Brassinosteriod Insensitive 2 (BIN2) acts as a downstream effector of the Target of Rapamycin (TOR) signaling pathway to regulate photoautotrophic growth in Arabidopsis

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Title: BIN2 acts as a downstream effector of the TOR signaling pathway to regulate photoautorophic growth in *Arabidopsis*

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Summary

·The components of Target of Rapamycin (TOR) signaling pathway have been well characterized in heterotrophic organisms from yeast to humans. However, due to the rapamycin insensitivity, embryonic lethality in *tor* null mutants and lack of reliable ways of detecting TOR protein kinase in higher plants, the key players upstream and downstream of TOR remain largely unknown in plants.

·Using engineered rapamycin-sensitive BP12-2 plants, the present study has shown that the combined treatment of rapamycin and active-site TOR inhibitors (asTORis) results in synergistic inhibition of TOR activity and plant growth in Arabidopsis.

·Based on this system, we reveal that TOR signaling plays a crucial role in modulating the transition from heterotrophic to photoautotrophic growth in *Arabidopsis*. *Ribosomal protein S6 kinase 2* (*S6K2*) was identified as a direct downstream target of TOR, and the growth of TOR suppressed plants can be rescued by upregulating *S6K2*. Systems, genetic, and biochemical analyses have revealed that *Brassinosteriod Insensitive 2* (*BIN2*) acts as a novel downstream effector of *S6K2*, and the phosphorylation of BIN2 depends on TOR-S6K2 signaling in *Arabidopsis*.

·By combining pharmacological with genetic and biochemical approaches, we have determined that the TOR-S6K2-BIN2 signaling pathway plays important roles in regulating the photoautotrophic growth of *Arabidopsis*.

Key words: Target of rapamycin; *Arabidopsis*; asTORis; photoautotrophic growth; *S6K2*; *BIN2*

Introduction

Target of rapamycin (TOR) is the central controller of cell growth and dynamically regulates metabolic homeostasis and readjustment by controlling key regulatory proteins for transcription and translation in response to nutrition and energy availability in eukaryotes (Henriques et al., 2014; Rexin et al., 2015; Xiong and Sheen, 2015; Dobrenel et al., 2016). The components of the TOR signaling pathway are highly conserved in heterotrophic organisms from yeast to humans, but the key players can differ considerably between heterotrophic species and photoautotrophic plants (Henriques *et al.*, 2014; Rexin et al., 2015; Xiong and Sheen, 2015; Dobrenel et al., 2016). For example: 1) in heterotrophic species, TOR protein interacts with two scaffold proteins, *regulatory-associated protein of mTOR* (*RAPTOR*) to form TOR complex 1 (TORC1) and *rapamycin-insensitive companion of mTOR* (*RICTOR*) to form TOR complex 2 (TORC2). However, *RICTOR* is missing from plants and whether plants have one or two TOR complex remains unclear; 2) although *ribosomal protein S6 kinase* (*S6K*) and *eiF4E-binding protein* (*4E-BP1*), which are downstream effectors of TOR, mediate TOR signaling for ribosome biogenesis and protein synthesis in heterotrophic species, no *4E-BP1* orthologs have been detected in plants; 3) the *tuberous sclerosis complex 1/2* (*TSC1/TSC2*) complex is a key upstream regulator of TOR in all examined animals, but it is also absent from plants; 4) all examined heterotrophic species are hypersensitive to rapamycin, whereas most plants are insensitive to rapamycin; 5) *glycogen synthase kinase 3β*(*GSK3β*) acts as a substrate of S6K in mammals, but little is known about their interaction in plants. These differences indicate that plants have probably evolved a distinct TOR signaling pathway that differs from that of heterotrophic species (Henriques *et al.*, 2014; Rexin et al., 2015; Dobrenel et al., 2016). Due to early lethality of *tor* null mutants and the rapamycin insensitivity of most plants, less progress has been

made in identifying new components of the TOR pathway in plants via conventional genetic approaches than in animals or other species. This has limited the understanding of TOR signaling in various biological processes in plants. However, significant advances have been made by using rapamycin (allosteric inhibitor of TOR) and active-site TOR inhibitors (asTORis) to decipher TOR signaling pathway via chemical genetic approaches in yeast and animals (Benjamin *et al.*, 2011; Laplante & Sabatini, 2012; Dobrenel *et al.*, 2016). It is possible that the key components of the TOR signaling cascade can be determined in plants by using rapamycin and asTORis.

Rapamycin and asTORis are two major kinds of TOR inhibitors with distinct modes of action (Albert *et al.*, 2010; Benjamin *et al.*, 2011). Rapamycin has been established as a potent antifungal, anticancer, and immunosuppressive natural product (macrocyclic lactone) since the 1960s. The impressive characteristics of rapamycin have fueled efforts in identifying its molecular targets in eukaryotic cells. Three classes of rapamycin-resistant mutants were initially identified through genetic screening in yeast. The first rapamycin resistance locus harbored defects in the *FK506-binding protein 12* (*FKBP12*), which is a receptor of rapamycin (Heitman *et al.*, 1991). The two other rapamycin resistance-conferring alleles occur at either *target of rapamycin 1 (TOR1)* or *target of rapamycin 2 (TOR2)*, and these result from the missense mutations, Ser1972Arg (tor1-1) and Ser1975Ile (tor2-1), respectively, which prevent the binding of FKBP12-rapamycin to the TOR protein (Kunz *et al.*, 1993; Helliwell *et al.*, 1994). *TORs* encode large Ser/Thr protein kinases that resemble phosphatidylinositol (PI)-3 kinases and are highly conserved in eukaryotes (Dobrenel *et al.*, 2016). Before binding to TOR, rapamycin first forms a complex with FKBP12, which then interacts with the FKBP12-rapamycin-binding (FRB) domain of TOR to form a heterogonous

ternary complex, and this results in preventing TOR from associating with its scaffold protein RAPTOR and phosphorylating its substrate proteins (Hara *et al.*, 2002). Several previous studies have shown that plants respond poorly to rapamycin largely because planta FKBP12s have lost amino acids critical to interaction with rapamycin (Xu *et al.*, 1998; Sormani *et al.*, 2007; Leiber *et al.*, 2010; Ren *et al.*, 2012; Deng *et al*., 2016). However, overexpression of yeast or human *FKBP12* can confer rapamycin sensitivity in *Arabidopsis* (Sormani *et al.*, 2007; Ren *et al.*, 2012; Deng et al., 2016). Importantly, this rapamycin-FKBP12 system provides a highly inducible, selective and reversible system for addressing TOR functions in plants. However, the phenotypic consequences generated from rapamycin exposure are substantially weaker than embryonic lethality and cell death observed in *Arabidopsis* lines in which TOR signaling has been knocked down or out (Menand *et al.*, 2002; Sormani *et al.*, 2007; Ren *et al.*, 2012; Caldana *et al.*, 2013), suggesting that the incomplete inhibition of TOR signaling by rapamycin exposure could severely limit its utility in dissecting the overall TOR functions.

To overcome the limitations of rapamycin-based approaches, in recent years, major research efforts have been directed to develop the novel ATP-competitive inhibitors against catalytic site of mTOR kinase (Albert *et al.*, 2010; Benjamin *et al.*, 2011; Fruman & Rommel, 2014). These novel inhibitors were named after active-site mTOR inhibitors (asTORis). More than 30 asTORis have been developed and several of these have been examined in clinical trials as potential therapeutic agents (Liu et al., 2009; Benjamin *et al.*, 2011; Fruman & Rommel, 2014). Unlike rapamycin, which only targets mTORC1, asTORis compete with ATP at the catalytic sites of both mTORC1 and mTORC2 complexes and thus could inhibit broader kinase dependent functions of mTOR than rapamycin (Benjamin *et al.*, 2011). Different asTORis

have different potency in cells. KU63794 (KU), Torin1 and AZD8055 (AZD) showed low, moderate and high potency in both animals and plants (Benjamin *et al.*, 2011; Montane & Menand, 2013). In plants, the inhibitory effects of asTORis on TOR kinase activity and plant growth were examined by independent groups. Results indicated that asTORis can efficiently inhibit plant growth by regulating the reprogramming and reinitiation of translation, selective protein synthesis, hormone response, and photosynthesis (Montane & Menand, 2013; Schepetilnikov *et al.*, 2013; Xiong *et al.*, 2013; Dong *et al.*, 2015, Li *et al*., 2015; Deng *et al*., 2016), suggesting that the interaction between asTORis and kinase domain of TOR is highly conserved across organisms. However, the IC50 of asTORis administered to plants are much higher than that in mammalian cells (Montane & Menand, 2013; Schepetilnikov *et al.*, 2013; Xiong *et al.*, 2013). High dosages of asTORis not only increased costs but also caused off-target risks which have been detected in mammalian cell lines (Liu *et al.*, 2012; Rodon *et al*., 2013; Fruman & Rommel, 2014). For example, Torin1, one of the most widely used asTORis, does not display off-target activities at concentrations below 1 μM, whereas strong off-target effects against MRCKa in the AGC family, PI3Kα in the PIKK family, and the members of DNA-PK family were observed when concentrations of 10 μM were used in mammalian cells (Liu et al., 2012).

Based on the different mode of action between rapamycin and asTORis, it is possible that synergistic inhibition of TOR by combining rapamycin with low concentrations of asTORis might reduce the costs, minimize the side effects and thus improve effective TOR inhibition. In this study we revealed that the synergistic growth inhibition of *Arabidopsis* was observed when combining rapamycin with low concentrations of asTORis treatment on BP12-2 plants, which were generated in our previous study (Ren *et al.*, 2012). A potent and

highly inducible TOR suppression system was established in *Arabidopsis*. *AtS6K2* was identified as a downstream effector of TOR to positively mediate the growth in *Arabidopsis*. Systems, genetic, and biochemical analyses unexpectedly revealed that AtS6K2 directly phosphorylated brassinosteroid insensitive 2 (BIN2). Our findings suggest that TOR signaling pathway plays pivotal roles in the transition from heterotrophic to photoautotrophic growth in *Arabidopsis*.

Materials and Methods:

Arabidopsis growth and transformation

In the present study, the wild-type (WT) *Arabidopsis* Columbia (Col-0) ecotype was used. *Arabidopsis* seedlings and plants were grown in growth chambers at 22 ºC and 16 h/8 h light/dark cycle settings unless indicated otherwise. Transgenic plants were generated by the floral dipping method (Zhang *et al.*, 2006). *Arabidopsis* growth, transformation, and screening of primary transformants were performed according to the published protocols (Zhang *et al.*, 2006). For seed surface sterilization, the mature seeds were treated with 70% ethanol for 2 min. The supernatant was then discarded and treated with 10% sodium hypochlorite containing 0.3% Tween-20 for 5 min. The samples were then centrifuged for 2 min at 4,000*g*, and the supernatant was discarded, followed by five rinses with sterile water. Finally, the seeds were suspended in 0.1% sterile agarose and kept at 4°C for 2 days.

Western blotting, kinase and phosphatase assay

Western blotting was performed as described previously (Ren *et al.*, 2011). Transient expression of protein in tobacco plants was performed according to previous reports (Sparkes et al., 2006; Shamloul et al., 2014). For the in vitro kinase assay, HIS-BIN2, HIS-S6K2, and HIS-TOR proteins were expressed in Escherichia coli BL21 strain and purified with affinity tags in accordance with

192 the manufacturer's protocol (GE Healthcare). HIS-TOR is a HIS tag fusion
193 protein containing amino acids 1,832–2,482 of the AtTOR-containing kinase
194 domain. *In vitro* kinase reactions were performed with 10 mM $MgCl_2$, 20 mM
195 Tris·HCl (pH 7.5), 10 μCi [γ-^{32}P] ATP (100 μM) and 100 mM NaCl. The 20-μL
196 reaction solutions were incubated at 30°C at least for 1 h. The reaction was
197 terminated by adding 20 μL of a 2× SDS/PAGE sample buffer. After boiling the
198 reaction samples for 5 min, SDS/PAGE gel was performed for protein analysis
199 and autoradiography. For gel shift assay, 10 μg proteins extracted from 10
200 DAG *Arabidopsis* seedlings treated with various inhibitors for 6h were
201 analyzed using 10% SDS-PAGE and visualized with high-affinity anti-HA
202 antibody (1:1,000 dilution, Earthox catalogue no. E022010-01). Calf intestine
203 alkaline phosphatase (CIAP) and CIAP inhibitor were purchased from
204 Sigma-ALDRICH with catalogue no. SRP6549-10UG and Product No. 10125,
205 respectively. CIAP and its inhibitor treatment were performed according to the
206 manufacturer's instructions. The polyclonal *Arabidopsis* TOR antibody was
207 generated as described in a previous study (Xiong & Sheen, 2012).

208 **RNA Isolation and real-time PCR analysis**

209 Total RNA was isolated by using RNAprep Pure Plant Kit (TianGen Biotech)
210 and RT-PCR was performed by using PrimeScript RT Reagent Kit (TAKARA
211 Biotech) following the manufacturer’s instructions. Real-time assays were
212 performed on a Bio-Rad CFX96 System by using the TransStart Top Green
213 qPCR Super Mix (TransGen Biotech). Primer sequences used for RT-PCR and
214 qRT-PCR analyses are listed in Tables S3 and S4.

215 **Combination index (CI) of rapamycin and asTORis**

216 The interactions between rapamycin and asTORis were quantitatively
217 measured by CI values. As defined by Chou (2006), CI > 1 represents
218 antagonism, CI = 1 indicates additive effects, and CI < 1 means synergism
219 (Chou, 2006). The sterilized seeds of BP12-2 plants were germinated on a

0.5× MS solid medium plate and incubated at 4°C in darkness for 3 d. Then, the plants were allowed to vertically grow under constant white light (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 22°C for 6 days. Next, the seedlings were transferred to 48-well plates containing 0.5× MS liquid medium supplemented with 0.1% DMSO, rapamycin, KU, Torin1, AZD, or a combination at varying concentrations ranging from 0.001 μM to 10 μM. Six seedlings were grouped in each well and six biological repeats were prepared for each treatment. The plants were grown under constant white light (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 22°C for 4 days. Growth inhibition was determined by testing the fresh weight of each treatment. Percent growth value was calculated by using the absorbance values of untreated plants (6 DAG seedlings) on day 0 (D0), DMSO-treated plants on day 4 (C4), and drug-treated plants (T4) as follows: [(T4-D0)/(C4-D0)] x 100. Inhibitory concentration 50 (IC_{50}) and Combination Indices (CIs) were calculated by using the CompuSyn software program (ComboSyn, Inc., Paramus, NJ, USA) (Chou and Talalay, 1984). Affected fraction (Fa) is defined as a growth inhibition level of a drug at a specific dose and can be calculated by using the program's instruction as follows: (100 - % growth value)/100.

Grafting experiments

Sterilized seeds were germinated on 0.5× MS plates and vertically grown in a tissue culture room under constant white light (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 22°C for 6 days. Grafting was performed under sterile conditions. Six DAG seedlings were cut at the middle of the hypocotyl by using a sharp blade. The detached scions and rootstocks were closely put together on 0.5× MS media supplemented with 0.5 μM rapamycin or 0.5 μM rapamycin plus 0.5 μM Torin1 under a microscope. To increase the success rate of grafting, the junction section was monitored under a microscope every 8 h for three times to ensure that the damaged part of the scion and rootstock remain in contact with each other.

Transmission electron microscopy

WT and BP12-2 *Arabidopsis* seedlings were grown on sugar-free 0.5× MS plates for 6 days, then the whole seedlings were transferred to sugar-free 0.5× MS media supplemented with DMSO, rapamycin, rapamycin+Torin1, and rapamycin+AZD. After growing for 3 days, leaf fragments were excised from the first true leaves and fixed by immersing in fixative comprised of 3% glutaraldehyde and 4% paraformaldehyde in 0.05 M potassium phosphate buffer (pH 7) followed precipitation of the samples by vacuum infiltration. The leaf fragments were then collected and stained. The subsequent steps for the examination of ultrathin sections were performed on a Philips CM120 Biotwin Lens transmission electron microscope as described elsewhere (Waters *et al.*, 2009). For each treatment, 10 sections were collected from four individual seedlings of WT and BP12-2 plants, respectively. The images presented in Fig. 3 were taken at a magnification of 11,000×, and 1-μm scale bars are shown.

Isolation and identification of *S6K2* T-DNA insertion lines

s6k2 (SALK_083818) mutants in Columbia background were obtained from the *Arabidopsis* Biological Resource Center (ABRC). The knockout lines were identified and confirmed by PCR using primers designed using the T-DNA Primer Design website (http://signal.salk.edu/tdnaprimers.2.html). The details of these primers are listed in Table S4.

Yeast two-hybrid assays

Yeast two-hybrid assays were performed as described elsewhere (Ren *et al.*, 2011), following the user manual of the Clontech yeast two-hybrid system, Matchmaker™ GAL4 Two-Hybrid System 3 and Libraries.

Measurement of chlorophyll content

Relative chlorophyll content was measured as described elsewhere (Ren *et al.*, 2012).

Results

Rapamycin and asTORis synergistically inhibit the photoautotrophic growth of *Arabidopsis*

Previous studies have indicated that rapamycin can significantly reduce the biomass of *ScFKBP12* transgenic *Arabidopsis* at a concentration below 1 μM, but a plateau/saturation effect for growth inhibition was observed when the concentration was above 1 μM (Ren et al., 2012). Plant growth was found to be inversely proportional to the concentration of asTORis and the inhibition displayed a clear-cut dose-dependent effect compared with rapamycin (Montane & Menand, 2013; Dong et al., 2015; Li et al., 2015). Recent observation has further shown that rapamycin can significantly reduce the IC50 of KU and enhance the drug potency of KU in ScFKBP12 transgenic *Arabidopsis* (Deng et al., 2016). In contrast to KU, Torin1 exhibits high potency and has been more widely used to assess TOR kinase activity in *Arabidopsis* (Schepetilnikov et al., 2013; Xiong et al., 2013). AZD is one of the strongest and most selective inhibitors of TOR in mammals and plants, and has much higher potency than Torin1 or KU (Chresta, 2009; Montané and Menand, 2013; Dong et al., 2015; Li et al., 2015). However, the potential combined effects of rapamycin and Torin1 or AZD have not yet been examined in plants and animals. To further examine whether the synergism effects can be generated when rapamycin is combined with Torin1 or AZD, dose-effect curves were developed for each drug alone and pairwise combination. The inhibitory concentration 50 (IC50) of rapamycin, Torin1, and AZD have been determined in WT and BP12-2 plants grown on sugar-free 0.5× MS media under continuous light condition for 10 days, respectively (Fig. S1 and Table S1). Results showed that rapamycin exhibit the highest potency against BP12-2 plants, follows by AZD and Torin1. The IC50 of rapamycin, AZD and Torin1 are 0.25–0.5 μM, 0.5–1 μM and 1–2 μM in BP12-2, respectively (Fig. S1 and Table

S1). Rapamycin, Torin1 or AZD alone failed to arrest cotyledon greening of BP12-2 and WT plants at their IC50 dose ranges (Figs. 1a-c). However, drug combination studies showed that the cotyledon greening of BP12-2 but not WT plants was blocked when treated with rapamycin+Torin1 or rapamycin+AZD (Figs.1 and 2a). Interestingly, Torin1+AZD failed to block the process of cotyledon greening in BP12-2 or WT plants (Fig. 1 and Fig. S1e). Fresh weight assays of pairwise combination were performed to investigate whether rapamycin combined with Torin1 or AZD could impart a synergistic effect on BP12-2 plant growth (Figs. 1b and 2b). Strong synergistic effects (CI<0.3) but not additive effects were those consisting of rapamycin+Torin1 or rapamycin+AZD against BP12-2 plants (Figs. 2b–c, Table S2a–b). The additive effects were detected in combined treatments of Torin1+AZD with combination index (CI) ranging from 0.9-1.1 in BP12-2 plants (Figs. 2a–c, Table S2a–b). The IC50 values of each drug when used in combination were significantly lower than that when used alone (Table S1), thereby indicating synergistic inhibitory effects at the effective dose (ED_{50}) (Table S1). The combination treatment of rapamycin+AZD generated the strongest synergistic effects in BP12-2 plants (Figs. 2a–c, Table S2a–b).

To further confirm the synergistic inhibitory effects of rapamycin+asTORis treatment on BP12-2 plants instead of the effects of copy number and insertion sites of yeast FKBP12, seven additional BP12 lines were examined on 0.5× MS sugar-free medium supplemented with RAP+Torin1, in contrast to BP12-2 and WT plants (Fig. S2). The phenotypes, including arrested brownish seedlings (Figs. S2 a–b), fresh weight (Fig. S2c), and cotyledon greening rate (Fig. S2d) were highly similar to that of BP12-2 but not WT plants. Furthermore, these particular phenotypes of independent BP12 lines were also observed on sugar-free medium containing with RAP+AZD, respectively (Figs. S2c–d).

These observations suggest that suppression of plant growth using the combination treatment of rapamycin and Torin1 or AZD can synergistically block the transition from heterotrophic to photoautotrophic growth during the seed-to-seedling stage in BP12-2 plants.

TOR is implicated in chloroplast formation in *Arabidopsis*

The formation of chloroplasts is one of the most fundamental forces that drive the transition of young plants from heterotrophic dependency on seed reserves to autotrophic creation of its own energy and food via photosynthesis. We therefore compared the ultrastructure of chloroplasts between BP12-2 and WT plants treated with rapamycin, asTORis, or their combinations by using transmission electron microscopy (TEM). Rapamycin reduced the number (1.63 ± 0.11-fold, n = 30) and size (0.66 ± 0.07-fold, n = 30) of chloroplast in BP12-2 true leaves and promoted the accumulation of amyloplasts, whereas these were not observed in WT plants (Fig. 3a). The rapamycin+Torin1-treated BP12-2 line showed a significantly lower number of chloroplasts (6.45 ± 0.79-fold, n = 30) that were also of smaller size (3.04 ± 0.38-fold, n = 30) compared to that of WT plants (Fig.3a). The thylakoid membrane networks and grana stacks were also poorly developed in rapamycin+Torin1-treated BP12-2 plants, with fewer and smaller stacked grana structures and stroma thylakoids than that in WT plants (Fig. 3a). Compared to WT plants, the double-membrane envelopes of chloroplasts were completely degraded, and the stroma thylakoids and grana thylakoids were totally replaced by huge amyloplasts particles in rapamycin+AZD-treated BP12-2 plants (Fig. 3a). These results indicated that TOR plays a crucial role in chloroplast formation in *Arabidopsis*. To further confirm that the chloroplast effects of pharmacological treatments were due to TOR suppression in *Arabidopsis*, 20 independent *TOR* RNA interference (RNAi) lines were generated. Twelve of these lines

displayed severely defective chloroplast formation (Fig. 3b), which phenocopied the BP12-2 plants grown on medium with rapamycin+asTORis. Transcriptional analyses showed that the mRNA levels of AtTOR were respectively reduced to 23%, 47% and 36% in TOR-RI3, TOR-RI4, and TOR-RI5 relative to that in WT plants. Next, we analyzed the AtTOR protein levels in these AtTOR RI lines by using anti-AtTOR antibody. The results showed that the AtTOR protein was almost undetectable in TOR RNAi plants compared to that in the WT (Fig. 3b), which was suggestive of the correlation between plant chlorophyll content and the level of expression of the AtTOR protein.

TOR signaling regulates the expression of chloroplast- and photosynthesis-associated genes.

To gain insights into the molecular mechanisms linking TOR to chloroplast formation and photoautotrophic growth, key marker genes associated with these processes were selected and their expression levels were measured in BP12-2 plants treated with DMSO, rapamycin, AZD, or RAP+AZD (Fig. 3c). These genes included *light harvesting complex gene 1* (*LHCA1*), which is located in the chloroplast and associated with photosystem I; *pheophytinase* (*PPH*), which is located in chloroplast stroma and is involved in the catabolism of chlorophyll; *RuBisCO small subunit 1A* (*RBCS1A*) and *RuBisCO large subunit* (*RBCSL*), which are located within the RuBisCO complex and acts as the limiting enzyme in the Calvin cycle; *HEME1*, which is involved in chlorophyll biosynthesis process and located in chloroplasts and *CHLM* involved in chlorophyll biosynthesis (Jarvis & Lopez-Juez, 2013). Ten DAG seedlings of BP12-2 plants were transferred to sugar-free 0.5× MS media supplemented with DMSO, 0.5 μM rapamycin, 0.5 μM AZD, and RAP+AZD (0.5+0.5μM) for 8 h, respectively. Rapamycin and AZD efficiently repressed the

expression of the *LHCA1, RBCS1A, RBCSL, HEME1*, and *CHLM*, which are all key positive regulators of chloroplast formation and photoautotrophic growth (Fig. 3c). The strong synergistic inhibitory effects of these genes were also observed when rapamycin was combined with AZD (Fig. 3c). However, the expression of *PPH*, the key negative regulator of chloroplast formation and photoautotrophic growth, was significantly upregulated in rapamycin and AZD-treated BP12-2 plants (Fig. 3c), and more remarkably induced by RAP+AZD (Fig. 3c). These observations may explain the observed defects in chloroplast formation and photoautotrophic growth in *TOR* suppressed plants in *Arabidopsis*.

TOR signaling spatially bridges the transition between heterotrophic and photoautotrophic growth in *Arabidopsis*

Unlike heterotrophic species, plants simultaneously possess both heterotrophic and photoautotrophic growth. Seed-to-seedling and root-to-shoot are two major points of transition between heterotrophic and photoautotrophic growth in plants. The observations given above indicated that TOR temporally bridges the transition from heterotrophic to photoautotrophic growth during the seed-to-seedling stage. We next examined whether TOR spatially bridges the signaling communication between heterotrophic and photoautotrophic organs (root-to-shoot). The specific inhibitory effect of rapamycin on BP12-2 plants but not on WT plants provides a useful tool to test this hypothesis by using grafting approaches. We grafted the WT scion onto the BP12-2 rootstock or vice versa to generate the WT/BP12-2 or BP12-2/WT chimeric plants. The grafting success rate of WT/WT was 63% and 30% on rapamycin and RAP+Torin1 medium plates, respectively (Fig. S3a), whereas the chimeric control BP12-2/BP12-2 could not be grafted on the medium in the presence of rapamycin or RAP+Torin1. The grafting success rates of the other

chimeric plants are shown in Fig. S3a. Expectedly, the detached shoots of BP12-2 plants did not survive in the presence of rapamycin or rapamycin+Torin1 (Fig. 4a), whereas WT roots complemented BP12-2 shoot growth in BP12-2/WT chimeric plants (Figs. 4a, S3b and c). Consistently, the detached BP12-2 roots were arrested (Fig. 4a), but WT shoots were able to rescue root hair and lateral root growth of BP12-2 plants in response to rapamycin or rapamycin+Torin1 inhibition (Figs. 4a and S3b and c). These results indicate that TOR likely mediates the crosstalk between shoots and roots.

Unexpectedly, the roots flourished from the hypocotyl of the detached WT plants but not in BP12-2 shoots in the presence of rapamycin or rapamycin+Torin1 (Fig. 4a). These results prompted us to further explore the functions of TOR in the crosstalk between shoot and roots by culturing the detached shoots of BP12-2 and WT plants in the presence of TOR inhibitors. The WT plants but not BP12-2 scions quickly generate adventitious roots from the hypocotyl and accumulated chlorophyll in leaves in the presence of rapamycin or rapamycin+Torin1 (Figs. 4b and c). Importantly, the number of adventitious roots was remarkably proportional to its chlorophyll levels (Figs. 4b and c), suggesting that TOR plays an important role in root system development in *Arabidopsis*. The presence of adventitious roots in WT plants and not in RAP-treated BP12 plants were in agreement with the previously established role of TOR in meristem formation (Xiong *et al.*, 2013).

In parallel, the detached roots of WT but not BP12-2 plants gradually accumulated chlorophyll and acquired a green coloration on the medium supplemented rapamycin (Fig. S4e). Torin1 significantly reduced the chlorophyll content of BP12-2 plants compared to that of WT plants when

combined with rapamycin (Figs. S3d). As a consequence of chloroplast accumulation, lateral roots and root hairs appeared on the detached roots of WT plants (Figs. S3d-e). Consistently, the chlorophyll content was highly proportional to the number of lateral roots (Figs. S3d), thereby suggesting the underlying mechanism that links TOR signaling to light-triggered root initiation and development in *Arabidopsis* (Bellini *et al.*, 2014).

Overexpression of *AtS6K2* partially rescues the photoautotrophic growth in TOR suppression lines

To identify the downstream events mediating heterotrophic to photoautotrophic conversion in the TOR signaling cascade, we introduced the well-established TOR downstream substrates AtS6K1 and AtS6K2 (Mahfouz *et al.*, 2006) into the BP12-2 background. A total of 36 independent transgenic *Arabidopsis* plants containing both P35S::*AtS6K1*-HA or P35S::*AtS6K2*-HA and P35S::*ScFKBP12*-Myc were obtained (see supplemental Methods and Materials). In the T3 generation, we selected nine independent lines (OE1–9) of BP12-2/*AtS6K1* and BP12-2/*AtS6K2* to perform rapamycin+Torin1 sensitivity assays (Figs. S4a and c). The lines of BP12-2/*AtS6K2-OE1-9,* but not BP12-2/*AtS6K1-OE1-9,* displayed RAP+Torin1-resistant phenotypes (Figs. S4a and c), indicating that *AtS6K2* but not *AtS6K1* significantly restored the defects in chlorophyll biosynthesis, root development, and fresh weight in BP12-2 plants in response to rapamycin+Torin1 treatments (Figs. S4a and c). Next, the expression level of *AtS6K1* and *AtS6K2* in BP12-2/*AtS6K1*-OE1-6 and BP12-2/*AtS6K2*-OE1-6 lines was confirmed by semi-quantitative PCR, western blot, and real-time PCR (Figs. 5a–b and S4b). The BP12-2/*AtS6K2*-OE1 plants were further subjected to treatments using rapamycin, asTORis alone, or its combination in contrast to BP12-2 and WT

(Fig. 5c). Consistently, these partially rescued the photoautotrophic growth of BP12-2 plants in the presence of rapamycin and asTORis (Figs. 5c and d).

We further isolated the T-DNA insertional mutants of *AtS6K1* and *AtS6K2*. The plants of *s6k1/s6k1* did not display any detectable defects as described in a previous report (Mahfouz *et al.*, 2006), whereas *s6k2/s6k2* showed an early embryo lethality phenotype (Figs. 6a–c). The phenotypes of the s6k2 mutants resembled *s6k1/s6k2* double-mutant phenotypes (Henriques *et al.*, 2010). Because *S6K1* (At3g08730) and *S6K2* (At3g08720) are closely linked in tandem on chromosome 3, we performed semi-quantitative RT-PCR to examine S6K1 expression in *s6k2* T-DNA mutant and showed that *S6K1* expression was not disrupted in *s6k2*/*s6k2* aborted seeds (Fig. S4d). Next, we introduced P35S::*AtS6K2* into the *S6K2/s6k2* background. P35S::*AtS6K2* was able to rescue the embryo lethality phenotypes of *s6k2/s6k2* (Fig. 6d), indicating that *AtS6K2* functionally complements the T-DNA insertion mutant s6k2 in *Arabidopsis*. A previous study showed that the Phospho-p70 S6 kinase [Thr(P)-389] polyclonal antibody detects TOR phosphorylation of Thr(P)-455 in AtS6K2 (Xiong *et al.*, 2013). *AtS6K2*-OE1/BP12-2 plants were used to detect TOR protein kinase activity under rapamycin, asTORis, and rapamycin+asTORis treatments (Fig. 6e). The signals of AtS6K2 Thr(P)-455 phosphorylation were significantly reduced in response to various treatments (Fig. 6e). Especially, the strong synergistic inhibitory effects of AtS6K2 Thr(P)-455 phosphorylation were observed in the combination of rapamycin and asTORis, suggesting that AtS6K2 is a key downstream effector of TOR signaling that regulates the photoautotrophic growth in *Arabidopsis*.

BIN2 acts as a novel downstream effector of TOR signaling to negatively regulate photoautotrophic growth

To identify the direct partner of AtS6K2, yeast two-hybrid screening was performed to identify the putative proteins that bind to the given bait protein, AtS6K2 (PGBK-*S6K2*), from 7-DAG *Arabidopsis* seedling cDNA libraries. A total of 612 positive clones were generated on stringency selection plates with SD-His-Leu-Trp media. Because RPS6 and RAPTOR are the well-known interacting proteins of S6K2, the specific PCR primer pairs of *RPS6* and *RAPTOR* were used to identify the positive yeast colonies. Seventy-five percent of these clones expressed *RPS6A*, *RPS6B*, *RAPTOR1*, and *RAPTOR2*. The rest of the clones (non-*RPS6/RAPTOR* clones) were amplified by using a PGADT7 vector-specific primer pair and sequenced. Six of these were BIN2. To confirm this observation, the well-known *AtS6K2* interacting proteins such as *RAPTOR*s, *RPS6*s, and different domains of *TOR* were used as positive control. The results showed that yeast colonies expressing the bait (BK-*S6K2*) and the preys (AD-*BIN2*) alone could not survive on the SD-His-Leu-Trp media, whereas *AtS6K2* interacting proteins and *BIN2* showed specific binding to the *AtS6K2* protein (Fig. S5). Furthermore, the SD-His-Leu-Trp+X-gal plate was used for X-gal staining to further verify the interaction between *AtS6K2* and *BIN2*, and paralleled with *RPS6A*, *RPS6B*, *RAPTOR1*, positive, and negative control (Fig. S5). The blue color shown in Fig. S5b indicates that *BIN2* is an important partner of *AtS6K2*.

BIN2 is a well-established gene that plays a major role in integrating brassinosteriod, auxin, and stress in *Arabidopsis* (Li *et al.*, 2001; Zhang *et al.*, 2009; Kim *et al.*, 2012; Saidi *et al.*, 2012); however, our understanding of its function in the plant TOR signaling pathway is limited. Importantly, *BIN2* is the homolog of human *GSK3β* and shares 62% identity, and GSK3β is phosphorylated by S6K and plays a crucial role in various diseases in animals and humans (Zhang *et al.*, 2006; Cybulski & Hall, 2009). Because the BIN2

protein shares a high sequence similarity with human GSK3β, we therefore overexpressed *HsGSK3β* and *BIN2* in *Arabidopsis* (Figs. S6a and b). Interestingly, both *HsGSK3β* and *BIN2* overexpression lines displayed exactly the same phenotypes such as dark green leaves (Figs. 7a and b), dwarf status (Figs. 7a and b), short primary roots (Figs. 7 b), and less lateral roots (Fig. 7c), indicating that the highly conserved functions of BIN2 and HsGSK3β was identified in *Arabidopsis*. In the T3 generation, six independent transgenic lines of HsGSK3β and BIN2 were selected to examine the mRNA and protein levels by semi-quantitative PCR, western blot, and real-time PCR analyses (Figs. S6a and b).

To explore the novel regulatory link between S6K and BIN2/GSK3β, we performed the kinase assay and showed that AtS6K2 directly phosphorylates BIN2 (Fig. 8a). Furthermore, HsS6K and AtS6K2 phosphorylated BIN2 and human GSK3β, respectively (Figs. 8b and S6c), which was suggestive of the highly conserved function of GSK3β and S6K2 in eukaryotic cells. To confirm the physical interaction between AtS6K2 and BIN2 in plant cells, we used tobacco (*Nicotiana benthamiana*) to transiently co-express AtS6K2 and BIN2 proteins in leaf cells, followed by a co-immunoprecipitation experiment (Fig. 8c). In the anti-Myc immunoprecipitate from AtS6K2-Myc and AtS6K2-Myc/AtBIN2-HA plants, AtS6K2-Myc was detected in both AtS6K2-Myc and AtS6K2-Myc/AtBIN2-HA plants using an anti-Myc antibody, whereas AtBIN2 was immunoprecipitated only in the presence of AtS6K2 (Fig. 8c). On the other hand, AtS6K2-Myc was co-immunoprecipitated by an anti-HA antibody when co-expressed with AtBIN2-HA (Fig. 8c), whereas the negative control showed that *AtS6K2* was not immunoprecipitated in the absence of AtBIN2 (Fig. 8c). These results were suggestive of a physical interaction between AtS6K2 and BIN2 *in vivo*. Most regulatory domains such

555 as the putative active site (AA45-205), ATP binding site (AA45-185), substrate
556 binding site (AA50-205), and activation loop region (AA180-205) overlapped at
557 the amino-terminal of BIN2 (Fig. S6d). To map the phosphorylation region(s) of
558 BIN2 that were targeted by AtS6K2, four N-terminus truncated *BIN2* mutants
559 (*MBIN1*:ΔAA1–45, *MBIN2*: ΔAA1–130, *MBIN3*: ΔAA1–180, and *MBIN4*:
560 ΔAA1–210) were generated (Fig. S6d). N-terminal mutants truncated at amino
561 acids 210 (MBIN4) showed significantly reduced phosphorylation compared to
562 MBIN1, MBIN2, and MBIN3 (Fig. S6e). These observations thus suggest that
563 the protein sequence consisting of amino acids 45–210 of BIN2 is the putative
564 phosphorylation region targeted by AtS6K2. In mammals, S6K is not controlled
565 by mTOR only and other proteins such as 3'-phosphoinositide-dependent
566 protein kinase 1 (PDK1) phosphorylate and regulate S6K. It is possible that
567 S6K2 regulates BIN2 independent of TOR. To test the involvement of TOR
568 and S6K2 in the phosphorylation of BIN2 *in vivo*, we examined the
569 phosphorylation status of BIN2 in BIN2-OE1 plants via gel shift assay.
570 Ten-day-old seedlings of BIN2-OE1 plants grown on a 0.5× MS medium were
571 transferred to 0.5× MS media respectively supplemented with DMSO,
572 LY2584702 (S6K selective inhibitor), GSK2334470 (PDK selective inhibitor),
573 KU, Torin1, or AZD and grown for 6 h. Protein blots from extracts of seedlings
574 treated with DMSO and GSK2334470 generated two additional, distinct high
575 molecular weight bands of BIN2-HA signals (Fig. 8d). When BIN2-OE1
576 seedlings were treated with LY2584702, KU, Torin1, or AZD, only a single
577 band of the expected size for BIN2-HA was produced (Fig. 8d). Importantly,
578 the higher molecular weight band shift of BIN2 was abolished by calf intestine
579 alkaline phosphatase (CIAP) treatment and imidazole (CIAP inhibitor) can
580 suppress the activity of CIAP efficiently (Fig. 8e), indicating that the observed
581 BIN2 mobility shift resulted from BIN2 phosphorylation. These results suggest
582 that phosphorylation of the BIN2 protein depends on TOR-S6K2 signaling.

583 Furthermore, the mass spectrometric analysis was performed to identify the
584 phosphorylated residues of BIN2 by S6K2. The results showed that Ser^{187},
585 Ser^{203}, and Thr^{273} of BIN2 are the putative phosphorylated targets of S6K2 (Fig.
586 8f and Fig.S7).
587
588 There are 10 *GSK3* homologs in *Arabidopsis* that can be phylogenetically
589 clustered into 4 subgroups (Fig. S8a). No RNA interference of *BIN2* was
590 reported in *Arabidopsis* to date, and thus *BIN2*-RNAi plants were generated in
591 this study. P35S::*BIN2*-RNAi constructs with kanamycin plant selection marker
592 were transformed into WT Colombia background, and 36 transgenic plants
593 containing P35S::*BIN2*-RNAi were obtained. A total of 20 independent lines
594 displayed bigger leaves, faster growth, and earlier flowering than that in WT
595 plants (Fig. 9a). Nine of these plants were selected for *BIN2* expression
596 analysis via real-time PCR, which showed a significant reduction in all of 9
597 lines (Fig. S8b). The fresh weight of *BIN2*-RI1 was 15.25 ± 1.32 and 3.05 ±
598 0.27 (n = 30) times higher than that in the *BIN2*-OE1 and WT plants (Fig. 9a),
599 respectively, thereby indicating that BIN2 acts as a negative regulator of
600 photoautotrophic growth in *Arabidopsis*. Because the function of *BIN2* is
601 similar to that of the other nine *GSK3* homologs in *Arabidopsis* (Yan et al.,
602 2009), it is possible that *BIN2* RNAi also targets the other family members. The
603 expression levels of other *BIN2* homologs were decreased, although this
604 reduction was not statistically significant (Fig. S8c). However, for *AtSK12*, one
605 of three members in the subgroup I showed a highly significant decrease in
606 expression level (Fig. S8c), which is likely caused by 26-bp continuous DNA
607 fragments of *AtSK12* that were targeted by BIN2-RNAi DNA (Fig. S8d). These
608 observations suggest that highly functional redundancy exists between
609 *AtSK12* and *BIN2*. A previous study showed that the behavior of *AtSK12*
610 exactly resembles that of *BIN2* (Kim et al., 2009). For example, overexpression

611 of *AtSK12* displayed the same phenotypes as the gain of function of *BIN2* (Kim
612 et al., 2009). It is reasonable that simultaneously reducing the expression level
613 of *AtSK12* and *BIN2* can generate phenotypes that were opposite to the gain
614 of function of *BIN2* and *AtSK12*.
615
616 Next, *BIN2*-RI1, *BIN2*-OE1, and WT plants were treated with Torin1, AZD, and
617 KU (Figs. 9b–c). Similar to the rapamycin-BP12-2 plants, *BIN2*-OE1 was more
618 sensitive to asTORis treatment than WT was (Figs. 9b–c). Interestingly, the
619 RNAi of *BIN2* displayed tolerance to asTORis compared with *BIN2*-OE1 and
620 WT controls (Figs. 9b–c). We therefore introduced *BIN2*-RI1 into the BP12-2
621 background by genetic crossing, which in turn resulted in the partial rescue of
622 rapamycin-BP12-2 growth inhibition by *BIN2*-RI1 (Figs. S9a and b). The
623 reduced fresh weight and cotyledon greening of rapamycin-BP12-2 plants
624 treated with asTORis were also effectively rescued by *BIN2*-RI1. These
625 findings suggest that BIN2 can mediate TOR signaling in a negative manner.
626
627 **Discussion**

628 TOR signaling plays an important role in plant growth by reprogramming
629 transcription, translation, and metabolism in *Arabidopsis* (Deprost et al., 2007;
630 Ahn *et al.*, 2011; Moreau *et al.*, 2012; Ren *et al.*, 2012; Caldana *et al.*, 2013;
631 Xiong *et al.*, 2013). However, our understanding of the roles of TOR signaling
632 in the transition from heterotrophic to photoautotrophic growth in plants is
633 limited. The present study revealed that the combined treatment of BP12
634 plants with low concentrations of rapamycin and asTORis results in synergistic
635 growth inhibition. A potent and highly inducible TOR suppression system with
636 rapamycin in combination with asTORis was established in *Arabidopsis*.
637 *AtS6K2* and *BIN2* were identified as the downstream effectors of TOR that

638 mediate growth in *Arabidopsis*. Based on these observations, we proposed a
639 working model that highlights the conserved and distinct nodes of the
640 TOR-S6K2-BIN2 signaling pathway between mammalian cells and plants (Fig.
641 S10). In mammals, the phosphorylation of S6K is controlled by both mTOR
642 and PDK1 (Fig. S10b). A previous study showed that GSK3β is constitutively
643 phosphorylated by S6K in mTOR active cells (Zhang *et al.*, 2006). However,
644 GSK3β also acts upstream of mTOR and is regulated by WNT signaling (Fig.
645 S10b) (Wu & Pan, 2010). Interestingly, a recent study has shown that S6K
646 activates mTOR signaling via positive feedback (Fig. S10b) (Ben-Hur *et al.*,
647 2013). These observations suggest that the crosstalk between these proteins
648 is quite complicated in mammalian cells. It is possible that plants also use
649 mechanisms similar to those observed in mammals, in which S6K could also
650 activate TOR via positive feedback. In plants, TOR signaling couples light,
651 energy, nutrition, and growth factors to promote photoautotrophic growth (Fig.
652 S10c) (Ahn *et al.*, 2011; Moreau *et al.*, 2012; Ren *et al.*, 2012; Caldana *et al.*,
653 2013; Xiong *et al.*, 2013). TOR inhibitors suppress photoautotrophic growth by
654 inhibiting the activity of TOR (Figs. S10a and c) (Montane & Menand, 2013;
655 Schepetilnikov *et al.*, 2013; Xiong *et al.*, 2013). S6K2 functions as a key
656 downstream readout of TOR signaling, whereas BIN2 integrates TOR-S6K2
657 signaling with photoautotrophic growth in a negative manner. Several feedback
658 loops of this pathway that have been described in mammalian cells remain
659 largely unknown in plants (Fig. S10c), but BP12-2 seedlings showed better
660 growth in the presence of rapamycin, asTORis, or RAP+asTORis when S6K2
661 was overexpressed, indicating that the evolutionarily conserved S6K-TOR
662 feedback loop likely exists in plants as well. Our findings advance the critical
663 understanding and provide new insights into TOR signaling during
664 photoautotrophic growth in *Arabidopsis*. Considering that TOR is an essential
665 gene in all eukaryotic cells, the synergism generated by RAP+asTORis may

likely in turn contribute to blocking TOR signaling in tumor cells, pathogenic fungi, and pests.

We showed that AtS6K2 rather than AtS6K1 physically interacted with BIN2. AtS6K1 is very similar to AtS6K2 in terms of protein sequence, although these show distinct subcellular localization and molecular functions in *Arabidopsis* (Mahfouz *et al.*, 2006). AtS6K1 is exclusively localized in the cytoplasm, whereas AtS6K2 is mainly confined to the nucleus or nucleolus (Mahfouz *et al.*, 2006). Importantly, BIN2 inactivates BZR1 and BES1, which are the key transcriptional factors of the BR signaling pathway, by phosphorylating these in the nucleus (Ryu *et al.*, 2007). Similarities in the nuclear localization of AtS6K2 and BIN2 raise the possibility that they can interact with each other. We examined whether AtS6K1 interacted with BIN2; however, we were unable to detect the interaction signals between AtS6K1 and BIN2. A previous study has shown that S6K directly phosphorylates GSK3β(Zhang et al., 2006), but these did not examine whether this activity was independent of PDK. Our results showing that the phosphorylation of BIN2 was dependent on TOR-S6K signaling, but not PDK-S6K signaling, have advanced our understanding on this complicated signaling network. By using rapamycin and asTORis, a widely used chemical genetic strategy has been employed to overcome the challenges resulting from embryo lethality of TOR mutations in *Arabidopsis*. Taking together, the BP12/RAP/asTORis system therefore opens up new avenues for identifying the new components of the TOR signaling pathway and provides a valuable platform to dissect the TOR signaling cascade in plants by integrating chemical, classic genetics, and functional genomics approaches.

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698

699 **Figures legends**

700 **Fig. 1 The combination of rapamycin and asTORis significantly enhances**
701 **growth inhibition of BP12-2, but not WT plants.**

702 **(a)** Representative images of WT and BP12-2 seeds germinated on sugar-free
703 0.5× MS medium supplemented with DMSO(0.1%), rapamycin(0.5 μM), AZD
704 (0.5 μM), and RAP(0.5 μM)+AZD (0.5 μM) for 10 days. **(b, c)** The fresh weight
705 **(b)** and cotyledon greening **(c)** of BP12-2 and WT plants grown on sugar-free
706 0.5× MS media supplemented with DMSO(0.1%), RAP(0.5 μM), AZD (0.5 μM),
707 Torin1(1μM), RAP(0.5 μM)+AZD (0.5 μM), RAP(0.5 μM)+Torin1(1μM), AZD
708 (0.5 μM)+Torin1(1μM). Thirty seedlings were pooled for each measurement at
709 10 DAG. The concentration of all drugs was set at their IC_{50} values (also see
710 Fig. S1 and Table S1). Each data point was averaged from three independent
711 biological replicates. Error bars indicate ± SD for triplicates. Asterisks indicate
712 significant differences by using the student's *t*-test compared to the DMSO
713 treatment (**P < 0.01, **** p <0.0001).

714

715 **Fig. 2 Treatment of BP12-2 plants with rapamycin+asTORis results in**
716 **synergistic inhibitory effects**

(a) Upper panel: BP12-2 plants grown on the sugar-free 0.5×MS media supplemented with RAP(0, 0.01, 0.1, 0.25 and 0.5 μM) and DMSO(0.1%); Lower panel: BP12-2 plants grown on the sugar-free 0.5×MS media containing RAP(0μM)+AZD(0.5 μM), RAP(0.01 μM)+AZD(0.5 μM), RAP(0.1 μM)+AZD(0.5 μM), RAP(0.25 μM)+AZD(0.5 μM), RAP(0.5 μM)+AZD(0.5 μM) and DMSO(0.1%) for 10 days, respectively. **(b)** The fresh weight of BP12-2 plants was measured after growing on sugar-free 0.5× MS medium containing with DMSO(0.1%), rapamycin(0.5 μM), AZD(0.5 μM), RAP(0.5 μM)+AZD(0.5 μM); and RAP(0.5 μM)+Torin1(1 μM), respectively, bars, SD. **(c)** BP12-2 plants treated with DMSO and varying concentrations for rapamycin, asTORis (AZD and Torin1), or a combination for plant growth assays and assessment of CI values as described in Materials and Methods. Computer-simulated Fa-CI curves show synergism (CI < 1), additive effect (CI = 1), or antagonism (CI > 1) for the indicated levels of growth inhibition (Fa) induced by the drug combination (see also Table S1). Circles (O) indicate the Fa-CI data points based on experimental values (see also Table S1). Asterisks indicate significant differences by using the student's *t*-test relative to that observed with DMSO treatment (**P < 0.01, **** p <0.0001).

Fig. 3 Suppression of *TOR* by pharmacological treatment or genetic manipulation results in chloroplast degeneration.

(a) Electron micrographs showing an increase in the accumulation of starch granules in the chloroplast of the first true leaves of WT and BP12-2 plants after treatment with DMSO (0.1%), rapamycin (0.5 μM), rapamycin (0.5 μM)+Torin1 (1 μM) and rapamycin (0.5 μM)+AZD (0.5μM), respectively. **(b)** RNA interference of *AtTOR* results in chloroplast degradation in rosette leaves (upper panel) and a significant decreasing in the expression of AtTOR proteins

744 (bottom panel). The total RNA and protein were extracted from the yellowing
745 seedlings marked with stars. Seedlings were grown on sugar-free 0.5×MS for
746 30 days. Scale bar: 1 cm. (c) Real-time PCR was utilized for the quantification
747 of the relative expression level of marker genes associated with chloroplasts
748 and photosynthesis in BP12-2 plants treated with DMSO(0.1%), rapamycin(0.5
749 μM), AZD(0.5 μM), and rapamycin(0.5 μM)+AZD(0.5 μM) for 8 h. At least 10
750 seedlings were pooled for total RNA extraction in each measurement. Data
751 were obtained and averaged from three independent biological replicates.
752 Error bars indicate ±SD for triplicates. Asterisks indicate significant differences
753 by using the student's *t*-test relative to that observed with DMSO treatment (*P
754 < 0.05, ** p <0.01).

755

756 **Fig. 4 TOR bridges the crosstalk between photoautotrophic and**
757 **heterotrophic organs in *Arabidopsis*.**
758 **(a)** The growth performance of the reciprocally grafted plants between BP12-2
759 and WT growing on sugar-free 0.5× MS media in the presence of 0.5 μM
760 rapamycin for 7 days. "X" indicates that the shoot failed to fuse with the root, "/"
761 means that the shoot fused with the root. Stars mark the injunction or junction
762 point between WT and BP12-2 plants. Thirty seedlings were used for each
763 independent grafting experiment. **(b)** The detached shoots of WT and BP12-2
764 plants grown on sugar-free 0.5× MS in the presence of DMSO (0.1%), 0.5 μM
765 rapamycin, and 0.5 μM rapamycin + 0.5μM Torin1 for 0 day (upper panel) and
766 10 days (bottom panel), respectively. The control seedlings are on the leftmost
767 and rightmost. **(c)** The chlorophyll content (upper panel) and lateral root
768 number (lower panel) of the WT and BP12-2 plants in B were measured (n =
769 30). Scale bar: 1 mm. Asterisks indicate significant differences by using the
770 student's *t*-test compared to that observed with DMSO treatment (**P < 0.01,

771 ****P < 0.0001). Values are expressed as the means ± SD of three
772 independent experiments.
773
774 **Fig. 5 Overexpression of *AtS6K2* partially rescues *TOR* suppression**
775 **phenotypes.**
776 **(a–b)** The expression level of *AtS6K2* in six independent
777 BP12-2/P35S::*AtS6K2* transgenic lines is confirmed by qRT-PCR (top panel in
778 a), western blot (bottom panel in a) and real-time PCR (b), *Actin 2* was used as
779 internal control. **(c)** *AtS6K2*-OE1 plants partially rescued the defective
780 chloroplast and photoautotrophic growth of BP12-2 plants. The seeds of WT
781 (upper panel), BP12-2 (middle panel), and *AtS6K2*-OE1/BP12-2 (bottom panel)
782 plants were germinated on sugar-free 0.5× MS supplemented with different
783 TOR inhibitors at its IC_{50} value for 10 days. Scale bar: 0.1 cm.
784 **(d)** The fresh weight and cotyledon greening rate of WT, BP12-2, and
785 *AtS6K2*-OE1/BP12-2 plants grown on medium supplemented with DMSO,
786 rapamycin (0.5 μM), and combining rapamycin (0.5 μM) with AZD (0.5 μM),
787 Torin1 (1 μM), and KU (5 μM), respectively. At least ten 10 seedlings were
788 pooled for each measurement at 10 DAG. Each data point was averaged from
789 three independent biological replicates. Error bars indicate ± SD for triplicates.
790 Asterisks indicate significant differences by using the student's *t*-test relative to
791 that observed with DMSO treatment (**P < 0.01, **** p <0.0001).
792
793 **Fig. 6 S6K2 functions as a downstream output of TOR signaling in**
794 ***Arabidopsis*.**
795 **(a)** The genome structure of *AtS6K2* and T-DNA insertion position of
796 Salk_083818. **(b)** Embryonic lethality caused by mutation of *AtS6K2*. The
797 silique of WT (left panel of b) and *S6K2/s6k2* (right panel of b) plants. The
798 white seed indicates the aborted embryo of the *s6k2/s6k2* mutant (right panel

of b). **(c)** The embryo developing phenotypes of S6K2. The developing embryos of *S6K2/S6K2* (left panel of c), *s6k2/s6k2* (middle panel of c), and the enlargement embryo image of *s6k2/s6k2* (right panel of c) were captured by using Nomarski optics. Scale bar = 0.01 mm. **(d)** Functional complementation assay in *S6K2/s6k2* background shows that overexpression of *AtS6K2* rescues the embryo lethal phenotype of the *s6k2/s6k2* mutant. Scale bar = 0.01 mm. **(e)** In *AtS6K2*-OE1/BP12-2 plants, the phosphorylation of T449-*AtS6K2* by TOR kinase is significantly inhibited by TOR inhibitors and their combinations compared to that of the control. HA and MYC antibody detected the *AtS6K2*-HA and *FKBP12*-MYC proteins, respectively.

Fig. 7 Functional characterization of *BIN2* and human *GSK3β* in *Arabidopsis*

(a) Representative images of the overexpression of *BIN2* (*BIN2*-OE1; left panel), *HsGSK3β* (*HsGSK3β*-OE1; central panel), and WT (right panel) plants grown in soil for 20 days under normal condition. Scale bar = 1 cm. **(b)** Comparison of inflorescence, leaves, stature, and architecture among WT, *BIN2*-OE, and *HsGSK3β*-OE plants. **(c)** The images show root system development in the *BIN2*-OE, *HsGSK3β*-OE, and WT plants. Scale bar = 1 cm.

Fig. 8 Phosphorylation of BIN2 is dependent on the plant TOR-S6K2 signaling pathway.

(a, b) *In vitro* kinase assays show that human *S6K* phosphorylates *Arabidopsis* BIN2 (a), and *AtS6K2* phosphorylates human *GSK3β* (b). The upper panel shows the results of autoradiography, and the bottom panel protein staining. **(c)** *AtS6K2* physically interacts with BIN2 in plant cells. Proteins transiently expressed in *Nicotiana benthamiana* leaves, immunoprecipitated (IP) with anti-Myc or anti-HA antibody, and immunoblotted

with anti-HA or anti-Myc antibody, respectively. **(d)** S6K2 kinase activity is required for BIN2 phosphorylation. Ten-DAG BIN2-OE1 (HA-tagged BIN2) seedlings were transferred to 0.5× MS medium with DMSO (0.1%), LY2584702 (1μM, S6K inhibitor), GSK2334470 (1μM, PDK inhibitor), Torin1 (5μM), KU (10μM), and AZD(2μM) grown for 6 h. Then, total proteins were extracted from the seedlings and subjected to SDS/PAG gel shift assays. (e) The S6K2-induced mobility shift was abolished by CIAP treatments (upper panel) and the activity of CIAP can be suppressed using alkaline phosphatase inhibitor (Imidazole) (lower panel). U=unit; %=volume/volume. (f) Mass spectrometric analysis for the putative phosphopeptides of BIN2 showing in vitro S6K2 phosphorylation site of BIN2 at Ser^{203} marked in red. His-BIN2 was incubated with His-S6K2 and ATP at 30 ºC for 1h. The protein were digested by trypsin and analysed by LC-MS/MS.

Fig. 9 *BIN2* mediates TOR signaling to modulate photoautotrophic growth in *Arabidopsis*.

(a) The growth performance of WT, *BIN2*-OE1, and *BIN2*-RI1 plants in soil for 25 days under normal growth conditions. **(b)** The seeds of the *BIN2*-RI1, WT, and *BIN2*-OE1 plants were germinated on sugar-free 0.5× MS supplemented with DMSO (0.1%), AZD (0.5 μM), Torin1 (1 μM) and KU (5 μM) and grown for 10 days in contrast to the DMSO control. **(c)** The fresh weight (FW; upper panel) and cotyledon greening (CG; bottom panel) of *BIN2*-RI1, WT and *BIN2*-OE1 seedlings were measured after the plants were grown on sugar-free 0.5× MS containing DMSO (0.1%), AZD (0.5 μM), Torin1 (1 μM), and KU (5 μM) for 10 days, respectively. Thirty seedlings were pooled for each measurement. Values are expressed as the means ± SD of three independent experiments. Asterisks indicate statistically significant differences by using the student's

t-test relative to that observed with DMSO treatment (*P < 0.05, ** p <0.01, **** p <0.0001).

Supporting information

Supplemental figures

Fig. S1. TOR modulates photoautotrophic growth in *Arabidopsis*.

Fig. S2. Synergistic inhibition of various BP12 plants after treatment with rapamycin+asTORis.

Fig. S3. TOR signaling mediates the crosstalk between shoots and roots.

Fig.S4 Functional analysis of AtS6K1 and AtS6k2 in TOR signaling pathway.

Fig. S5 *BIN2* acts as a putative interactor of *AtS6K2*.

Fig. S6 The interaction between TOR-S6K and BIN2.

Fig. S7 Phosphorylation of BIN2 by S6K2 in vitro kinase assay.

Fig. S8 Phylogenetic analysis of *GSK3* homologs in *Arabidopsis* and its expression level in BIN2-RI1 plants.

Fig. S9 BIN2 RNA interference rescues the photoautotrophic growth of TOR suppression plants.

Fig. S10 The working model of TOR-S6K2-BIN2 in regulating the photoautotrophic growth of *Arabidopsis*.

Supplemental tables

Table S1: IC_{50} values of rapamycin and asTORis in either single or combinative treatments.

Table S2 Combination Index (CI) values of TOR inhibitor combinations.

Table S3 The PCR primers used in gene cloning.

Table S4: Real-time PCR and gene identification primers used in the study.

Supplemental Methods

Methods S1: Generation of overexpression and RNA interference constructs.

Methods S2: Cotyledon greening and fresh weight measurements.

Methods S3: Generation of various overexpression and RNA interference lines.

Methods S4: Generation of BP12-2/*S6K1*-OE, BP12-2/*S6K2*-OE, *BIN2*-RI, and BP12-2/*BIN2*-RI1 lines.

Methods S5: Mass Spectrometric Analysis

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