Rapamycin and Glucose-Target of Rapamycin (TOR) Protein Signaling in Plants*[S]

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Background: Plants possess conserved TOR PK but appear to display rapamycin resistance.
Results: Rapamycin effectively inactivates *Arabidopsis* TOR PK and retards glucose-mediated growth.
Conclusion: Integrative analyses with TOR-S6K phosphorylation, rapamycin, and estradiol-inducible *tor* and *fkp* mutants unravel the central roles of glucose-TOR signaling in diverse plant cells and organs.
Significance: Rapamycin and estradiol-inducible *tor* mutants facilitate chemical genetic dissection of plant TOR signaling networks.

Target of rapamycin (TOR) kinase is an evolutionarily conserved master regulator that integrates energy, nutrients, growth factors, and stress signals to promote survival and growth in all eukaryotes. The reported land plant resistance to rapamycin and the embryo lethality of the *Arabidopsis tor* mutants have hindered functional dissection of TOR signaling in plants. We developed sensitive cellular and seedling assays to monitor endogenous *Arabidopsis* TOR activity based on its conserved S6 kinase (S6K) phosphorylation. Surprisingly, rapamycin effectively inhibits *Arabidopsis* TOR-S6K1 signaling and retards glucose-mediated root and leaf growth, mimicking estradiol-inducible *tor* mutants. Rapamycin inhibition is relieved in transgenic plants deficient in *Arabidopsis* FK506-binding protein 12 (FKP12), whereas FKP12 overexpression dramatically enhances rapamycin sensitivity. The role of *Arabidopsis* FKP12 is highly specific as overexpression of seven closely related FKP proteins fails to increase rapamycin sensitivity. Rapamycin exerts TOR inhibition by inducing direct interaction between the TOR-FRB (FKP-rapamycin binding) domain and FKP12 in plant cells. We suggest that variable endogenous FKP12 protein levels may underlie the molecular explanation for longstanding enigmatic observations on inconsistent rapamycin resistance in plants and in various mammalian cell lines or diverse animal cell types. Integrative analyses with rapamycin and conditional *tor* and *fkp12* mutants also reveal a central role of glucose-TOR signaling in root hair formation. Our studies demonstrate the power of chemical genetic approaches in the discovery of previously unknown and pivotal functions of glucose-TOR signaling in governing the growth of cotyledons, true leaves, petioles, and primary and secondary roots and root hairs.

The evolutionarily conserved protein kinase (PK), target of rapamycin (TOR),[3] is encoded by orthologous genes from yeast to plants and humans and likely plays central roles in integrating nutrients and energy availability with other environmental signals to coordinate survival, growth and development (1–4). In *Saccharomyces cerevisiae*, TOR is activated under favorable conditions to promote cell growth by maintaining a robust rate of ribosome biogenesis, translation initiation, and nutrient import (4). In mammals, TOR operates as a hub of the signal transduction network that controls metabolism, growth, and division by diverse signals including nutrient, energy, stress, hormones, and mitogens (2–7). Modulation of TOR expression levels suggests a correlation with the cell and organ size, seed yield, and stress resistance in *Arabidopsis* (8, 9). Studies of the *Arabidopsis* TOR interaction partner RAPTOR and a downstream effector TAP46 also suggest their vital roles in growth and development, stress adaptation, autophagy, and nitrogen mobilization (10–12). Despite the importance of TOR functions in eukaryotes, little is known about the plant TOR signaling network and its upstream regulators due to the lack of molecular and biochemical assays for endogenous TOR PK activity and the embryo lethality of null *Arabidopsis tor* mutants (1).

Rapamycin, a natural antibiotic produced by the soil bacterium *Streptomyces hyproscopicus*, can specifically inactivate TOR PK in yeast and mammals. The inhibitory effect of rapamycin is mediated by the formation of a specific protein complex, in which rapamycin forms noncovalent bonds between FKP12 and the FRB domain of TOR proteins (4). Recent data suggest that FKP12-rapamycin inhibition of TOR PK function is achieved by causing dissociation of RAPTOR from TOR (13) or by blocking a specific subset of TOR complex1 (TORC1) substrates, but not TORC2 functions (14). In contrast to yeasts and mammals, studies over the past decade with land plants generated the prevailing view that plants are insensitive to rapa-

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[3] The abbreviations used are: TOR, target of rapamycin; CLUC, C-terminal firefly luciferase; FKP12, FK506-binding protein 12; FRB domain, FKP-rapamycin binding domain; mTOR, mammalian TOR; NLUC, N-terminal firefly luciferase; RAPTOR, regulatory associated protein of TOR; S6K, S6 kinase; TORC1, TOR complex 1; TORC2, TOR complex 2.

mycin because this drug does not inhibit *Arabidopsis* growth at concentrations that are effective in yeast and mammalian cells (1, 15). Yeast two-hybrid studies suggested that *Arabidopsis* FKP12 is unable to form a complex with rapamycin and TOR, whereas the *Arabidopsis* TOR-FRB can still bind to yeast or human FKP12 in the presence of rapamycin (15–17). It was proposed that *Arabidopsis* FKP12 had evolved structural changes to prevent the formation of the inhibitory complex with TOR and rapamycin (1, 15).

A main obstacle in elucidating the plant TOR signaling network is the lack of convenient and reliable molecular and biochemical assays to monitor plant TOR PK activities. The embryo lethality of null *tor* mutants (1, 15) further limits the molecular dissection of TOR functions in higher plants in the past decade. A key substrate and mediator of TOR PK is S6K, which is evolutionarily conserved in plants and humans (16). We report here that site-specific phosphorylation of *Arabidopsis* S6Ks can serve as a reliable and sensitive molecular and biochemical marker to monitor endogenous TOR PK activity in *Arabidopsis*. Unexpectedly, using this sensitive assay we found that rapamycin can effectively inhibit *Arabidopsis* TOR PK activation by glucose. Rigorous genetic analyses using independent transgenic *Arabidopsis* plants and cellular assays with reduced or increased *FKP12* expression provide compelling evidence for the specific role of endogenous FKP12 protein in mediating rapamycin inactivation of TOR PK activity. The establishment of the S6K1 Thr-449 phosphorylation-based TOR PK activity *in vivo* assay, the conditional *tor* mutants, and the discovery of the effectiveness of rapamycin in *Arabidopsis* unravel the central roles of glucose-TOR signaling in diverse plant cells and organs and open new possibilities to molecular dissect the TOR signaling networks in plants.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions—Col-0 wild-type (WT) *Arabidopsis* plants were used in this study, and all transgenic plants generated are in the Col-0 background. Plants were grown at 23 °C/20 °C, 65% humidity, and 75 μmol m^{-2}s^{-1} light intensity under a 12-h light/12-h dark photoperiod condition. Plants were grown in soil for 4 weeks for mesophyll protoplast isolation. For phenotypic analysis of rapamycin effects on seedling growth, seeds were germinated and grown in 6-well plates containing 1 ml of liquid medium (0.5 × MS and 0.5% sucrose, adjusted to pH 5.7 with KOH) with 1–10 μM rapamycin. In glucose experiments for seedling and root hair growth, 0.5% sucrose was replaced without or with 30 mM glucose. For long term rapamycin treatments, the medium was changed with fresh rapamycin every 2 days to ensure the rapamycin effect.

Plasmid Constructs—For *S6K1* (*At3g08730*) and *S6K2* (*At3g08720*) constructs: cDNAs were amplified from leaf RNA, fused to the FLAG tag, and cloned between a ^{35}S-driven promoter and *NOS* terminator (18, 19). The S6K1 mutant (T449A) and S6K2 mutant (T455A) were generated by PCR-based site-specific mutagenesis (20). For *FKP12* (*At5g64350*), *FKP15.1* (*At3g25220*), *FKP15.2* (*At5g48580*), *FKP20.1* (*At3g55520*), *FKP42* (*At3g21640*), *FKP62* (*At3g25230*), *FKP65* (*At5g48570*), and *FKP72* (*At3g54010*) constructs, cDNAs were amplified from leaf RNA, fused to the HA tag, and cloned between a

^{35}S-driven promoter and *NOS* terminator. All primers used are listed in supplemental Table 1.

Protoplast Transient Expression Assay—Protoplast transient expression assays were carried out as described previously (21). Data were generated from at least three independent experiments with consistent results. Protoplasts (1–4 × 10^4) were incubated for 10 h in 1 ml of mannitol (0.5 M) and KCl (20 mM) buffer (4 mM MES, pH 5.7) in 6-well tissue culture plates (1-mm depth) and treated with rapamycin for 30 min in the indicated concentrations. Protoplasts were harvested by centrifugation and suspended in SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, and 50 mM DTT) for SDS-PAGE and protein blot analysis.

Antibodies and Protein Blot Analysis—Phospho-p70 S6 kinase (Thr(P)-389) polyclonal antibody (Cell Signaling, catalog no. 9205) was used to detect TOR PK phosphorylation of Thr(P)-449 in S6K1 and Thr(P)-455 in S6K2. HA- or FLAG-tagged proteins were detected by anti-HA (Roche Applied Science) or anti-FLAG (Sigma) monoclonal antibodies using standard techniques. Polyclonal *Arabidopsis* TOR antibody was generated using a synthetic peptide, CTLN-RVIADLCSRGNPKEGAP. The antibody was affinity-purified using a SulfoLink matrix (Pierce).

Split Luciferase Assay—Protoplasts were co-transfected with NLUC-FRB and CLUC-FKP12s for 8 h, then incubated for 30 min with luciferin (0.25 μg/μl) and finally incubated for 5 min with rapamycin. Samples were transferred to 96-well plates, and the output was read using a modulus microplate reader (Turner Biosystems).

RT-PCR Analysis—Total RNA was isolated with TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from 1 μg of total RNA with MuLV reverse transcriptase (Promega). All quantitative RT-PCR analyses were performed by CFX96 real time PCR detection system with iQ SYBR Green supermix (Bio-Rad). *TUB4* (*AT5G44340*) was used as a control gene.

Transgenic Plants—To generate transgenic *FKP12* and *S6K1* overexpression lines, the coding region was cloned into an expression vector derived from the pCB302 minibinary vector (19). To generate the *fkp12* RNAi lines, the last 332 bp of the coding region were cloned in two opposite orientations in the pHANNIBAL plasmid (22) under the control of the ^{35}S-driven promoter. To generate the estradiol-inducible *tor* RNAi lines, the 480-bp coding region (560–1040) was cloned in two opposite orientations in the pHANNIBAL plasmid, and the whole silencing cassette was shifted to estradiol-inducible vector pLB12 (23). Estradiol (10 μM) was used to induce gene silencing effects.

Phylogenetic Tree—Protein sequences were aligned with ClustalW, using the SDSC Workbench, to generate the phylogenetic tree.

RESULTS

Sensitive Cell-based Assay to Monitor Endogenous Arabidopsis TOR PK Activity—To investigate the molecular and biochemical mechanisms underlying the regulations and functions of *Arabidopsis* TOR, we first established a sensitive cell-based assay to monitor TOR PK activity. *Arabidopsis* TOR shares high amino acid sequence similarity with human TOR, espe-
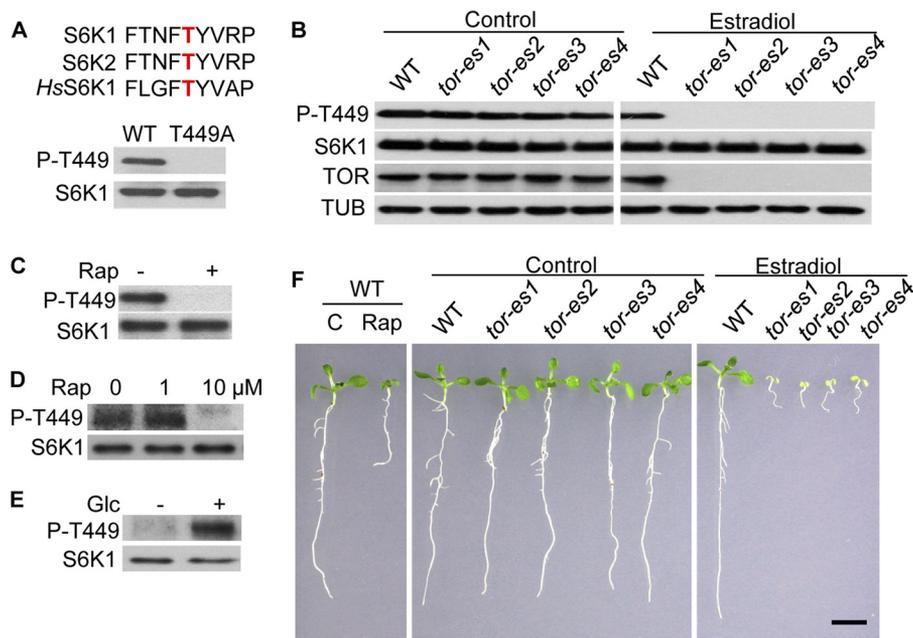
FIGURE 1. **Monitor TOR activity based on Thr-449 phosphorylation of S6K1.** *A*, conserved TOR phosphorylation of S6K proteins: conserved TOR phosphorylation motif in human and *Arabidopsis* S6Ks (*upper*) and Thr-449 phosphorylation specificity of S6K1 (*lower*). FLAG-tagged WT or mutant S6K1 (T449A) was transiently expressed in protoplasts. Total proteins were analyzed by protein blot analysis using anti-Thr(P)-T389 (*P-T455*) or anti-FLAG (*S6K1*) antibody. *B*, Thr-449 phosphorylation abolished in *tor* mutants. WT or estradiol-inducible *tor* mutants (*tor-es*) protoplasts expressing FLAG-tagged S6K1 were treated without or with estradiol (10 μM). Total proteins were analyzed by protein blotting probed with anti-TOR (*TOR*), anti-Thr(P)-389 (*P-T449*), or anti-FLAG (*S6K1*) antibody. Tubulin (*TUB*) was used as a loading control. *C*, inhibition of Thr-449 phosphorylation by rapamycin in leaf cells. WT protoplasts expressing FLAG-tagged S6K1 were treated with rapamycin (*Rap*, 1 μM) for 30 min. *D*, inhibition of Thr-449 phosphorylation by rapamycin in seedlings. Transgenic S6K1-overexpressing seedlings were treated with rapamycin (1 or 10 μM) for 30 min. *E*, activation of Thr-449 phosphorylation by glucose. Transgenic S6K1-overexpressing seedlings were grown with or without glucose (*Glc*, 30 mM) for 4 days. *F*, rapamycin and *tor* mutants retarding seedling growth. Rapamycin (10 μM) or estradiol (10 μM) was added at the time of germination for 9 days. *Scale bar*, 5 mm.

cially for the PK domain (supplemental Fig. S1), suggesting that they may have similar PK properties and share protein substrates. The direct TOR substrate and major effector S6K are highly conserved in *Arabidopsis* and humans. Intriguingly, the TOR phosphorylation motif FLGFTYVAP in human S6K1 appears to be conserved in the *Arabidopsis* orthologs, S6K1 and S6K2 (Fig. 1*A* and supplemental Fig. S2). Using a specific antibody against the Thr(P)-389 peptide in human S6K1 phosphorylated by TOR (4), we developed a cell assay to monitor endogenous *Arabidopsis* TOR PK activity based on the conserved S6K phosphorylation, Thr-449 and Thr-455 in *Arabidopsis* S6K1 and S6K2, respectively, as molecular and biochemical markers (Fig. 1*A*). With this sensitive assay, the endogenous PK activity for S6K phosphorylation is clearly detected in nondividing and fully differentiated leaf cells expressing the WT S6K1 or S6K2, but not the mutant S6K1(T449A) or S6K2(T455A) (Fig. 1*A* and supplemental Fig. S3*A*). To provide direct genetic and biochemical evidence for the endogenous TOR PK activity, we generated estradiol-inducible *tor* transgenic *Arabidopsis* plants (*tor-es*) to circumvent the embryo lethality of null *tor* mutants and produced a peptide antibody that specifically recognizes *Arabidopsis* TOR. Depletion of TOR expression by estradiol treatment eliminated Thr-449 and Thr-455 phosphorylation of S6K1 and S6K2, respectively (Fig. 1*B* and supplemental Fig. S3*B*). These studies provide the first evidence that S6K1 and S6K2 are true *Arabidopsis* TOR substrates and that Thr-449 phosphorylation of S6K1 or Thr-455 phosphorylation of S6K2 can be used to monitor the TOR PK activity in plants. The data reveal active TOR signaling in nondividing and fully differenti-

ated and expanded leaf cells, which is not previously recognized.

Rapamycin Inhibits TOR-S6K Signaling in Arabidopsis—Rapamycin, as a specific inhibitor of TOR PK, has been an instrumental tool in dissecting the complex TOR signaling network in yeasts and animals (2, 3). Research during the past decade generated the prevailing view that land plants are resistant to rapamycin due to evolutionary changes in the *Arabidopsis FKP12* gene (1, 15). We reexamined this hypothesis using the new sensitive cell assay. Surprisingly, we discovered that rapamycin effectively inactivates endogenous TOR PK activity revealed by Thr-449 and Thr-455 phosphorylation of S6K1 and S6K2, respectively (Fig. 1*C* and supplemental Fig. S3*C*), although at a higher concentration than was shown in mammalian and yeast cells (100–1000 nM *versus* 10–50 nM). Although S6K phosphorylation was clearly detected using the antibody against the Thr(P)-389 peptide in human S6K1 when overexpressed in *Arabidopsis* leaf cells, we failed to detect a reliable signal for the endogenous S6K1. It is possible that the endogenous S6K protein level was too low for efficient analysis in intact plants, which explained the difficulty in generating any *Arabidopsis* TOR PK assay *in vivo* in the past decade. Also, the antibody probably has low affinity for the *Arabidopsis* S6K proteins as it was generated against the Thr(P)-389 peptide motif in human S6K1, which has three amino acid variations in *Arabidopsis* S6Ks (Fig. 1*A*). As an alternative approach to detect endogenous TOR PK activity in planta, we generated transgenic S6K1-overexpressing plants and demonstrated that rapamycin also effectively inactivates Thr-449 phosphorylation of

S6K1 in *Arabidopsis* seedlings (Fig. 1*D*). Because rapamycin completely blocked the phosphorylation of overexpressed S6K1 in cells and seedlings, it is suggested that the endogenous S6K1 is similarly inhibited in planta.

Glucose-TOR Signaling in Arabidopsis Seedling Growth—Using S6K1 Thr-449 phosphorylation-based TOR PK activity assay, we screened for potential upstream nutrient signals and discovered that glucose strongly activates TOR PK activity in *Arabidopsis* seedlings (Fig. 1*E*). Because our previous studies have indicated that the growth of *Arabidopsis* seedlings strictly relies on glucose after germination and photomorphogenesis in the liquid culture medium (24), we showed that *Arabidopsis* seedlings are arrested in standard liquid culture medium without any sugar, whereas 30 mM glucose promotes vigorous growth (supplemental Fig. S4). We examined the role of glucose-TOR signaling in the growth of diverse organs in the presence of rapamycin. As shown in Fig. 1*F*, rapamycin strongly retards many key aspects of organ growth in seedlings after germination, including cotyledon expansion, true leaf development, petiole elongation, as well as primary and lateral root growth. Importantly, these phenotypes closely resemble the inducible *tor* mutant plant grown in the presence of glucose (Fig. 1*F*), supporting pivotal roles of glucose-TOR signaling in the growth of diverse plant organs. Interestingly, TOR activity may not be required for germination and photomorphogenesis because WT seedlings without or with rapamycin and *tor-es* seedlings look alike in the absence of sugar (Fig. 1*F* and supplemental Fig. S4). Contrary to previous studies and conclusions, these data strongly indicate that rapamycin can effectively inhibit *Arabidopsis* growth by suppressing TOR PK activity, which is not limited to embryo, endosperm, and meristems (15).

Inhibitory Effect of Rapamycin Requires Endogenous FKP12—It has been shown that rapamycin forms an inhibitory complex involving FKP12 and the FRB domain of TOR (25). To determine whether the inhibitory effect of rapamycin observed in *Arabidopsis* seedlings and leaf cells is dependent on FKP12, multiple *fkp12* transgenic lines were generated using RNA interference (RNAi) (Fig. 2*B*). In *fkp12* leaf cells, the inhibitory effect of rapamycin on Thr-449 phosphorylation of S6K1 is severely compromised compared with that in WT leaf cells (Fig. 2*A*). The inhibitory effect of rapamycin in whole seedling growth is also much reduced in *fkp12* plants (Fig. 2*C* and supplemental Fig. S5*A*). These studies strongly support the specific role of endogenous FKP12 protein in mediating rapamycin inactivation of TOR PK activity.

Overexpression of Arabidopsis or Human FKP12 Significantly Enhances Rapamycin Efficacy—*Arabidopsis* was thought to be resistant to rapamycin for a lack of the FKP12 binding to TOR-FRB in a rapamycin-dependent manner observed in yeast and human FKP12 (1). To facilitate the study of TOR functions using the rapamycin-based chemical genetics in *Arabidopsis*, we intended to genetically engineer the rapamycin sensitivity in *Arabidopsis* leaf cells by ectopic expression of human FKP12 (*Hs*FKP12). *Arabidopsis* FKP12 was also overexpressed in leaf cells to serve originally as a negative control. Unexpectedly, we found that overexpression of either *Arabidopsis* FKP12 or *Hs*FKP12 dramatically enhances cell sensitivity to rapamycin
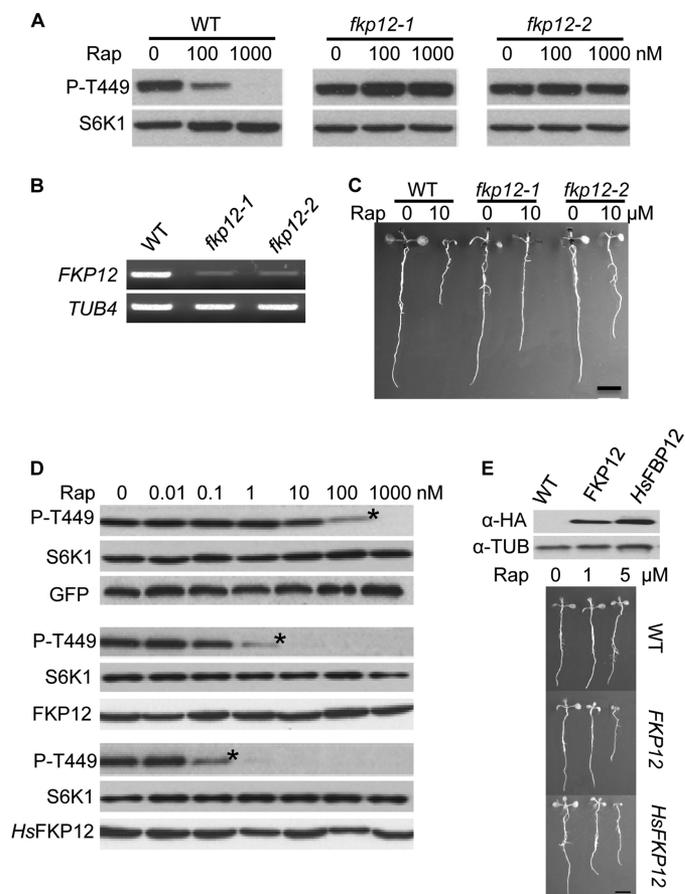
FIGURE 2. **A crucial role of FKP12 for rapamycin inactivation of TOR.** *A*, FKP12 requirement for inhibition of Thr-449 phosphorylation by rapamycin (*Rap*). WT or *fkp12* mutant protoplasts expressing FLAG-tagged S6K1 were treated with rapamycin. Total proteins were analyzed by protein blotting probed with anti-Thr(P)-389 (*P-T449*) or anti-FLAG (S6K1) antibody. *B*, RT-PCR analysis of *FKP12* expression. *C*, FKP12 requirement for inhibition of seedling growth by rapamycin. Rapamycin was added at the time of germination for 9 days. *D*, overexpression of *Arabidopsis* or human FKP12 enhancing the rapamycin inhibition effect on Thr-449 phosphorylation in leaf cells. FLAG-tagged S6K1 was co-expressed with HA-tagged FKP12s or GFP in protoplasts and treated with rapamycin for 30 min. *E*, overexpression of *Arabidopsis* or human FKP12 enhancing rapamycin inhibition effect on seedling growth. Rapamycin was added at the time of germination for 9 days. *Scale bar*, 5 mm.

100–1000-fold (Fig. 2*D*). Transgenic *Arabidopsis* seedlings with elevated *Arabidopsis* or human FKP12 expression also exhibit enhanced growth retardation at lower rapamycin concentrations (Fig. 2*E* and supplemental Fig. S5*B*). These data suggest that, in contrast to previous studies, rapamycin inhibits *Arabidopsis* TOR PK activity and this inhibition can be further enhanced by overexpressing *Arabidopsis* or human FKP12 protein.

Action of Arabidopsis FKP12 Is Highly Specific—In addition to FKP12, it has been suggested that human FKP38 (*Hs*FKP38) is also involved in TOR regulation (26). We further investigated the function of seven closely related *Arabidopsis* FKP proteins (Fig. 3*A*) in mediating the inhibitory effect of rapamycin on the endogenous *Arabidopsis* TOR PK activity. Despite equal level of protein expression, none of these seven FKPs can increase rapamycin sensitivity or display function similar to that of *Hs*FKP38 to inhibit TOR PK activity directly (Fig. 3*B*). The findings demonstrate the specificity of *Arabidopsis* and human FKP12 in rapamycin-dependent TOR PK inhibition.
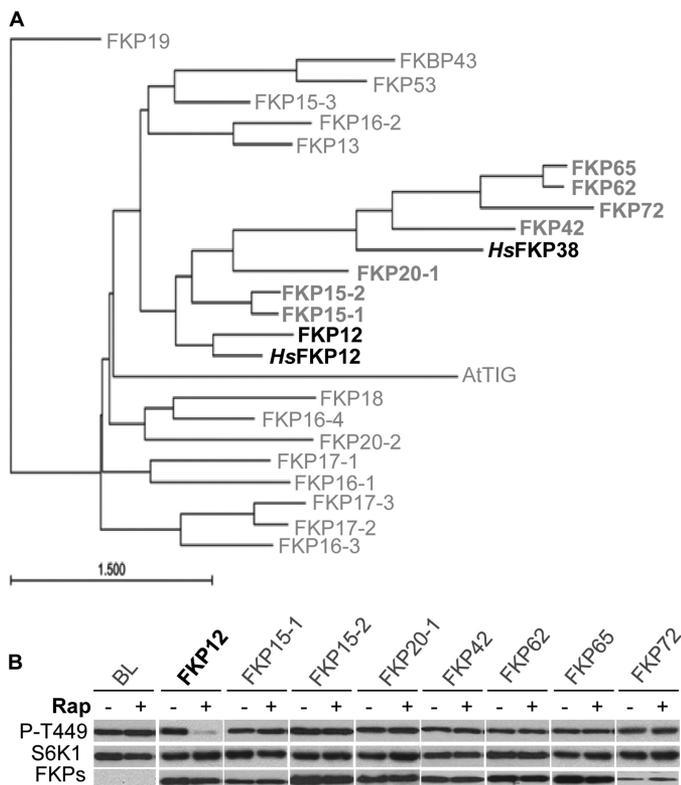
FIGURE 3. **Specificity of FKP12 in rapamycin-dependent TOR inhibition.** *A,* phylogenetic comparison of *Arabidopsis* FKPs with human FKP12 and FKP38. The nomenclature is based on Ref. 34. *B,* unique role of FKP12 in enhancing *Arabidopsis* leaf cell sensitivity to rapamycin. HA-tagged FKPs and FLAG-tagged S6K1 were co-expressed in protoplasts and treated with rapamycin (*Rap*, 1 nM) for 30 min. Total proteins were analyzed by protein blotting probed with anti-Thr(P)-389 (*P-T449*), anti-FLAG (S6K1), or anti-HA (FKPs) antibody.

Rapamycin Induces Interaction between Arabidopsis TOR-FRB Domain and FKP12s in Plant Cells—Comparison of FKP12 coding sequences and the FRB domains between human TOR and *Arabidopsis* TOR reveals high sequence conservation (supplemental Fig. S6). Because our comprehensive analyses have suggested that the inhibitory effect of rapamycin depends on FKP12, we next examined whether FKP12 associates with *Arabidopsis* TOR-FRB domain in a rapamycin-dependent manner by employing the split luciferase complementation assay for protein-protein interaction in intact and active *Arabidopsis* leaf cells (27). The N- and C-terminal fragments of firefly luciferase (NLUC and CLUC) were translationally fused to TOR-FRB and FKP12, respectively (Fig. 4*A*), and the constructs were co-expressed in leaf protoplasts. The emitted luminescence reflects the intensity of protein interaction. As shown in Fig. 4*B*, there is only minimal background luminescence signal in leaf cells co-transfected with NLUC-FRB and CLUC-FKP12 or empty control vectors in the absence of rapamycin. In contrast, rapamycin even at 1 nM (a low concentration level) can quickly and significantly trigger the luminescence intensity increase, which reaches the maximum at 1 μM, correlating with complete inhibition of TOR phosphorylation of S6K1 by rapamycin (Fig. 2*A*). Rapamycin does not affect the expression of NLUC-FRB and CLUC-FKP12 proteins (Fig. 4*C*). These results strongly indicate that rapamycin induces the direct interactions between the *Arabidopsis* TOR-FRB domain and both *Arabi-*
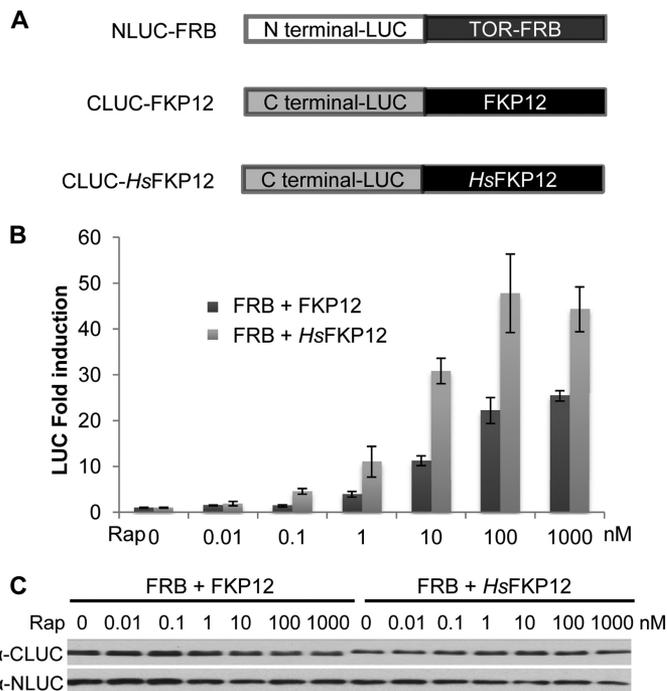
FIGURE 4. **Rapamycin-dependent interactions between *Arabidopsis* TOR-FRB domain and FKP12s.** *A,* NLUC-FRB and CLUC-FKP12 constructs. *B,* relative interaction intensity between TOR-FRB domain and FKP12s. NLUC-FRB and CLUC-FKP12 or CLUC-*Hs*FKP12 were co-expressed in protoplasts for 6 h and treated with rapamycin (*Rap*) for 5 min. Rapamycin induces the direct interactions between *Arabidopsis* TOR-FRB domain and both *Arabidopsis* and human FKP12 in a concentration-dependent manner. Results represent means ± S.D. (*error bars*). *n* = 3. *C,* protein expression level of NLUC-FRB and CLUC-FKP12s.

dopsis and human FKP12 in a concentration-dependent manner in plant cells.

Glucose-TOR Signaling Is Central to Root Hair Development—To explore other possible roles of glucose-TOR signaling further, we thoroughly examined *Arabidopsis* seedlings grown in the presence or absence of glucose and found profound differences in root hair patterns (Fig. 5*A*). Root hairs are long tubular-shaped extensions from single epidermal cells to aid plants in absorbing nutrients and water, interacting with microorganisms, and physically anchoring plants to the soil. Although it is established that root hair development is controlled by hormones and other signals, the effect of glucose is previously unknown (28). Importantly, rapamycin completely prevents root hair growth in WT seedlings in the presence of glucose, but the inhibitory effect of rapamycin is diminished in *fkp12*-deficient seedlings (Fig. 5*A*). Consistently, FKP12-overexpressing transgenic seedlings are hypersensitive to rapamycin (Fig. 5*B*), and the estradiol-inducible *tor-es* mutants abolish root hair growth mediated by glucose-TOR signaling (Fig. 5*C*). Because this glucose regulation is unaffected in the hexokinase 1 glucose sensor mutant *gin2* (supplemental Fig. S7), glucose-TOR signaling in controlling root hair development acts in the hexokinase 1-independent pathway (24, 29).

DISCUSSION

TOR PK is a central integrator of nutrient sensing and signaling in all eukaryotes (1–4). The TOR signaling network is involved in various growth-related processes by mostly
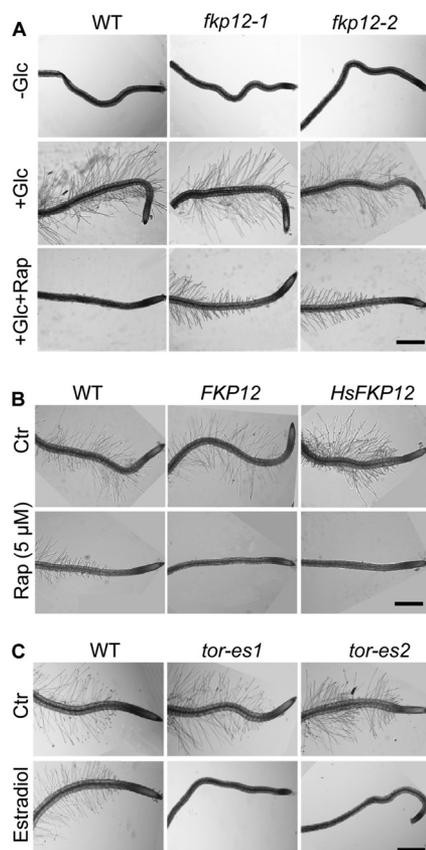
FIGURE 5. **A pivotal role of glucose-TOR signaling in root hair formation.** *A*, rapamycin inhibition of glucose-mediated root hair growth. Seedlings were germinated with or without glucose (30 mM) for 4 days. Rapamycin (*Rap*, 10 μM). *B*, enhancing the rapamycin inhibition effect on root hair formation by overexpressing *Arabidopsis* or human FKP12. Seedlings were germinated with 30 mM glucose for 4 days. *C*, retardation of root hair growth in *tor* mutants. Seedlings were germinated with 30 mM glucose for 4 days. *Scale bar*, 500 μm.

unknown mechanisms in plants (1). Although the *Arabidopsis TOR* transcript is detected in most tissues, it has been reported that the expression patterns of *Arabidopsis* TOR::GUS fusion protein are limited to proliferating cells in embryo, endosperm, and primary meristems (15). This raised the question for how nonproliferating cells regulate their growth-related processes through TOR signaling or whether TOR has any functions in fully differentiated cells in plants. The analyses with conditional *tor* mutant plants and an antibody to a specific *Arabidopsis* TOR peptide presented here provide compelling evidence that the TOR protein is expressed and active in fully differentiated leaves, and its PK activity is responsible for Thr-449 and Thr-455 phosphorylation of S6K1 and S6K2, respectively (Fig. 1 and supplemental Fig. S3). Thus, TOR may have distinct functions tailored and integrated in specific cellular contexts and environments in both dividing and nondividing cells. The use of liquid culture in protoplast transient expression and seedling assays facilitates the uptake and replenishment of rapamycin for effective and flexible TOR PK manipulation in future exploration of the plant TOR signaling network.

Although structural studies have demonstrated the formation of an inhibitory complex involving rapamycin, FKP12 and TOR-FRB, several studies using either the yeast two-hybrid method or direct binding assay had failed to show that *Arabi-*dopsis FKP12 can interact with TOR-FRB (15–17). The use of *in vivo* split luciferase complementation assay clearly illustrates that it is possible to detect physiologically relevant, direct, and quick interactions between the *Arabidopsis* TOR-FRB domain and *Arabidopsis* or human FKP12 induced by rapamycin in a concentration-dependent manner (Fig. 4). These results suggest that the interaction between FKP12 and TOR-FRB may be transient and weak or need posttranslational modification, making it difficult to be detected using *in vitro*, nonphysiological, or nonplant assays.

Previous studies have suggested successful "restoration" of the rapamycin susceptibility by ectopic expression of *Saccharomyces* FKP12 in *Arabidopsis* (17). Our results clearly suggest that overexpression of FKP12 actually enhances rather than restores the rapamycin sensitivity. Therefore, variable endogenous FKP12 protein levels may offer a molecular explanation underlying varied rapamycin resistance at low rapamycin concentrations (nanomolar) and provide a feasible strategy to improve rapamycin efficacy for clarifying TOR functions and advancing TOR research in plants, various mammalian cell lines, or diverse animal cell types and model systems (2, 3).

Glucose is the preferred energy source in all organisms from bacteria, yeasts, plants, to humans. In plants, glucose has emerged as the key regulators of many vital processes, including root, stem and shoot growth; reproduction; stress responses; and senescence (29, 30). How glucose modulates complex responses remains largely enigmatic. Integrative analyses with TOR-S6K phosphorylation, rapamycin, and conditional *tor* and *fkp* mutants uncover the central roles of glucose-TOR signaling in controlling the growth of diverse plant organs and root hairs. In addition to glucose, plant organ growth and root hair development are also controlled by many genetic, nutritional, hormonal, and environmental factors (28–31). Future studies will determine how the glucose-TOR signaling is coordinated and integrate with these multiple signals to control plant organ and root hair growth.

Taking advantage of chemical genetic tools and approaches, the established cellular and seedlings assays will facilitate the understanding of molecular and biochemical mechanisms of TOR signaling in plants, circumventing limitations inherent to lethal and conditional mutants. A new generation of mammalian TOR (mTOR) PK active site inhibitors (*e.g.* PP242 and Torin1) has recently been developed (32, 33). These inhibitors directly target the ATP site of mTOR PK and have shown to be effective for suppression of animal cell growth and proliferation. Unlike rapamycin, which is suggested to be specific for TORC1, PP242 and Torin1 inhibit a broader spectrum of mTOR functions through inactivation of both TORC1 and TORC2 (32, 33). It will be of great interest to combine and compare the effective range and specificity of these distinct inhibitors in future molecular dissections of the plant TOR PK signaling network.

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