functional aspects. Using insertional mutations in Arabidopsis, genetic complementation, and physical characterization of TOR-bound DNA from the chromatin, we have advanced our understanding of Arabidopsis TOR.

The spatial distribution of the 12 T-DNA insertions over 14.2-kb genome region and the embryo-lethal phenotypes of all but the FATC domain insertions allow us to conclude that the HEAT, FAT, and FRB domains are not sufficient for embryogenesis. Full rescue of the tor-10/tor-10 embryo defect by complementation with the kinase domain (Fig. 2C; Supplemental Table S7) suggest the essentiality of the kinase domain. However, the kinase domain alone was not sufficient because it failed to achieve full restoration of embryogenesis in tor-3/tor-3 mutant (Supplemental Table S6) that was defective for HEAT, FAT, FRB, kinase, and FATC. Remarkably, the kinase domain alone, in the absence of all other domains, was able to support the growth and development of embryo from 16 to 32 cell stage to late heart stage in tor-3/tor-3. This established that TOR is not essential for the initial four or five cell divisions after fertilization. Whether HEAT, FAT, or FRB alone in combination with the kinase domain would suffice is unknown. Menand et al. (2002) also showed that T-DNA insertion preceding FRB domain, which is predicted to disrupt kinase domain further downstream, causes embryo lethality. This suggests that all lesions involving expression of the downstream kinase domain result in a phenotype very similar to the tor-3 mutant, reflecting that kinase domain is essential for Arabidopsis embryogenesis. An intact kinase domain is also essential for TOR function in yeast cell growth and metabolism (Alarcon et al., 1999), indicating that the function of kinase domain is critical and essential in diverse eukaryotic species.

The FATC domain of plant TOR is not essential for TOR function as evident from the normal life cycle of three independent insertion mutants (Supplemental Table S4). Restoration of normal phenotype in tor-10/tor-10 with kinase domain constructs without FATC domain further support this conclusion. However, the FATC domain is essential for TOR functions in yeast and mammalian system (Takahashi et al., 2000; Dames, 2010). It is possible that FATC domain plays a role that is not required for plant life cycle under the laboratory conditions employed in our study. Alternatively, its function is redundant to that of other unknown proteins. Our observations further indicate that the carboxy domain and the amino domain of TOR are quite permissive as GFP fusions at the carboxy or amino end did not abolish TOR function in Arabidopsis (Supplemental Figs. S6 and S7). This contrasts the observations with yeast TOR where N- or C-terminal GFP fusions abolish TOR function (Sturgill et al., 2008). These results show that TOR as a canonical eukaryotic protein (De Virgilio and Loewith, 2006a) does show some functional diversity.

So far, only one study has shown that overexpression of TOR (T-DNA activation-tagged lines) altered Arabidopsis development (Deprost et al., 2007). In those four T-DNA activation lines, however, the TOR expression level increased less than 2 times in two lines, and decreased around 50% in the other two lines in leaves relative to wild-type control (Deprost et al., 2007). It also should be noted that TOR expression levels are quite variable between leaves and roots in these four T-DNA activation lines (Deprost et al., 2007). So far, likely due to the large size of TOR full-length transcript (approximately 7.5 kb), transgenic plants have not been successfully produced with this gene. In this study, we were able to overcome this bottleneck and generate transgenic lines with overexpression constructs using the full-length TOR cDNA coding sequence driven by strong constitutive promoter cauliflower mosaic virus 35S. The highest TOR expression level increased 80-fold in transgenic plants relative to wild-type control (Supplemental Table S9). The endogenous TOR gene expression levels are not affected in the overexpression lines, whereas the TOR as transgene show higher levels of expression (Supplemental Table S10). We also observed that P35S: AITOR can rescue the lethal phenotype in the segregating homozygous recessive mutant embryos after introducing it into TOR/tor-3 and TOR/tor-10 backgrounds, indicating the functional TOR protein was produced with our constructs in the transgenic lines.

In this study, although the transgenic lines of TOR kinase domain overexpression lines shared some phenotypes with full-length TOR lines, the phenotypes of delayed flowering, delayed senescence, and tuberous roots were found only in kinase domain overexpression lines (Fig. 3, D–F). Interestingly, with kinase domain deletion construct that contain HEAT repeats, FAT, and FRB domains, the transgenic lines showed early senescence (Fig. 3, J and K). Taken together these results suggest that higher activity of the kinase or HEAT-FAT-FRB domains confer contrasting outcomes, whereas with normal (wild-type) levels of TOR expression, their functions are coordinated to produce normal development. The overexpression lines may provide us with useful visual markers to further dissect the roles of different TOR domains.

Our work shows that TOR binds to 45S rRNA promoter and activates expression and that enhanced activation requires the 5’-ETS. The demonstration of the Leu zipper region of the HEAT domains as the DNA-binding domain, along with the observation that the kinase domain contains an active NLS, it is conceivable that the large TOR protein gains entry to the nucleus with the involvement of NLS-6 in the kinase domain and it activates rRNA expression by binding to the promoter via the HEAT domain’s Leu zipper motif. Consistent with these observations, overexpression of kinase domain in wild-type plants produced higher levels of rRNA expression, indicating that the DNA-binding function of HEAT domain is required for this activation and kinase domain alone can inter-
act with full-length or truncated TOR protein (Supplemental Fig. S2) containing Leu zipper to restore TOR activity in Arabidopsis. Interestingly, high conservation of motifs especially corresponding to NLS-6 and Leu zipper DNA-binding domains was found in TOR proteins from diverse eukaryotes (Supplemental Figs. S9 and S10). The observation that truncated TOR can restore full TOR function insofar as embryogenesis is concerned, suggests that overexpression of kinase domain might recruit more TOR into the nuclei and activate rRNA transcription. This study showed that TOR protein is able to directly interact with the promoters of ribosome RNA genes to regulate rRNA expression as shown in human and murine cells (Tsang et al., 2010), indicating that this function is conserved among diverse eukaryotic species.

We have found that RNA levels were dramatically reduced in TOR mutant embryos. The reason why TOR mutant embryos fail to develop beyond the 16 to 32 cells stage might be due to a shortage of ribosomes to support protein synthesis. Ribosomal RNAs are the major components (approximately 65%) of ribosomes and the dosage of rRNAs is rate limiting for ribosome biogenesis, cell division, and cell growth (Byrne, 2009). It is possible that the mutant embryos survive up to 16 to 32 cell stage by making use of maternally inherited ribosomes or a low level of ribosome assembly in the zygote.

Many developmental defects in different organs ranging from root, hypocotyl, stem, leaf, and meristem were observed in TOR overexpression lines. Ribosome biogenesis requires coordination of the ribosomal components that include four different rRNAs and approximately 130 ribosome proteins (Byrne, 2009). This coordinated program is likely disturbed when RNA levels are altered. Our data suggests that TOR plays a key role to control this coordination and to tightly regulate development in an ordered temporal and spatial pattern. These observations will provide framework for future investigations to address other functions of TOR signaling in plant developmental and signaling pathways.

**MATERIALS AND METHODS**

**Arabidopsis Growth and Transformation**

Arabidopsis (*Arabidopsis thaliana*) plants and plants were grown in growth chambers set at 22°C, with a 16-h/8-h light/dark cycle. Wild-type plants were of the ecotype Col. Transgenic plants were generated by the floral-dipping protocols of the ecotype Col. Transgenic plants were generated by the floral-dipping protocol. The full-length cDNA of the wild-type TOR (7.4 kb) and various truncated fragments were amplified by RT-PCR using the Advantage 2 polymerase mix kit (Clontech, cat. no. 639201) following the manufacturer's instructions. RT-PCR primer pairs for amplification of the full-length TOR gene from Arabidopsis were designed based on the cDNA sequence (http://www.arabidopsis.org) of TOR. These primers were designed to introduce a NotI site at the 5' end of forward primers and an XmnI site at the 3' end of reverse primers. Three overlapping fragments of the TOR gene were amplified and fused together by using restriction enzymes followed by ligation (NotI, BspEI, BpiI, and XmnI) to generate the full-length clone (7.4 kb). The PCR products, with the 5' NotI site and 3' XmnI site, were cloned into the TA cloning vector pCR2.1-TOPO (Invitrogen, cat. K450001). The recombinant clones were verified by DNA sequencing. Recombinant plasmids were digested with NotI and XmnI and cloned into p8GWG (see below) to generate the gateway system-based Entry vector (Earley et al., 2006).

**Cloning p8GWG into Gateway Entry Vectors**

The construction of the Gateway entry vector p8GWG is based on the pCR8/GW/TOPO (Invitrogen, cat. K2500-20) plasmid, comprising a TOPO AT cloning site flanked by attL1 and attL2 sites. To create p8GWG, a linker (forward primer: GCCGGCGCAAACACCCGGGA, reverse primer: CCCGGCGTTTITGCAGCCA, NotI and XmnI sites undelimited) was synthesized and directly inserted into pCR8/GW/TOPO. The resulting plasmid, p8GWG, was sequenced to confirm that the cloning sites were in frame between attL site and linker (attL1/NotI/AAAATA/XmnI/attL2). TOR and its truncated fragments were cloned into pCR2.1-TOPO (Invitrogen, cat no. K4500-01), sequenced, then removed by NotI and XmnI digestion, and then reinserted into the p8GWG NotI/XmnI cassette to generate Gateway entry vector constructs.

**Generation of Overexpression Constructs**

The Gateway system (Invitrogen) was employed for creating various expression constructs. Various plant, *Escherichia coli*, and yeast (*Saccharomyces cerevisiae*) expression constructs were made by transferring the full-length and different deletion derivatives of TOR from p8GWG into the appropriate pEarleyGate vectors through LR recombination reactions in E. coli (Earley et al., 2006). The resulting plasmids were used to transform wild-type Arabidopsis plants (Col), TOR/tor-3, TOR/tor-10 plants, and yeast.

**Generation of PrRNA:5' -ETS-GFP Constructs**

A 813-bp GFP cDNA (Invitrogen) was PCR amplified using forward primer GFF incorporating 5' Asid and NotI sites (Supplemental Table S4) and reverse primer GFP incorporating a 3' AscI site (Supplemental Table S4) and inserted into pCR8/GW/TOPO using the TA cloning kit (Invitrogen, cat. K2500-20). Sequencing was performed to verify that the coding sequence was in frame in the correct relative orientation between attL1 and attL2 sites. The 45S rRNA promoter (2,885 bp), and the 45S rRNA promoter plus 5'-ETS sequences (4,162 bp), were amplified using forward primer PrRNAF incorporating a 5' Asid site and reverse primes PrRNAR1 and PrRNAR2, respectively, incorporating 3' NotI sites (Supplemental Table S4). The PCR products were cloned into the TA cloning vector pCR2.1-TOPO (Invitrogen, cat. K2000-01) for sequence verification. After digestion with Asid and NotI, the PCR products were subcloned into the Asid/NotI restriction sites upstream of the GFP coding region in the vector p8GWG to generate clones PrRNA-GFP and PrRNA-ETS-GFP. These recombinant constructs were transferred into pEarleyGate303 (Earley et al., 2006) through LR recombination reactions (as described above).

**Isolation of DNA, Purification of Total RNA, and Cloning of TOR cDNA**

Genomic DNA and total RNA were isolated from Arabidopsis (ecotype Col) samples using DNeasy plant mini kit (QIAGEN, cat. no. 69104) and RNeasy plant mini kit (QIAGEN, cat. no. 74904) following the manufacturer’s instructions. A SMART RACE cDNA amplification kit (Clontech, cat. no. 639414) was used for cDNA amplification following the manufacturer’s instructions. The full-length cDNA of the wild-type TOR (7.4 kb) and various truncated fragments were amplified by RT-PCR using the Advantage 2 polymerase mix kit (Clontech, cat. no. 639201) following the manufacturer’s instructions. RT-PCR primer pairs for amplification of the full-length TOR gene from Arabidopsis were designed based on the cDNA sequence (http://www.arabidopsis.org) of TOR. These primers were designed to introduce a NotI site at the 5' end of forward primers and an XmnI site at the 3' end of reverse primers. Three overlapping fragments of the TOR gene were amplified and fused together by using restriction enzymes followed by ligation (NotI, BspEL, BpiI, and XmnI) to generate the full-length clone (7.4 kb). The PCR products, with the 5' NotI site and 3' XmnI site, were cloned into the TA cloning vector pCR2.1-TOPO (Invitrogen, cat. K450001). The recombinant clones were verified by DNA sequencing. Recombinant plasmids were digested with NotI and XmnI and cloned into p8GWG (see below) to generate the gateway system-based Entry vector (Earley et al., 2006).

**Isolation and Characterization of T-DNA Insertion Lines**

We obtained the following SALK lines from the Arabidopsis Biological Resource Center: tor-3 (SALK_036379), tor-4 (SALK_007054), tor-5 (SALK_147473), tor-6 (SALK_017177), tor-7 (SALK_028697), tor-8 (SALK_016286), tor-9 (SALK_013925), tor-10 (SALK_138622), tor-11 (SALK_043130), tor-12 (SALK_056253), tor-13 (SALK_108347), and tor-14 (SALK_056209). The knockout lines were identified and confirmed by PCR using primers designed from the T-DNA primer design Web site: http://signal.salk.edu/tdnaprimer.2.html. These primers are listed in Supplemental Table S2.
Generation of PTOR:TOR/Deletion Derivatives and Constructs for Complementation Assays

Based on the pCR8/GW/TOPO plasmid (Invitrogen, cat. K2500-20), a linker (GCCATGGCCAGCCGACGCCCTAAAACCCCGGT; NolI site at the 5' end, and Xmal site at the 3' end underlined) was synthesized and directly inserted into pCR8/GW/TOPO and sequenced to confirm the in-frame attL sequence with the linker (attL/AaisI/AAAAAAA/NolI/AAAAAAA/Xmal/attL2). TOR and its deletion derivatives were digested with NolI and Xmal and cloned into the NolI/Xmal sites. The 3.4-kb TOR promoter from Arabidopsis genomic DNA was amplified with oligo primers to introduce AaisI at the 5' end of forward primers and a NolI at the 3' end of reverse primers (see Supplemental Table S4), and the respective PCR products were examined under a ZEISS SteRED Lumar V12 fluorescence microscope. The tissues of onion samples was performed as described in McLean et al., (1990). The tissues were harvested in 16-h light/8-h dark at 24°C. Onions scales were incubated for 24 to 48 h in the growth chamber. Real-time PCR was performed as described by Mouritzen et al. (Mouritzen et al., 2009) using the TaKaRa long-range PCR system and Invitrogen mutagenesis kit. The cDNAs of P45SrRNA:GFP and P45SrRNA-5.8S:rRNAs were used as internal controls and to assess the efficiency of cDNA synthesis.

ChIP Assays

The ChIP assays were performed as described in Tsang et al. (2003) and Xie et al. (2004) with minor modifications (see below). Primers used in the ChIP assays are listed in Supplemental Table S11. A total of 1.5 g of 10 DAG Arabidopsis whole-plant tissues were harvested, ground in liquid nitrogen, then used using 37 mL 1% formaldehyde solution for 30 min to cross-link proteins to DNA, followed by the addition of 2.5 mL 2M Gly to stop the cross-linking reaction. The cross-linked chromatin was fragmented by sonication. The resulting input sample is immunoprecipitated with GFP antibody (Invitrogen, 33-2600) that specifically recognizes the fusion proteins GFP-TOR or TOR-GFP, to enrich for DNA sequences that associate with the TOR protein in vivo. Chromatin preparation, pre-clearing, IP, collection, washes and elution of immune complexes, reverse cross-linking, and DNA cleanup were performed as described previously (http://www.epigenome-noe.net/researchtools/protocol.php?protid=13). The quantity of the R7 PCR fragment was arbitrarily set at 100%. The qPCR machine used this value as control to quantify relative binding by TOR in other regions. The ChIP experiments were performed with three biological replicates. Gels were stained using GelRed dye solution (BioImmun, cat. 41003).

GFP Transient Expression and Particle Bombardment in Plant Cells

Particle bombardment was performed as described in Giglione et al. (2000), with minor modifications (see below). The model Biolistic delivery system from Bio-Rad (PDS-1000/He) was used to deliver micron gold (Bio-RAD: cat no. 1652263)-coated pure plasmid DNA (purified with Qiagen mini-prep kits) spotted on microcarriers (Bio-RAD: cat no. 1652333) to transform onion (Allium cepa) epidermal cells incubated on Murashige and Skoog media. Both stopping screens (cat no. 1652336) and rupture disks 1,100 psi (cat no. 1652329) were from Bio-RAD. Bombardment parameters were as follows: vacuum, 25 inches Hg; distance to target, 6 cm; helium pressure, 1,300 p.s.i. Onion scales were incubated for 24 to 48 h in the growth chamber 16-h light/8-h dark at 24°C. Epidermal tissues were removed and washed on glass slides for microscopy. The 4',6-diamino-phenylindole staining of the onion samples was performed as described in McLean et al., (1990). The tissues were examined using a ZEISS SteRED Lumar V12 fluorescence microscope.

Quantitative Real-Time PCR

Real-time PCR was performed as described by Mouritzen et al. (Mouritzen et al., 2005) with minor modifications. Total RNAs were isolated from Arabidopsis (ecotype Col) samples using RNeasy pPlant mMini Kit (QIAGEN: cCat. n° 7904) following manufacturer’s instructions. The QuantiTect RTR Reverse Transcription Kit (QIAGEN; cCat. n° 205331) was used for cDNA amplification following the manufacturer’s instructions. Dyes were purchased from Applied Biosystems (PowerSYBR qGreen PCR master mix; pPart n° 436759). AACTIN2 was used as internal control. GenScript rReal-time PCR (TaqMan) Primer dDesign (https://www.genscript.com/ssl-bin/app/primer) was used for primer design. The sStep oOne rReal-time PCR system from Applied Biosystems was used for real-time PCR. Comparative CT eEperiments of sStep oOne v1.0 software was used for data analysis. Six specific primer pairs of the 5S, 18S, and 25S rRNAs for quantitative RT-PCR were used (Supplemental Table S8). We have used 45S rRNA 3 (specific primers: 5RSSTR: 5'-CTGAAATCTAGCCGCAAAAGGC-3'; 18STRTR: 5'-CATGATCTTCGAGGCAGTTCA-3'; and 5.8RTR: 5'-TTCGTGACACCGACGGACGACTG-3') to synthesize the first strand of cDNA of rRNAs. We used rRNA-specific primers plus oligo dT derived first-strand cDNA as the template for quantitative RT-PCR analysis of rRNAs and mRNAs. Among 5S, 18S, and 25S rRNAs, they share similar expression pattern in mutant and overexpression lines. We therefore used the average values of 18S, 5.8S, and 25S rRNA to reflect 45S rRNA expression in cells. To quantify the GFP expression level of P45SrRNA-GFP and P45SrRNA-5.8S:rRNA-GFP in various transgenic Arabidopsis, we used GFP sequence-based GFP-F primer (Supplemental Table S4) plus oligo dT to synthesize the first strand of cDNA as the template to perform real-time PCR. The actin 2 (AT3g18700) and β-6-tubulin (AT5g12250) genes were used as internal controls and to assess the efficiency of cDNA synthesis.

Yeast Two-Hybrid Assays

Based on the pCR8/GW/TOPO (Invitrogen, cat. K2500-20) plasmid, a linker (GCCATGGCCAGCCGACGCCCTAAAACCCCGGT; NolI site at the 5' end, and Xmal site at the 3' end underlined) was synthesized and directly inserted into pCR8/GW/TOPO to generate P8GWY. The cDNAs of TOR and its deletion derivatives were generated by RT-PCR as described above, cloned them into P8GWY NolI/Xmal sites, then transferred them into pGADT7 (Ampicillin resistance) and pGBK7 (Kanamycin resistance) vectors by using the NolI and Xmal restriction site in pGADT7 and pGBK7, followed by transformation of the recommended plasmids into yeast host strains AH109 for interaction assays. All the yeast two-hybrid procedures were performed according to the manufacturer’s instructions (Clontech; cat. no. 630303).

Microscopy

For phenotyping mutant embryos, the ovules were cleared in chloral hydrate solution (8:1:2, chloral hydrate:glycerol:water; w/v/v). The embryos were viewed under Leica DMR compound microscope with differential interference contrast (Nomarski) optics. Images were captured using the Optronics Microfite camera and were edited in Adobe Photoshop CS.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. FATC domain shows high conservation among nonplant species while high diversity was found between plant and yeast/animal species.

Supplemental Figure S2. Yeast two-hybrid analysis of interactions of Arabidopsis TOR domains.

Supplemental Figure S3. Overexpression of kinase domain (P35S:TOR2031-2350) phenocopies the short hypocotyl phenotype of full-length TOR overexpression lines (P35S:TOR) in low-expression transgenic lines.

Supplemental Figure S4. Root phenotypes of transgenic Arabidopsis TOR kinase domain overexpression lines.

Supplemental Figure S5. Agarose gel analysis of rRNAs in different TOR overexpression lines.
Supplemental Figure S7. GFP fusion at the carboxyl or amino end of AITOR did not abolish AITOR function in Arabidopsis.

Supplemental Figure S8. ChIP assay of genomic loci representing five Arabidopsis chromosomes.

Supplemental Figure S9. Conservation of putative NLS in kinase domain among yeast, animals, human, and different plant species.

Supplemental Figure S10. Multiple alignment analysis of TOR protein DNA-binding domain region present in HEAT repeat.

Supplemental Table S1. PCR primers used for confirmation of different T-DNA insertion lines.

Supplemental Table S2. RT-PCR primers used for detecting transcript sequences in different tor mutant lines.

Supplemental Table S3. Phenotypic analysis of FATC domain T-DNA mutant lines.

Supplemental Table S4. PCR primers used for cloning and mutagenesis.

Supplemental Table S5. Analysis of tor-3/tor-3 and tor-10/tor-10 embryo phenotypes in comparison with embryos in wild type.

Supplemental Table S6. Functional complementation assays in TOR/tor-3 background.

Supplemental Table S7. Functional complementation assays in TOR/tor-10 background.

Supplemental Table S8. Primers used for real-time PCR.

Supplemental Table S9. Expression levels of TOR in the P535:TOR (full-length protein-coding cDNA) transgenic lines.

Supplemental Table S10. The endogenous TOR and transgene expression levels in various overexpression lines.

Supplemental Table S11. PCR primers used for ChIP assay.

Supplemental Materials and Methods S1. Analysis of T-DNA lines.

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