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Molecules and Cells

Possible Dual Regulatory Circuits Involving AtS6K1 in the Regulation of Plant Cell Cycle and Growth

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The role of *Arabidopsis* S6 Kinase 1 (AtS6K1), a downstream target of TOR kinase, in controlling plant growth and ribosome biogenesis was characterized after generating transgenic plants expressing AtS6K1 under auxin-inducible promoter. Down-regulation of selected cell cycle regulatory genes upon auxin treatment was observed in the transgenic plants, confirming the negative regulatory role of AtS6K1 in the plant cell cycle progression reported earlier. Callus tissues established from these transgenic plants grew to larger cell masses with more number of enlarged cells than untransformed control, demonstrating functional implication of AtS6K1 in the control of plant cell size. The observed negative correlation between the expression of AtS6K1 and the cell cycle regulatory genes, however, was completely reversed in protoplasts generated from the transgenic plants expressing AtS6K1, suggesting a possible existence of dual regulatory mechanism of the plant cell cycle regulation mediated by AtS6K1. An alternative method of kinase assay, termed "substrate-mediated kinase pull down", was employed to examine the additional phosphorylation on other domains of AtS6K1 and verified the phosphorylation of both amino- and carboxy-terminal domains, which is a novel finding regarding the phosphorylation target sites on plant S6Ks by upstream regulatory kinases. In addition, this kinase assay under the stress conditions revealed the salt- and sugar-dependencies of AtS6K1 phosphorylations.

INTRODUCTION

The target of rapamycin (TOR) pathway is a central regulator of cell growth and energy homeostasis, which incorporates various regulatory signals including nutrients, stress, energy status, hormones, and growth factors (Ma and Blenis, 2009; Zoncu et al., 2010). Dysregulation of the TOR pathway has been often implicated in a number of cellular ailing conditions such as cancer, diabetes and ageing, underscoring its critical role in the control of cell growth and development. First discovered in yeast and mammals by genetic screens and biochemical characterization, TOR is a member of the phosphoinositide 3-kinase (PI3K)-related protein kinase family and found to be conserved in all eukaryotic genomes deciphered to date, including protists, fungi, metazoans, and plants (Soulard et al., 2009). In animals and yeasts, TOR exists as two distinct functional complexes, TORC1 (TOR complex 1) and TORC2, which are characterized by two different interacting proteins RAPTOR and RICTOR, respectively. TORC1 in both animals and yeasts is sensitive to the immunosuppressant drug rapamycin, which forms an inhibitory complex with RAPTOR (Rapamycin-Associated Protein of TOR) subunit of the TORC1 after binding to a cellular peptidyl-prolylisomerase, FKBP12 (Loewith et al., 2002). On the other hand, the TORC2, in which RICTOR (Rapamycin-Insensitive Companion of TOR) replaces RAPTOR and mainly involved in the maintenance of the cytoskeleton, is not inhibited by rapamycin (Sarbassov et al., 2004).

Upon nutrients and mitogen signals, the growth stimulatory effects of TORC1 in animal cells are transduced primarily through the TOR kinase-dependent phosphorylation of the two downstream effectors, S6K1 (p70 ribosomal S6 kinase 1) and 4E-BP (eIF 4E-binding protein), leading to the promotion of protein synthesis and ribosome biogenesis (Ma and Blenis, 2009). Like Akt and PDK1, which play key regulatory roles in the upstream of the TOR pathway (Burgering and Coffer, 1995; Pullen et al., 1998), S6K1 also belongs to the AGC kinase family of serine/threonine kinase. Phosphorylation of S6K1 at its carboxy-terminal regulatory domain by TOR appears to be a prerequisite for its full activation, which requires the PDK1-dependent phosphorylation at the activation loop of the catalytic domain (Belham et al., 1999). In addition to its well-known target, ribosomal protein S6 (Chung et al., 1992; Price et al., 1992), several other substrates of S6K1 have been also identified recently in mammalian cells, the roles of which are all implicated in the protein synthesis as well (Ma and Blenis, 2009). Perhaps being closely related with its regulatory role in protein synthesis, S6K1 is also known to be involved in controlling cell size. In *Drosophila*, deficiency of dS6K leads to embryonic lethality or reduced body size, which is caused by decreased cell size rather than by reduction in cell number (Montagne et al., 1999). In yeast, the S6K1 ortholog, SCH9, which is phos-

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phorylated by TORC1 during promotion of protein synthesis and ribosome biogenesis in a similar manner to that in animal cells, controls life span and cell size (Arsham and Neufeld, 2006; Sengupta et al., 2010).

In plants, the significance of TOR pathway in maintaining proper growth and development is largely conserved, too; knockout mutant of TOR in *Arabidopsis* (*AtTOR*) induced embryonic lethality (Menand et al., 2002), and RNAi-mediated repression of *AtTOR* resulted in reduced shoot and root growth whereas overexpression of it conferred enhanced growth and stress tolerance (Deprost et al., 2007). *Arabidopsis* S6K1 (AtS6K1) was shown to operate downstream of AtTOR as a kinase phosphorylating the *Arabidopsis* ribosomal S6 protein (AtRPS6) and to be phosphorylated by AtPDK1, demonstrating the basic components and mode of action of the TOR pathway is also conserved in plants (Mahfouz et al., 2006). Recently, Xiong and Sheen (2012) demonstrated that the phosphorylation on T449 of AtS6K1 was inhibited by rapamycin treatment in dose-dependent manner. TAP46, a regulatory subunit of PP2A, was shown to be critical for TOR-mediated plant growth as a putatively direct target of TOR as in yeast and mammalian cells (Ahn et al., 2011). One interesting and a well-anticipated feature of the plant TOR signaling is the incorporation of phytohormones as its regulatory signal components. AtS6K1 has been reported to be activated by auxin and cytokinin in suspension cells (Turck et al., 2004), and silenced and overexpressed lines of *AtTOR* showed close correlation with the plants' sensitivity to ABA (Deprost et al., 2007).

Recently, it has been reported that AtS6K1 positively regulates cell size by inhibiting expression of the mitotic cyclin *CYCB1;1,* as demonstrated in transgenic *Arabidopsis,* in which the RNAi-based suppression of AtS6K resulted in the reduction of cell size and ploidy (Henriques et al., 2010). The association of AtS6K1 with the plant homologue of RB (Retinoblastoma) protein, RBR1, was shown as a possible underlying mechanism for the observed repression of cell proliferation in the study. In our previous study, we have also characterized transgenic *Arabidopsis* overexpressing *AtS6K1* under CaMV 35S promoter (Mahfouz et al., 2006). Although the transgenic plants in the study had unusually high rate of embryonic lethality, perhaps due to a constitutive overexpression of *AtS6K1*, which have likely disrupted the normal developmental programming, the progenies of those survived the vegetative growth stages nonetheless showed apparent normal phenotype. However, these plants exhibited hypersensitivity to osmotic stress, a phenotype that has been also observed in animal cells overexpressing the positive regulator of TOR, RHEB (Inoki et al., 2003).

In the present study, we set out to further characterize the role of AtS6K1 in regulating cell cycle and cell size, by generating transgenic *Arabidopsis* expressing *AtS6K1* in response to auxin treatment. Our results were basically in agreement with the data of Henriques et al. (2010) in that the induction of AtS6K1 by auxin in the transgenic plants resulted in suppression of the cell cycle-regulatory genes and increased cell mass in the calli derived from the transgenic plants. However, this relationship appears to be completely reversed in protoplast cells such that the induction of AtS6K1 in protoplast cells made from the transgenic plants caused increased expression of the cell cycle regulatory genes, suggesting that different regulatory circuits might be in operation under the control of AtS6K1 depending upon different physiological or spatial status of the cells. During this study, we also identified that, unlike the mammalian counterpart, AtS6K1 is phosphorylated on its amino-terminal acidic domain in addition to the carboxy-terminal domain, phosphorylation of which by the mTOR kinase activity is well characterized in mammalian S6K1. Determining whether this N-terminal phosphorylation of AtS6K1 is also subject to the catalytic activity of AtTOR, or it is mediated by a yet to be identified kinase would be an intriguing subject of the future researches on characterizing the plant TOR signaling pathway.

MATERIALS AND METHODS

Plant materials and growth

Arabidopsis plants were grown in growth chamber at 22°C under 16-h light/8-h dark photoperiods at 150 μmol/m^2 s. For induction of *AtS6K1* driven by DR5 promoter, plants were treated with 1 μM NAA in 1 N NaOH. For auxin treatment, 14-day-old seedlings were transferred to liquid media with 1 μM NAA and 30-day-old leaves were treated with 10 μM NAA by spray. To induce callus, seeds were germinated on callus induction medium (CIM) containing MS salts with 0.5 mg/L 2,4-D, 0.05 mg/L kinetin and 0.5 g/L MES at 22°C. For osmotic and sugar starvation treatments of suspension cells, *Arabidopsis* suspension cells (Columbia) maintained under standard condition at their active growth stage (5 days after subculture) were transferred into a new culture media with each treatment (150 mM NaCl, no sugar added, or both) and grown further for 24 h before harvesting the cells for total soluble protein isolation.

Cloning of *Arabidopsis* S6K1-NT (AtS6K1-NT), AtS6K1-CT (AtS6K1-CT), and P_{DR5}*::HA-AtS6K1* cassette

GST-AtS6K1-NT was generated by cloning the region of AtS6K1 spanning the first 130 amino acids into a modified pGEX-KG vector by PCR amplification using GST-AtS6K as a template. Similarly, GST-AtS6K1-CT was generated by PCR cloning of the AtS6K1 corresponding to the amino acids residues 392-465. For constructing P_{DR5}*::HA-AtS6K1*, a full-length *AtS6K1* cDNA was cloned into pENTR1A vector and then transferred to a modified pEarleyGate201 destination vector (Earley et al., 2006) in which the CaMV (Cauliflower mosaic virus) 35S basal promoter was replaced with seven copies of DR5 element (Ulmasov et al., 1997). The resulting construct consisted of CaMV 35S enhancer and 7X DR5 as a regulatory region, and vector-derived HA (human influenza hemagglutinin epitope tag) fused in-frame to *AtS6K1* cDNA.

Substrate-mediated kinase pull down and kinase assay

Equal amount of total soluble proteins prepared (250 μg - 1 mg) were incubated with replica samples of 10 to 20 μl of the GST-fusion protein substrate resin at room temperature for 15 to 30 minutes by gentle rotation. After the incubation, the resins were spun down and washed with 1 ml of a buffer containing 20 mM Hepes (pH 7.5), 125 mM NaCl, 1 mM DTT, 50 mM NaF, 1 mM Na_3VO_4, 5 mM Benzamidine, and 1 mM PMSF by quick vortexing. The wash was routinely repeated 10 times, and after final wash, 50 to 100 μl of kinase reaction buffer containing 20 mM Hepes (pH 7.5), 125 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$, 5 mM $MnCl_2$ and 100 μM ATP with 0.1 μCi γ-P^{32}-ATP (3,000 Ci/mmol, Perkin-Elmer, USA) was added to each resin sample and the kinase reaction was carried out at room temperature for 20 min. The reaction was terminated by addition of 10 mM EDTA and the GST-fusion substrate resins were pelleted by quick centrifugation. After discarding the supernatant, the GST-fusion proteins were released from the agarose resin by addition of SDS sample buffer and by heating the sample at 95°C for 3 min, and the kinase activity was monitored by SDS-PAGE followed by autoradiography.

Table 1. Primers for Real-time PCRs in this study

Primer name	Sequence
ACTIN2-F	5′-TTGTTTTCGTTTCTATGATGCACTTGT-3′
ACTIN2-R	5′-TTGCAAAGAGTTTCAAGGTTTTTCTTC-3′
AtS6K1-F	5′-TGATGCCAAGTTTCAAGCCGGAAGTATC-3′
AtS6K1-R	5′-CATGTGTCAGAAGGAATCTAGAACCAAAG-3′
AtS6K2-F	5′-TACAACCAAGTTTCAAGCCGGCGGTTT-3′
AtS6K2-R	5′-ACCGTTGATTTTGTGCATGTTCATATG-3′
HA-AtS6K1-R	5′-GCACCACTTTGTACAAGAAAGCTGGGT-3′
CYCD3;1-F	5′-TCGTTTCTGTCGCGTGTCTCTCAT-3′
CYCD3;1-R	5′-GATAATGTGGTCTACGAACGAAATTGG-3′
CYCB1;1-F	5′-AGCAAGATCTTCTCTCCGCCAAGT-3′
CYCB1;1-R	5′-TGTTGCTTCCATTGCTGATACGCC-3′
RNR2-F	5′-TCCACTGTGACTTTGCGTGTCTCT-3′
RNR2-R	5′-ACCAGAAGACGGTCAGCGACAAAT-3′
CDKB1;1-F	5′-TCCACCAACTGCTCTTCGTGAGAT-3′
CDKB1;1-R	5′-TCACGGTGAAGCACACCATGACTA-3′
pre-rDNA-F	5′-GCGTTTGAGAGGATGTGGCGGGGAAT-3′
pre-rDNA-R	5′-TAAATGCGTCCCTTCCATAAGTCGGG-3′
AtpBrp-F	5′-TGAGGTAACTCTACGCAAAGTCTATAA-3′
AtpBrp-R	5′-GTTCATGACACTGGCAGTTCCA-3′

Real-time PCR

Total RNA was isolated from young seedlings and mature leaves of plant using RNeasy plant mini kit (Qiagen, USA) and reverse transcription of total RNA was carried out using MMLV reverse transcriptase (Promega, USA). To examine the levels of rDNA transcription, pre-rDNA specific primers and oligo-dT were used. All reactions were normalized using *ACTIN2*, a housekeeping gene, as an internal control. Primers used in this study are listed in Table 1. Data shown in Figs. 3 to 5 are representative of three independent experiments.

Protoplast isolation

Protoplasts were isolated according to Yoo et al. (2007) with a little modification. To prepare protoplasts from mature leaves, we selected fresh leaves from 30-DAG-leaves of *Arabidopsis*, and selected leaves were cut into about 1-mm strips by blades. For digestion, leaf strips were incubated in an enzyme solution under the dark condition for at least 3 h. To isolate protoplasts from callus, 50 mg of calli were incubated in an enzyme solution under the dark condition for 5 h. Released protoplasts were washed and incubated in W5 solution for 16-24 h before harvesting.

RESULTS AND DISCUSSION

Phosphorylation of N-terminal and C-terminal regions of AtS6K1 identified by the substrate-mediated pull down approach

The *Arabidopsis* genome has two paralogs of S6K, which are respectively named AtS6K1 and AtS6K2, of which the AtS6K1 has been suggested as a major downstream effector of the TOR pathway in the cytoplasm (Mahfouz et al., 2006). The catalytic domain of AtS6K1 is highly homologous (up to 74% identity in amino acids sequences) to the other orthologs found in animals and yeasts with a salient AGC kinase signature and the consensus PDK1 phosphorylation target motif. However, the amino-terminus of AtS6K1 preceding the catalytic domain is more than twice as long as that of animal S6K1 (i.e., p70-S6K), and although it is also enriched with acidic amino acids (pI 4.13), it does not bear any noticeable sequence similarity with that of mammalian counterpart, including its apparent absence of the TOS signaling motif, which is a highly conserved five amino acid residues present in the amino-termini of all animal S6K1s and found to be essential for the RAPTOR-dependent activation of S6K1 by TOR (Nojima et al., 2003; Schalm and Blenis, 2002). The carboxy-terminus of AtS6K1, which immediately follows the catalytic domain, on the other hand, is much shorter in length compared with that of the animal S6K1s (Fig. 1A). The autoinhibitory domain, which is present in animal S6K carboxy-terminus and contains several phosphorylation sites, is missing in this region. Nonetheless, the region itself shows a significant homology with the corresponding region of the animal S6K1 carboxy-terminus, where four amino acid residues were found to be phosphorylated in a rapamycin-sensitive manner, thus they are likely to be a direct target of the TOR kinase activity in both animals and plants.

Although no phosphorylation sites have been reported in the N-terminal acidic domain of animal S6Ks, the extended region of AtS6K1 N-terminus may contain some phosphorylation sites, compensating the missing phosphorylation sites in the C-terminus. To inquire this possibility, the N-terminal and C-terminal domains of AtS6K1 were separately cloned by PCR amplification of the full-length AtS6K1 to generate GST-fusion proteins of each domain, GST-AtS6K1-NT (N-terminal domain) and GST-AtS6K1-CT (C-terminal domain), respectively. Both of these GST-fusion proteins were used as the substrates for the endogenous kinase assays, which were performed by the substrate-mediated kinase pull down method as described in Materials and Methods. The rationale of the method is to trap and pull down a specific kinase(s) present in the cell extracts by using a substrate protein bound to the GST-agarose beads, in a similar manner to the immunoprecipitation procedure. In order to stabilize the interaction between the kinase and the substrate, EDTA was added (up to 10 mM) to suppress the kinase catalytic activity during the incubation period (Fig. 1B). Both GST-AtS6K1-NT and GST-AtS6K1-CT were used as the pull down substrates in this assay and were incubated with the increasing amount of the soluble proteins extracted from the same *Arabidopsis* suspension culture cells and subjected to the kinase assay *in vitro,* after rigorous washing. It was evident from the result that both N-terminal and C-terminal regions of the AtS6K1 were phosphorylated, an observation that has not been found in the S6Ks of any other systems (Fig. 1C). The intensities of the phosphorylation were proportional to the amount of the cellular proteins added to the incubation step, while the GST protein included as a negative control in the assay did not pick up any phosphorylation signal, indicating that they were mediated by specific kinases present in the cell extracts and coprecipitated with the substrate proteins used in the assay, GST-AtS6K-NT and GST-AtS6K-CT. In effect, these kinase activities readily disappeared if ATP and Mg^{2+} were provided during the wash step (Fig. 1D, lanes 5 and 8), demonstrating the importance of the EDTA, which was included in the incubation mixture, in keeping the kinase and the substrate bound together during the incubation and pull-down steps (see legend for Fig. 1B). Recently, phosphorylation on T449 of AtS6K1 (see Fig. 1A) was detected in fully differentiated leaf cell, whereas rapamycin, a specific inhibitor of TOR, inhibited the phosphorylation, and in addition, an inducible *tor* transgenic plant failed to phosphorylate the residue of AtS6K1, proving that TOR does phosphorylate AtS6K1 in plants (Xiong and Sheen, 2012).

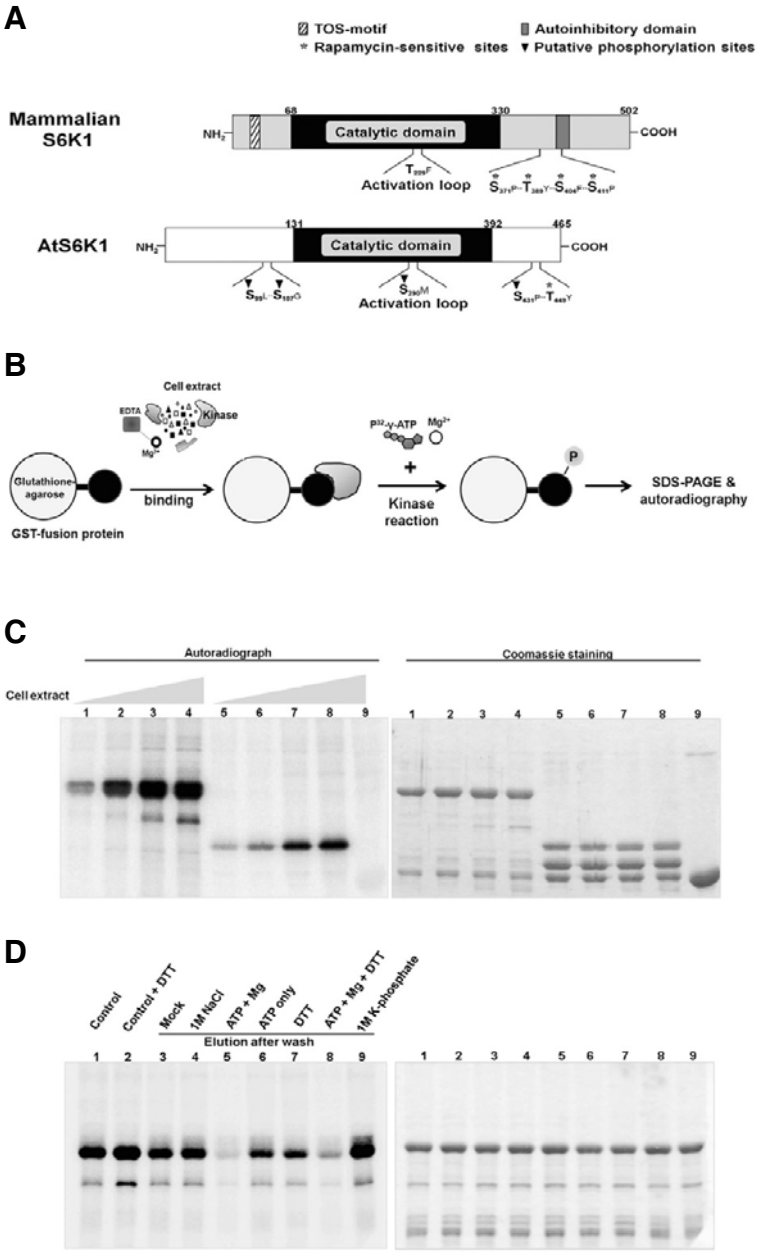

Fig. 1. (A) Comparison of mammalian S6K1 and *Arabidopsis* S6K1 (AtS6K1). Mammalian S6K1 contains TOS-motif, catalytic domain, and autoinhibitory domain whereas AtS6K1 has a putative catalytic domain. Possible phosphorylation sites in AtS6K1 (▼) were predicted based on the comparison with phosphorylation sites of mammalian S6K1 including rapamycin-sensitive residues (*). (B) A scheme for substrate-mediated kinase pull down method. After binding of the cognate kinase in cell extract to the GST-fusion substrate, the kinase-substrate-GST complex is washed in the presence of EDTA. After extensive washes, the kinase reaction is carried out by adding the reaction buffer containing γ-P^{32}- ATP and Mg^{2+}. Stable maintenance of the enzyme-substrate complex by EDTA-dependent ATP quenching is shown. (C) Phosphorylation of AtS6K1-NT and AtS6K1-CT according to the substrate-mediated kinase pull down method. The substrate-dependent kinase pull down assay was performed with respect to the input of the enzyme (endogenous kinase). GST-fusion protein substrates (AtS6K1-NT or AtS6K1-CT) were allowed to bind to the increasing amount of cellular soluble proteins as indicated. Left panel; autoradiograph of the kinase reactions with increasing amount of the *Arabidopsis* cellular protein input: 25 μg (lanes 1 and 5), 100 μg (lanes 2 and 6), 500 μg (lanes 3 and 7), and 1 mg (lanes 4 and 8) of cell extract were incubated in the kinase reaction, respectively, using GST-AtS6K1-NT (lanes 1 to 4) or GST-AtS6K1-CT (lanes 5 to 8) as substrates. Lane 9 is the negative control kinase reaction with 1 mg of *Arabidopsis* protein input using GST protein as substrate. Right panel; Coomassie stained gel of the reaction PAGE from which the autoradiograph shown in left panel was generated. (D) Assay for the best kinase elution method. The best condition for releasing the endogenous kinases from the pull down substrate was tested in a kinase assay method as described in text. Different samples subjected to different elution condition during wash steps of the kinase reaction. Left panel; Autoradiograph of the kinase reaction SDS-PAGE gel. Lane 1, control; lane 2, control + DTT in the kinase reaction; lane 3, elution control (mock elution with kinase reaction buffer); lane 4, 1 M NaCl elution; lane 5, ATP + Mg elution; lane 6, ATP-only elution; lane 7, DTT elution; lane 8, ATP + Mg + DTT elution; lane 9, 1 M K-Phosphate elution. Right panel; Coomassie stained gel of the reaction PAGE from which the autoradiograph shown in the left panel was generated.

Whether both of these phosphorylation events are catalyzed, either directly or indirectly, by the TOR kinase activity remains to be determined.

We find that the kinase assay method introduced here is very reliable and versatile, especially when used against the kinase activities of unknown identity. In principle, it also should be able to provide a means of identifying the kinase for a specific target substrate by combining this method with a proteomic identification method.

Osmotic and nutrient stresses act independently to regulate the activities phosphorylating AtS6K1 and AtRPS6 in suspension cells

In order to obtain a better insight into the effects of stress signals in regulating the AtS6K1 under a proliferative condition of the cell cycle, endogenous kinase activities of *Arabidopsis* suspension cells phosphorylating the N-terminal and C-terminal domains of AtS6K1 were examined by the substrate-mediated kinase pull down using GST-AtS6K1-NT and GST-AtS6K1-CT as the pull-down substrates. GST-AtRPS6-CT, which has carboxy-terminal 100 amino acids of the *Arabidopsis* ribosomal protein S6 (residues 150 to 249) fused to GST, was also used as a reference in this study, as we have shown previously that phosphorylation of the AtRPS6-CT was sensitive to osmotic stress in mature plant leaves (Mahfouz et al., 2006). *Arabidopsis* suspension cells were treated with osmotic stress (150 mM NaCl) or sugar starvation (no sucrose in the culture media), or both and total soluble proteins isolated from them were used as the kinase source for the substrate-mediated pull down reaction. Although the cells subjected to either of the two stress treat-

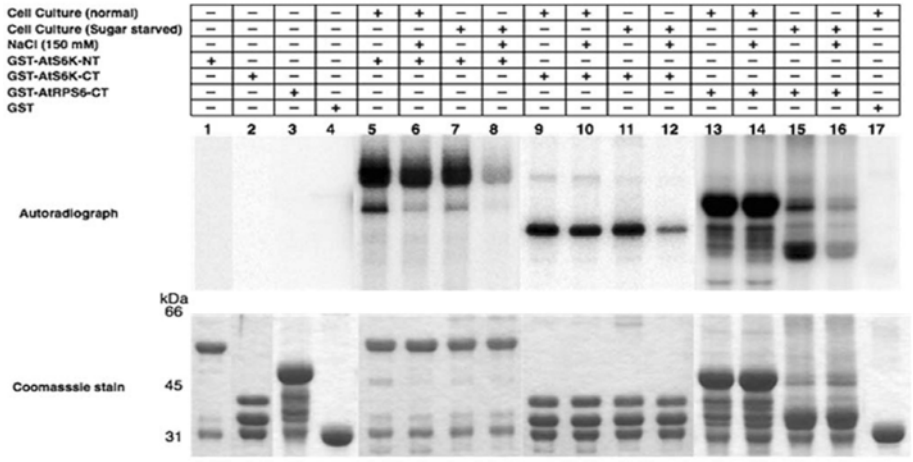

Fig. 2. Effects of nutrient and salt stress on the phosphorylations of AtS6K and AtRPS6 in *Arabidopsis* suspension cells. GST fusion proteins of the N-terminal and C-terminal domains of AtS6K1 (GST-AtS6K1-NT & GST-AtS6K1-CT) and the ribosomal S6 protein C-terminus (GST-AtRPS6-CT) were used as substrates in the substrate-mediated kinase pull down reaction to study the effect of starvation and osmotic stresses imposed on *Arabidopsis* suspension cells. Cells were maintained and treated as described in the "Material and Method". Upper panel: Autoradiograph of the kinase reaction SDS-PAGE gel. Lanes 1-4, kinase reaction with only the GST-fusion protein substrate (negative control for autophosphorylation), GST-AtS6K1-NT, GST-AtS6K1-CT, GST-AtRPS6-CT, and GST, respectively; lanes 5-8, GST-AtS6K1-NT subjected to phosphorylation by proteins from control, osmotic stressed, sugar starved, and osmotic stressed + sugar starved *Arabidopsis* suspension cells, respectively; lanes 9-12, GST-AtS6K1-CT subjected to the phosphorylation reaction in the same manner as for the samples of the lanes 5-8; lanes 13-16, phosphorylation of GST-AtRPS6-CT in the same manner as for the samples of the lanes 5-8; lane 17, GST protein subjected to phosphorylation by proteins from the control cells (negative control for non-specific kinase activity). Bottom: Coomassie staining of the same gel shown in the above autoradiogram. Starvation-induced specific degradation of AtRPS6 is apparent both in the coomassie stained gel and the autoradiograph.

ments exhibited noticeable growth retardation, neither starvation nor osmotic stress alone had any significant inhibitory effect on the kinase activities responsible for phosphorylations of the N- and C-terminal domains of AtS6K1 and AtRPS6 (Fig. 2, lanes 5, 6 and 7 for the N-terminal domain of AtS6K1; lanes 9, 10 and 11 for the C-terminal domain of AtS6K1; lanes 13, 14 and 15 for the AtRPS6). In contrast, phosphorylations on these domains became markedly decreased when both osmotic and starvation stresses were treated together. These unexpected observations do not appear to support the currently established role of S6K1 in cellular growth stimulation and the phosphorylation status of both S6K and RPS6 that show close correlation with the cells' active physiological conditions (Ma and Blenis, 2009). We think that one possible explanation for this apparent discrepancy is the presence of high concentration of auxin, which was included as a standard component of the culture media for maintenance of the suspension cells' proliferative potency. Auxin has been shown to stimulate AtS6K activity as well as the maize RPS6 phosphorylation (Reyes de la Cruz et al., 2004; Turck et al., 2004), thus it is conceivable that the activities phosphorylating AtS6K1 and AtRPS6 in the suspension cells remain high even under stress conditions. What is still intriguing, however, is that either osmotic stress or starvation alone did not have any effect on these phosphorylation activities, but when these stresses were combined together, they were able to override the stimulatory signal for the phosphorylation of AtS6K1 and AtRPS6, which suggests the two stress signals operate independently but converge on to inhibit this stimulatory signal. Another interesting observation from the data is a starvation-induced specific degradation of AtRPS6 (Fig. 2, lanes 15 and 16). While the other two substrates exposed to the exactly same condition did not show any proteolysis, GST-AtRPS6-CT subjected to the incubation with the sugar starved *Arabidopsis* suspension cell extracts became degraded in a reproducible manner. This indicates that a specific protease activity targeting the carboxy-terminal motif of the AtRPS6 is turned on by starvation stress in plant cells, which plays as an additional mechanism for a tight translational regulation under such condition. Determining exactly at which points these stress signals are incorporated into the plant TOR pathway affecting the phosphorylation of AtS6K1 and AtRPS6 would be a key step toward elucidating the structural framework of the plant TOR signaling pathway, and will be the primary focus of our next study to follow in the immediate future.

Repression of cell cycle-regulatory genes in P_{DR5}::*AtS6K1* transgenic plants after auxin treatment

We opted to study the role of AtS6K1 in a transgenic plant overexpressing the gene under an inducible promoter. In doing so, we chose the auxin-inducible synthetic promoter, DR5 (Ulmasov et al., 1997) so that the transcriptional induction of *AtS6K1* and the activation of AtS6K1 protein could be achieved at the same time under such system, as auxin is a positive regulator of the AtS6K1 activity (Turck et al., 2004). Transgenic *Arabidopsis* expressing an *AtS6K1* cDNA under a chimeric promoter cassette of the DR5 promoter element fused to downstream of the CaMV 35S enhancer region (Fig. 3A) was generated and three independent lines of homozygous progenies were obtained. They were then treated with 1 μM NAA at 14 days after germination (DAG) or at 30 DAG and examined firstly for changes in the expression of endogenous *AtS6K1* and *AtS6K2*. Throughout the study, we used NAA as the source of the exogenous auxin treatment because unlike the natural auxin, IAA, delivery of NAA to plant cells is not dependent upon the PIN auxin transporters, thus potentially maximizing the induction effect of auxin on the *AtS6K1* transgene in these plants. We observed that treatment of auxin at a concentration of higher than 10 μM resulted in substantial induction of the endogenous *AtS6K1* and *AtS6K2* as well (Supplementary Fig. 1). However, the 1 μM of auxin treatment to these plants at 14 DAG caused a minimal effect on the expressions of endogenous *AtS6K1* and *AtS6K2* (with about two-fold increase in *AtS6K2* expression only) while strong induction of the transgenic *AtS6K1* (*HA-AtS6K1*) was observed under the same

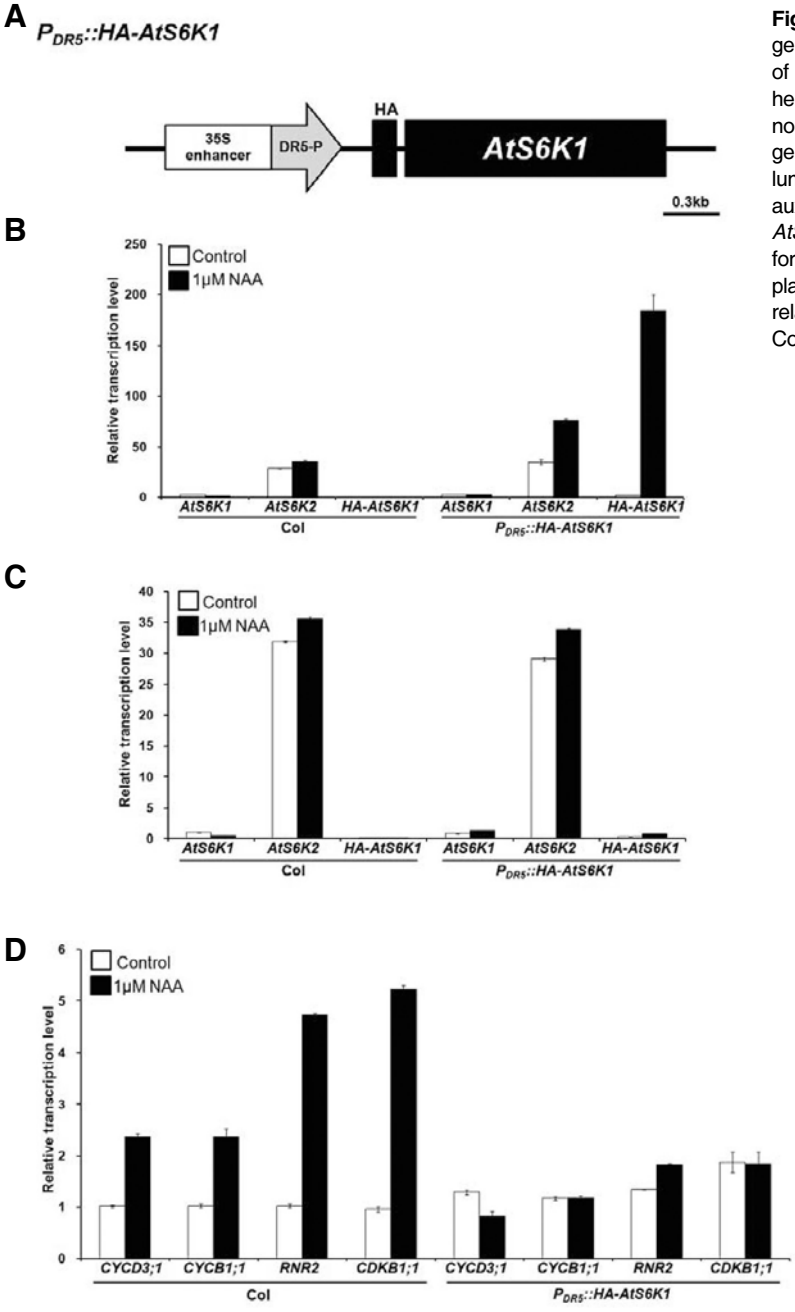

Fig. 3. Induction of P_{DR5}*::HA-AtS6K1* cassette in transgenic plant with auxin treatment. (A) Schematic diagram of P_{DR5}*::HA-AtS6K1* cassette. HA, human influenza hemagglutinin epitope tag. (B) Expression of endogenous *AtS6K1*, *AtS6K2* and *HA-AtS6K1* in 14 days after germination (14 DAG)-seedlings of untransformed Columbia or *HA-AtS6K1*-expressing transgenic plants after auxin treatment. (C) Expression of endogenous *AtS6K1*, *AtS6K2* and *HA-AtS6K1* in 30 DAG-leaves of untransformed Columbia or *HA-AtS6K1*-expressing transgenic plants after auxin treatment. (D) Expression of cell cycle-related genes in 14 DAG-seedlings of untransformed Columbia or *HA-AtS6K1*-expressing transgenic plants.

condition (Fig. 3B). By contrast, at 30 DAG, the expression of *AtS6Ks* in these plants displayed no responsiveness to the auxin treatment; not only did the endogenous *AtS6Ks* show no increased expression, with higher basal expression level observed only in *AtS6K2*, even the transgenic *HA-AtS6K1* under the auxin-inducible promoter did not show any increase in the expression (Fig. 3C). Thus, we chose to use the auxin-treated plants at 14 DAG to examine the effect of the increased *AtS6K1* on the expression of some selected cell cycle-regulatory genes; the G1-specific *CYCD3;1* gene, the S phase-specific *RNR2* gene, the G2/mitotic-specific *CYCB1;1* gene, and the plant-specific *CDKB1;1* (Fig. 3D). In control plants, these cell cycle-related genes were found to be up-regulated upon auxin treatment without concomitant induction of the endogenous *AtS6K* expression. On the other hand, the auxin treatment and the induction of the *AtS6K1* transgene expression (*HA-AtS6K1*) resulted in significant suppression of these genes in the transgenic plants (Fig. 3D). Transgenic plants did not show any particular morphological phenotype during all phases of their life-cycle, except for longer root length observed in the seedlings (Supplementary Fig. 2), which is likely to be caused by the induction of *HA-AtS6K1* in the root tip, where the endogenous auxin concentration is high.

While independently working on this project, it has been recently reported by Henriques et al. (2010) that cell cycle progression is down regulated by AtS6K activities resulting in the stimulation of endoreduplication and increased cell size, which was demonstrated by RNAi-mediated suppression of both

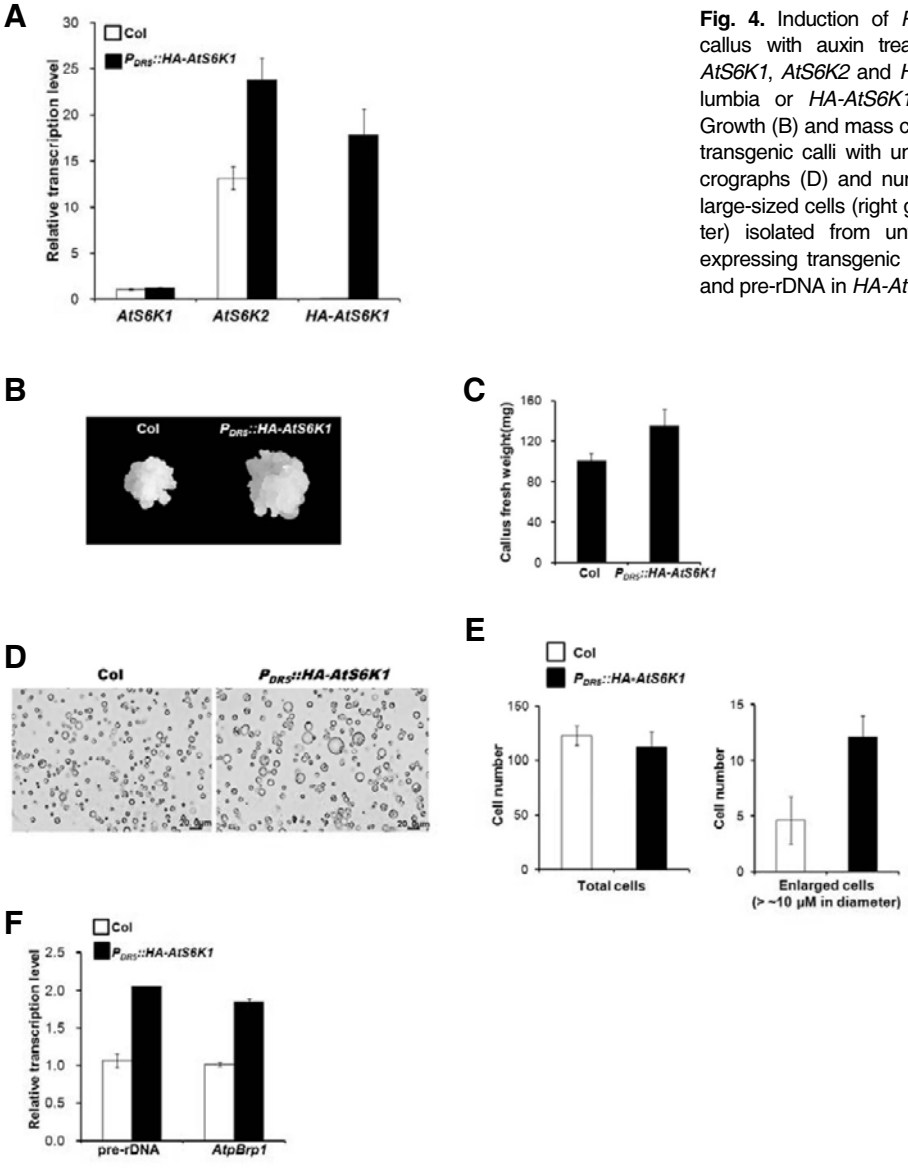

Fig. 4. Induction of P_{DR5}*::HA-AtS6K1* cassette in transgenic callus with auxin treatment. (A) Expression of endogenous *AtS6K1*, *AtS6K2* and *HA-AtS6K1* in calli of untransformed Columbia or *HA-AtS6K1*-expressing transgenic plants. (B, C) Growth (B) and mass comparison (C) of *HA-AtS6K1*-expressing transgenic calli with untransformed Columbia. (D, E) Light micrographs (D) and numbers (E) of total cells (left graph) and large-sized cells (right graph, larger than about 10 μm in diameter) isolated from untransformed Columbia or *HA-AtS6K1*-expressing transgenic calli. (F) Transcription levels of *AtpBrp1* and pre-rDNA in *HA-AtS6K1*-expressing transgenic callus.

AtS6K1 and *AtS6K2* in transgenic *Arabidopsis*. Through overexpression of the AtS6K1 only, our data basically confirmed their results and also showed that, in addition to the *CDKB1;1* gene, which was found to be the primary target of the AtS6Ks (Henriques et al., 2010), additional cell cycle regulatory genes are also under the control of AtS6K1. Although the induction of the transgenic HA-AtS6K1 expression was readily observed by RT-PCR in tissues isolated from the transgenic plants, we have not been able to successfully detect the AtS6K expression at protein level even though the transgene contained a copy of the HA-fusion tag. This could be simply due to the problem of having only a single copy of such fusion tag, which have been reported more often than not in the plant expression systems. We are currently working on several measures to resolve this issue.

Increased cell mass and rDNA transcription observed in P_{DR5}*::HA-AtS6K1* transgenic callus

In order to monitor a prolonged effect of the AtS6K1 on growth of undifferentiated tissues, which might be potentially different from that observed in tissues undergoing a normal developmental programming, seeds of the transgenic *Arabidopsis* expressing *HA-AtS6K1* under the DR5 promoter were induced to form callus by germinating on media containing 0.5 mg/L 2,4-D and 0.05 mg/L kinetin and grown for 2 months. As expected from the presence of auxin in the callus inducing media, transcript level of the transgenic *HA-AtS6K1* was dramatically increased in the calli derived from the transgenic plants (Fig. 4A). Interestingly, the increased transcription of the transgenic *AtS6K1* also appears to cause a slight increase of the endogenous *AtS6K2* transcription, a same trend also observed in the transgenic *Arabidopsis* plants of 14 DAG (Fig. 3B), suggesting presence of a cross-talk regulatory mechanism between the expression of the two genes. Although the transgenic calli expressing *HA-AtS6K1* did not seem to grow any faster than the control callus tissues, they consistently show larger cell mass as compared with the control by about 30% on average (Figs. 4B and 4C). In order to distinguish whether this increased cell mass was a result of an increase in cell size, or due to a higher

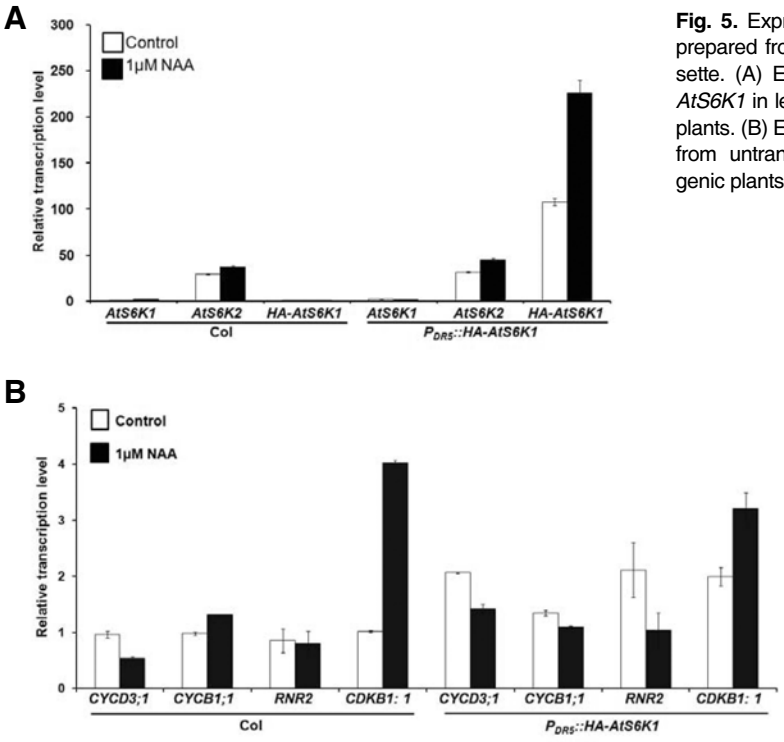

Fig. 5. Expression of cell cycle-related genes in leaf protoplasts prepared from transgenic plants containing P_{DR5}*::HA-AtS6K1* cassette. (A) Expression of endogenous *AtS6K1*, *AtS6K2* and *HA-AtS6K1* in leaf protoplasts from *HA-AtS6K1*-expressing transgenic plants. (B) Expression of cell cycle-related genes in leaf protoplasts from untransformed Columbia or *HA-AtS6K1*-expressing transgenic plants.

rate of cell proliferation, the size of individual cells in calli was compared after generating protoplasts from the calli. We found that cells with larger than 10 μm in diameter were more frequently found in the protoplast cells derived from the transgenic calli expressing *HA-AtS6K1* than in untransformed control (Figs. 4D and 4E). In addition, the transgenic calli showed concomitant decrease in transcriptions of the cell cycle-regulatory genes (Supplementary Fig. 3), supporting our experimental data with the transgenic *Arabidopsis* plants of 14 DAG described in Fig. 3, as well as the results of Henriques et al. (2010).

The observed increase in cell mass of the *HA-AtS6K1* expressing callus led us to consider a possible involvement of stimulation of ribosomal RNA synthesis in conferring the phenotype, as mTOR signaling pathway has been shown to affect many processes for cellular growth including ribosome biogenesis (Mayer and Grummt, 2006). As upstream binding factor (UBF) acts as a general transcription factor for rDNA transcription of mammalian cells in an S6K1-dependent manner (Hannan et al., 2003), AtpBrp1, an *Arabidopsis* orthologue of UBF (Imamura et al., 2008), may also mediate AtS6K1-dependent rDNA transcription. To explore this possibility we first measured rDNA transcription in the *HA-AtS6K1*-expressing callus. The rDNA transcript level was indeed increased by about 2-fold in the transgenic callus (Fig. 4F). Next, we examined the transcripts level of *AtpBrp1* in the transgenic callus and found about 2-fold increase in the expression of *AtpBrp1* (Fig. 4F) implying that AtpBrp1 may regulate the rDNA transcription in AtS6K1-dependent manner. It is possible that AtpBrp1 contributes to the ribosome genesis for the growth of plant cell after AtS6K1-dependent activation processes such as phosphorylation, as reported in mammalian cells and yeast (Berger et al., 2007; Hannan et al., 2003), although it still remains to be learned a lot about how AtpBrp1 is activated before it functions as a general transcription factor for rDNA transcription in *Arabidopsis*.

Constitutive expression of *HA-AtS6K1* in transgenic protoplasts and the concomitant induction of cell cycle-related genes

Induction of *AtS6K1* in both transgenic plants at 14 DAG and transgenic callus with a cassette of P_{DR5}*::HA-AtS6K1* resulted in suppression of cell cycle-related genes although we did observe the increase of callus weight in the transgenic callus. We then decided to use leaf protoplast which may be better to see the effect of AtS6K1 on cell cycle, free from the developmental context. To our surprise, leaf protoplasts prepared from the transgenic *Arabidopsis* expressing *HA-AtS6K1* at 30 DAG showed remarkably elevated level of *HA-AtS6K1* expression (~ 80 times) even before auxin treatment (Fig. 5A). Auxin treatment also increased its expression by two fold as well as the expression of endogenous *AtS6K2*. The observed deregulation of the auxin-dependent expression pattern of the P_{DR5}*::HA-AtS6K1* appears to be due to a promoter-specific, because a reporter construct having GUS under the standard DR5 promoter without the 35S promoter enhancer region still showed an auxin-dependent expression under the same condition (Supplementary Fig. 4). When we examined the expression of cell cycle-related genes in these transgenic protoplasts, two-fold increased expression of those genes was observed in the absence of auxin treatment (Fig. 5B). Their expression with auxin treatment, however, became decreased, which was similarly observed in the auxin-treated plants at 14 DAG (Fig. 3D). We also isolated protoplasts from the calli expressing *HA-AtS6K1* in order to examine whether cell cycle-related genes in the calli may become de-repressed with the transition into protoplasts, as observed in the case of the leaf tissue. While the induction of the *HA-AtS6K1* expression in these calli-derived protoplasts was comparable to that of the leaf-derived protoplasts, endogenous *AtS6K2* expression was found to be increased by more than 4 times than control, (Supplementary Fig. 5A), which might have confounded the effect of *HA-AtS6K1* on

the expressions of cell cycle-related genes. Despite this potential complication, de-repression of the cell cycle-related genes was also observed in the protoplasts generated from the transgenic calli expressing *HA-AtS6K1*, as the transcription of these genes was not found to be repressed as significantly as in the transgenic calli (Supplementary Figs. 3 and 5B). Although the magnitude of repression was not as dramatic as that observed in the leaf-driven protoplasts, this is probably due to the condition of calli maintenance, which requires presence of high concentration of auxin that would have affected expressions of both endogenous and transgenic *AtS6Ks* as well as the cell cycle-related genes over the period of the calli growth. These data appear to indicate the reversed role of AtS6K1 in regulating the expression of cell cycle-related genes in protoplasts, probably not specific to mature leaf tissues of a specific stage. However, more in-depth analyses to solidify this finding and to explain the underlying mechanism would be necessary before drawing any conclusion on the existence of such contrasting roles of AtS6K1 in regulating cell cycle. This *AtS6K1*-dependent increase in the cell-cycle genes expression in the protoplasts appears not be maintained under a stress condition, as both the transgenic and the control leaf protoplasts subjected to 150 mM NaCl showed marked decrease in the expression of these genes (Supplementary Fig. 6).

These data demonstrate that AtS6K1 may up-regulate the expression of genes for cell cycle progression under different developmental and/or physiological conditions, such as in protoplasts, which are devoid of the cell wall structural components and undergoing the procedure of reestablishing cell fate determination (Grafi, 2004) although it was suggested that AtS6K1 may act as a repressor of cell proliferation (Henriques et al., 2010), which were also supported in our experimental data described above. However, *CDKB1;1* transcript level in the auxin-treated protoplasts from transgenic line expressing *HA-AtS6K1* was less than that in auxin-treated protoplasts from untransformed Columbia (Fig. 5B). This was also found in auxin-treated 14-DAG samples (Fig. 3D). Since *CDKB1;1* expression was increased in cells with *AtS6K* RNAi (Henriques et al., 2010), the role of AtS6K1 in protoplasts may be selective in activating the expression of various cell cycle-related genes. A high level of the endogenous *AtS6K2* induction observed throughout these experiments makes it ambiguous whether the increase in these cell-cycle genes expression was solely due to the expression of *HA-AtS6K1*. Since there has not been an intensive study on S6K2 function in plants including *Arabidopsis*, it is difficult to know if *AtS6K2* expression affects the cell cycle progression acting synergistically with AtS6K1 or has different function from AtS6K1. Further characterization of AtS6K2 as well as AtS6K1 should reveal their specific functions.

All in all, the results obtained from our present study showcase the complexity of the regulatory circuits involving S6K as a component of TOR signaling pathway in modulating growth and development of plants, and our transgenic plants expressing *AtS6K1* in response to auxin can be used as one of many valuable tools for the ongoing efforts to uncover such complex regulatory mechanisms underlying plant growth regulation.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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