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

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Background: Plants possess conserved TOR PK but appear to display rapamycin resistance. Rapamycin effectively inactivates Arabidopsis TOR PK and retards glucose-mediated growth. Intensive 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. Rapamycin and estradiol-inducible tor mutants facilitate chemical genetic dissection of plant TOR signaling networks.

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 hygroscopicus, 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 FKBP11 and the FRB domain of TOR proteins (4). Recent data suggest that FKBP12-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; FKBP12, FK506-binding protein 12; FRB domain, FKBP-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, unveiling 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 mol m⁻³ H₂O) 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 modus microplate reader (Turner Biotysystems).

**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 minivector binary 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 35S-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-
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FIGURE 1. Monitor TOR activity based on Thr-449 phosphorylation of S6K1. A, conserved TOR phosphorylation motif 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 min) 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.

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. 1C and supplemental Fig. 3C), 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. 1A). As an alternative approach to detect endogenous TOR PK activity in plants, we generated transgenic S6K1-overexpressing plants and demonstrated that rapamycin also effectively inactivates Thr-449 phosphorylation of...
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 FK12 (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 FK12. Arabidopsis FK12 was also overexpressed in leaf cells to serve originally as a negative control. Unexpectedly, we found that overexpression of either Arabidopsis FK12 or HsFK12 dramatically enhances cell sensitivity to rapamycin S6K1 in Arabidopsis seedlings (Fig. 1D). 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. 1E). 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. 1F, 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. 1F), 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. 1F 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 fkpl2 transgenic lines were generated using RNA interference (RNAi) (Fig. 2B). In fkpl2 leaf cells, the inhibitory effect of rapamycin on Thr-449 phosphorylation of S6K1 is severely compromised compared with that in WT leaf cells (Fig. 2A). The inhibitory effect of rapamycin in whole seedling growth is also much reduced in fkpl2 plants (Fig. 2C and supplemental Fig. S5A). These studies strongly support the specific role of endogenous FK12 protein in mediating rapamycin inactivation of TOR PK activity.

Action of Arabidopsis FK12 Is Highly Specific—In addition to FK12, it has been suggested that human FKP38 (HsFKP38) is also involved in TOR regulation (26). We further investigated the function of seven closely related Arabidopsis FK12 proteins (Fig. 3A) 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 HsFKP38 to inhibit TOR PK activity directly (Fig. 3B). The findings demonstrate the specificity of Arabidopsis and human FK12 in rapamycin-dependent TOR PK inhibition.

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 fkpl2 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 FK12 expression. C, FK12 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 FK12 enhancing the rapamycin inhibition effect on Thr-449 phosphorylation in leaf cells. FLAG-tagged S6K1 was co-expressed with HA-tagged FK12s or GFP in protoplasts and treated with rapamycin for 30 min. E, overexpression of Arabidopsis or human FK12 enhancing rapamycin inhibition effect on seedling growth. Rapamycin was added at the time of germination for 9 days. Scale bar, 5 mm.
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**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. 4A), and the constructs were co-expressed in leaf protoplasts. The emitted luminescence reflects the intensity of protein interaction. As shown in Fig. 4B, there is only minimal background luminescence signal in leaf cells co-transfected with NLUC-FRB and CLUC-FKP12 or CLUC-HsFKP12 (a human control) 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. 2A). Rapamycin does not affect the expression of NLUC-FRB and CLUC-FKP12 proteins (Fig. 4C). These results strongly indicate that rapamycin induces the direct interactions between the Arabidopsis TOR-FRB domain and both Arabidopsis 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. 5A). 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. 5A). Consistently, FKP12-overexpressing transgenic seedlings are hypersensitive to rapamycin (Fig. 5B), and the estradiol-inducible tor-es mutants abolish root hair growth mediated by glucose-TOR signaling (Fig. 5C). 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...
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 mutants 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 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.

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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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