

## A Heat-Sensitive *Arabidopsis thaliana* Kinase Substitutes for Human p70<sup>s6k</sup> Function In Vivo

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**In mammalian cells, mitogen-induced phosphorylation of ribosomal protein S6 by p70<sup>s6k</sup> has been implicated in the selective translational upregulation of 5'TOP mRNAs. We demonstrate here that the homologous *Arabidopsis thaliana* protein, AtS6k2, ectopically expressed in human 293 cells or isolated from plant cells, phosphorylates specifically mammalian and plant S6 at 25°C but not at 37°C. When *Arabidopsis* suspension culture cells are shifted from 25 to 37°C, the kinase becomes rapidly inactivated, consistent with the observation that heat shock abrogates S6 phosphorylation in plants. Treatment with potato acid phosphatase reduced the specific activity of immunoprecipitated AtS6k2 threefold, an effect which was blocked in the presence of 4-nitrophenyl phosphate. In quiescent mammalian cells, AtS6k2 is activated by serum stimulation, a response which is abolished by the fungal metabolite wortmannin but is resistant to rapamycin. Treatment of mammalian cells with rapamycin abolishes in vivo S6 phosphorylation by p70<sup>s6k</sup>; however, ectopic expression of AtS6k2 rescues the rapamycin block. Collectively, the data demonstrate that AtS6k2 is the functional plant homolog of mammalian p70<sup>s6k</sup> and identify a new signalling pathway in plants.**

Protein kinases are common components of signal transduction pathways in all eukaryotes and have been adapted in different species to couple distinct stimuli to specific physiological responses (15). This paradigm is exemplified by the mitogen-activated protein (MAP) kinase family, whose existence has recently been identified in plants, in which they have been linked to signal transduction pathways implicated in wounding, pathogenesis, and abiotic stresses, as well as those that respond to the plant hormones such as abscisic acid, auxin, and ethylene (14). In contrast to the MAP kinase signalling pathways, homologs of the mammalian p70<sup>s6k</sup> and p85<sup>s6k</sup> (p70<sup>s6k</sup>/p85<sup>s6k</sup>) signalling components have not yet been identified in plants. In mammalian cells, p70<sup>s6k</sup>/p85<sup>s6k</sup> mediates the phosphorylation of S6, an integral protein of the 40S ribosomal subunit. Increased S6 phosphorylation has been implicated in the translational upregulation of an essential family of mRNAs encoding components of the protein synthetic apparatus (16, 17, 31). This family of mRNA transcripts is characterized by an oligopyrimidine tract at their transcriptional start site and is collectively referred to as 5'TOP mRNAs (20).

Recently, it has been shown that the p70<sup>s6k</sup>/p85<sup>s6k</sup> signalling pathway bifurcates from the MAP kinase pathway at the level of the receptor (22) with phosphatidylinositol-3 OH kinase, protein kinase B, and mTOR/FRAP identified as possible upstream signalling components (2, 6). The activities of the two isoforms appear to be regulated coordinately and are generated by a common transcript through alternative translational initiation start sites, with the larger isoform constitutively targeted to the nucleus (26). Discounting the nuclear targeting sequence at the amino terminus of p85<sup>s6k</sup>, both isoforms (1, 19) can be divided into four domains: a 65-amino-acid-long acidic

N-terminal region, which confers rapamycin sensitivity (35), followed by a conserved catalytic domain containing all the hallmarks of Ser/Thr kinases (13), a linker domain, and finally a C-terminal region containing a stretch of residues thought to function as an autoinhibitory domain (1, 10). Mitogenic activation of p70<sup>s6k</sup>/p85<sup>s6k</sup> is associated with multiple phosphorylation at Ser and Thr residues (8). Initial studies led to the identification of four clustered Ser/Thr-Pro phosphorylation sites, which reside in the autoinhibitory domain of the kinase and appear to modulate kinase activity (8, 12). In contrast, a second set of phosphorylation sites which are flanked by large aromatic residues was subsequently identified (25). These sites are the target of p70<sup>s6k</sup>/p85<sup>s6k</sup> selective dephosphorylation and inactivation by the immunosuppressant rapamycin and by the fungal metabolite wortmannin (12, 25), agents which operate via distinct mechanisms (5). Two of these sites, along with a more recently identified phosphorylation site, S371 (24), appear critical for kinase function: T229 (25, 34) in the activation loop and T389 (25) in the linker region, coupling the catalytic and autoinhibitory domains. Of these two sites, T389 has been demonstrated to be the principal target of rapamycin- and wortmannin-induced p70<sup>s6k</sup> dephosphorylation and inactivation (5, 25).

Despite the fact that p70<sup>s6k</sup>/p85<sup>s6k</sup> has not been detected in plants, it is clear that plants contain a homolog to ribosomal protein S6, whose level of phosphorylation appears to be tightly regulated. Indeed, in the case of heat shock, it has been demonstrated that cultured tomato cells exhibit rapid and reversible dephosphorylation of a basic ribosomal protein with an  $M_r$  of 30,000 (30K) presumed to be S6 (27). Similarly, treatment of detached pumpkin cotyledons with 6-benzylaminopurine, which induces rapid polysome formation, also leads to increased phosphorylation of a ribosomal protein with an equivalent molecular weight, whereas abscisic acid, which causes polysome disassembly, inhibits the cytokinin-induced phosphorylation of the same protein (36). Consistent with the proposed role of S6 phosphorylation in protein synthesis, the translation of 5'TOP mRNAs in wheat germ extracts is regulated in a manner equivalent to that previously shown for

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mammalian cells (28), indicating that many of the control elements implicated in this process may be conserved between mammals and plants.

Here, we have screened a genomic library from *Arabidopsis thaliana* to determine whether potential homologs of p70<sup>S6k</sup> exist in plants. We also examined (i) whether the corresponding cDNAs could be ectopically expressed in human 293 cells, (ii) whether they exhibited S6 kinase activity, and (iii) whether specific antibodies derived against the expressed proteins would immunoprecipitate an endogenous S6 kinase activity from *A. thaliana*. Most importantly, we determined whether the *Arabidopsis* S6 kinase could substitute for the mammalian p70<sup>S6k</sup> in signalling to S6 in mammalian cells.

## MATERIALS AND METHODS

**Library screens.** *Arabidopsis* genomic and cDNA libraries constructed in ZAPII vector were purchased from Stratagene. Recombinant clones ( $2.5 \times 10^8$ ) were screened by plaque hybridization using a random-primer-labelled fragment of the cDNA encoding the catalytic domain of rat p70<sup>S6k</sup> as a probe (19). Hybridization was performed according to standard procedures at 55°C. Positive ZAPII clones were isolated and processed according to the manufacturer's protocols. Isolation of cDNAs encoding the *Arabidopsis* ribosomal protein S6 was performed as described above, except that an end-labelled 48-mer oligonucleotide corresponding to the conserved S6 box (amino acids 52 to 68) was used as a probe and hybridization was carried out at 42°C.

**Protein expression in *Escherichia coli*, antibody generation, and protein purification.** The pQE expression system (Qiagen) was used to express a truncated form of AtS6k2 (amino acids 48 to 248) in *E. coli*. Growth, induction, preparation of cell extracts, and purification of overexpressed proteins by affinity chromatography on nitrilotriacetic acid (NTA)-chelating agarose were performed according to the manufacturer's protocols. The purified protein was injected into rabbits with Freund's complete adjuvant. The antisera obtained (from rabbits B and C) were purified by affinity chromatography using NTA-agarose-bound antigen as described previously (11). For Western blot analysis, 20 µg of protein from cell extracts was analyzed. Proteins blotted onto polyvinylidene difluoride membranes were incubated with a 1/1,000 dilution of purified antibodies (B2 or C1), labelled with an anti-rabbit secondary antibody, and revealed by using alkaline phosphatase.

**Mammalian cell culture, transfections, chemical treatment, and extract preparation.** Human embryonic kidney cells were maintained and transfected as described previously (9). The next day, the cells were washed twice and then deprived of serum for 24 h. After preincubation for 15 min with either rapamycin (20 nM) or wortmannin (200 nM) for 15 min, the cells were stimulated with 10% serum for 30 min prior to extraction as described elsewhere (5).

***Arabidopsis* suspension cell culture and plant cell extract preparation.** *Arabidopsis* suspension cells (21) were subcultured weekly at a 1/30 dilution in a medium containing Musharigge and Skoog medium with minimal organics (MSMO)-salt mixture (Sigma) supplemented with 0.5 mg of  $\alpha$ -naphthalene acetic acid per liter, 0.05 mg of kinetin per liter, and 3% sucrose in a 12-h light-dark period at 24°C under constant shaking (120 rpm). For heat shock experiments, 4-day-old cultures were transferred to a 37°C incubator for the time indicated below. Cells were harvested by filtration and resuspended (per g [fresh wt]) in 5 ml of ice-cold plant cell extraction buffer (50 mM HEPES [pH 7.6]-50 mM pyrophosphate-5 mM EDTA-15 mM EGTA-1 mM benzamidine-25 mM NaF-1 mM sodium molybdate-1.5% polyvinylpyrrolidone [PVPP]; prepared 24 h before use. Labile compounds (200 mM mannitol, 2 mM dithiothreitol [DTT], 0.2 mM phenylmethylsulfonyl fluoride, 5 µg of leupeptin per ml, and 5 µg of antipain per ml) were added immediately prior to use. Cells were ruptured by passage through a French pressure cell at 14,000 lb/in<sup>2</sup>, and the extracts were centrifuged at 10,000 × g for 20 min at 4°C. The supernatants were filtered through a double layer of mira cloth and further cleared by being spun at 160,000 × g for 1 h at 4°C.

**Preparation of plant ribosomes.** For substrate preparation, 20 ml of a heat-shocked *Arabidopsis* suspension culture was resuspended in 6 ml of plant ribosomal lysis buffer (100 mM KCl-10 mM MgCl<sub>2</sub>-5 mM EGTA-1 mM DTT-20 mM Tris [pH 7.4]-1% deoxycholate [DOC]-1% Triton X-100). The suspension was incubated at room temperature until thawed and cleared by two successive centrifugations at 12,000 × g for 20 min at 4°C. The supernatant was transferred to 38.5-ml Quick-seal centrifuge tubes (Beckman), underlaid with 4 ml of light sucrose (0.5 M sucrose in 500 mM KCl-5 mM MgCl<sub>2</sub>-2.5 mM EGTA-1 mM DTT-5 mM Tris [pH 7.4]-1% DOC-1% Triton X-100) followed by 4 ml of heavy sucrose (1 M sucrose in the same buffer). Ribosomes were pelleted by centrifugation at 230,000 × g for 16 h at 2°C and then resuspended in Staehelin A buffer (100 mM KCl-5 mM MgCl<sub>2</sub>-1 mM DTT-20 mM Tris [pH 7.4]). Preparation of ribosomes for two-dimensional electrophoresis was performed as described above except that 14-ml gradients containing 2 ml of each cushion were used. Ribosomal proteins were extracted from the ribosomes with acetic acid as described elsewhere (29).

**Immunoprecipitation and S6 kinase assays.** Total protein extract from transfected 293 cells (20 µg) was diluted in dilution buffer (20 mM morpholinepropanesulfonic acid [MOPS] [pH 7.2]-1 mM DTT-0.2% Triton X-100-10 mM MgCl<sub>2</sub>) containing 30 mM *p*-nitrophenylphosphate (pNpp) to a final volume of 200 µl and then subjected to immunoprecipitation by addition of 3 µl of AtS6k-specific antibody (B or C) as described previously (25). Immunoprecipitation from plant extract (1 mg) was performed accordingly except that the extracts were diluted to a final volume of 1 ml in plant extraction buffer without PVPP. S6 kinase activity was measured by using 40S subunits prepared from rat liver or polysomes prepared from *Arabidopsis* suspension cells as a substrate. Kinase assays were performed as described previously (25) but at 25°C unless indicated otherwise.

**Potato acid phosphatase treatment.** Immunoprecipitates of ectopically expressed AtS6k2 were incubated for 20 min at 20°C in 100 µl of dilution buffer supplemented with 5 µg each of antipain and leupeptin per ml under constant shaking. Potato acid phosphatase (30 mU) or phosphatase and pNpp (30 mM) were added to the samples as indicated. The reaction was stopped by diluting the samples in 1 ml of ice-cold dilution buffer supplemented with pNpp and subsequent washing of the beads with the same buffer. S6 kinase assays were performed as described above.

**<sup>32</sup>P<sub>4</sub> labelling and preparation of ribosomes.** After transfection, human embryonic kidney cells were quiesced for 24 h in Dulbecco modified Eagle medium lacking phosphate. The cells were incubated for 1 h in <sup>32</sup>P<sub>4</sub> (0.4 mCi/5 ml) and then pretreated with rapamycin (20 nM) for 30 min prior to addition of serum. After an additional 30 min, cells were harvested in ribosome lysis buffer (100 mM KCl-10 mM MgCl<sub>2</sub>-1 mM DTT-20 mM Tris [pH 7.4]-1% DOC-1% Triton X-100). The lysates were centrifuged at 12,000 × g for 10 min, and the supernatants were transferred to 3.5-ml Quick-seal centrifuge tubes (Beckman) underlaid with 600 µl of light sucrose cushion (0.5 M sucrose in 500 mM KCl-5 mM MgCl<sub>2</sub>-1 mM DTT-5 mM Tris [pH 7.4]-1% DOC-1% Triton X-100) followed by 600 µl of heavy sucrose cushion (1 M sucrose in the same buffer). Ribosomes were pelleted by centrifugation at 230,000 × g for 16 h at 2°C and then resuspended in Staehelin A buffer. Ribosomal proteins were extracted as described previously (29) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

## RESULTS

**Isolation of the AtS6k1 and AtS6k2 genes.** To identify the *Arabidopsis* homologs of p70<sup>S6k</sup>, a genomic library was exhaustively screened by using the catalytic domain of mammalian p70<sup>S6k</sup> as a probe (19). Multiple clones of a single genomic locus containing two nearly identical kinases were obtained, and the corresponding full-length cDNAs were isolated from a cDNA library and designated *AtS6k1* and *AtS6k2* (Fig. 1). Unexpectedly, these clones were found to be identical to two previously identified clones termed Atpk1/ATPK6 (23, 37) and ATPK19 (23). Southern blot analysis further revealed that the two kinases had no apparent close relatives in the *Arabidopsis* genome (37). Biochemical characterization of Atpk1 had suggested that this kinase phosphorylated ribosomal proteins of the 60S subunit but not S6 (38). Nevertheless, database analyses show a high level of conservation with p70<sup>S6k</sup> in the catalytic domain, with up to 74% similarity for both AtS6k1 and AtS6k2 (Fig. 1). Furthermore, this high similarity extends through the domain homologous to the p70<sup>S6k</sup> linker region (45%), which has been recently noted to be present in many members of the second-messenger family of serine-threonine kinases (13). However, the amino terminus, which is highly acidic and confers rapamycin sensitivity to p70<sup>S6k</sup> (4, 5, 35), is much longer in the plant kinases and exhibits less than 25% identity. The plant kinases also lack the region equivalent to the carboxy terminus of mammalian p70<sup>S6k</sup>, including the autoinhibitory domain (1, 8, 10), and do not contain an obvious nuclear targeting motif as found in the p85<sup>S6k</sup> isoform (26). Nevertheless, three of the phosphorylation sites essential for mammalian p70<sup>S6k</sup> activity, T229 (25, 34), S371 (24), and T389 (25), are conserved in AtS6k1 and AtS6k2 as S290, S431, and T449 and S296, S437, and T455, respectively (Fig. 1).

**Ectopic expression of *Arabidopsis* AtS6k2 in human 293 cells.** Given the high homology and absence of other S6 kinase clones, the enzymatic properties of AtS6k1 and AtS6k2 were reassessed by transiently expressing both cDNA clones in hu-

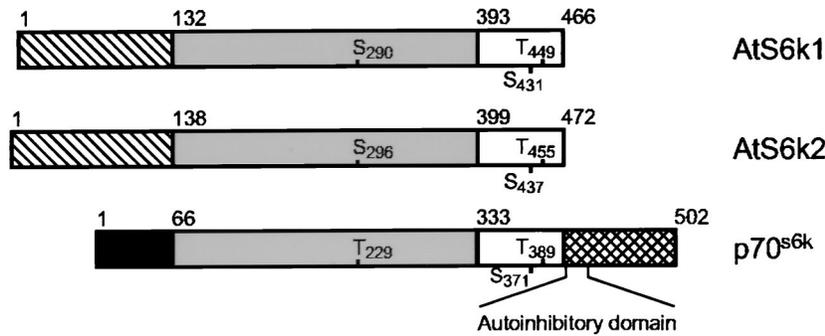


FIG. 1. Structural comparison of *Arabidopsis* AtS6k1 and AtS6k2 with mammalian p70<sup>s6k</sup>. The *Arabidopsis* N-terminal domain (▨), mammalian N-terminal domain (■), catalytic domain (□), *Arabidopsis* C-terminal domain mammalian linker region (□), and mammalian C-terminal domain (▩) are indicated. Numbers above the scheme denote amino acid positions. Phosphorylated serines (S) and threonines (T) known to be essential for the activity of the mammalian p70<sup>s6k</sup> and conserved in the plant proteins are indicated. AtS6k1 and AtS6k2 are also referred to as Atpk1 (37) or ATPK6 (23) and ATPK19 (23), respectively.

man 293 cells. Expression of the plant kinases was monitored in Western blots utilizing an affinity-purified polyclonal antibody directed against a conserved portion of the two proteins. The results show that the antibody did not cross-react with mammalian p70<sup>s6k</sup> and that AtS6k2 but not AtS6k1 is expressed in human cells (Fig. 2A). Transient expression of AtS6k1 led to the detachment and death of most cells (unpublished data), possibly explaining why the protein product could not be detected. Indeed, in subsequent studies employing epitope-tagged variants of both kinases, no AtS6k1 could be detected in cells transiently expressing this construct (data not shown). In extracts of cells transfected with *AtS6k2* cDNA, the antibody recognized a 60K protein. The expected molecular weight of full-length AtS6k2 is 52K. Therefore, the 60K band most likely represents the full-length protein, which is detected as a triplet, reminiscent of the pattern for differentially phosphorylated p70<sup>s6k</sup> (8, 12). Previously, it was reported that baculovirus-expressed AtS6k1 did not phosphorylate S6 in vitro but phosphorylated two small 60S ribosomal proteins, speculated to be homologs of mammalian small acidic ribosomal proteins P1 and P2 (38). Consistent with this finding, extracts derived from 293 cells transfected with the *AtS6k2* cDNA or the empty vector, in contrast to the p70<sup>s6k</sup> cDNA, had no measurable S6 kinase activity at 37°C when 40S mammalian ribosomes were employed as a substrate (Fig. 2B). Thus, even though the *Arabidopsis* kinase is expressed, it is catalytically inactive in vitro towards S6 at 37°C.

**The *Arabidopsis* AtS6k2 gene encodes a ribosomal protein S6 kinase.** The temperature employed in these kinase assays is known to cause heat shock in plants and has been demonstrated to induce dephosphorylation of S6 in vivo (27). This raised the possibility that the plant kinase, unlike its mammalian counterpart, may be catalytically inactive at the higher temperature. To examine this possibility, extracts were re-assayed at 25°C. The results show that the activity of mammalian p70<sup>s6k</sup> kinase is reduced at this temperature compared to that at 37°C, whereas AtS6k2 kinase activity can now be readily observed (Fig. 3A). To assess the specificity of AtS6k2 for S6, extracts from 293 cells transfected with either the empty vector, p70<sup>s6k</sup>, or AtS6k2 were tested for their ability to phosphor-

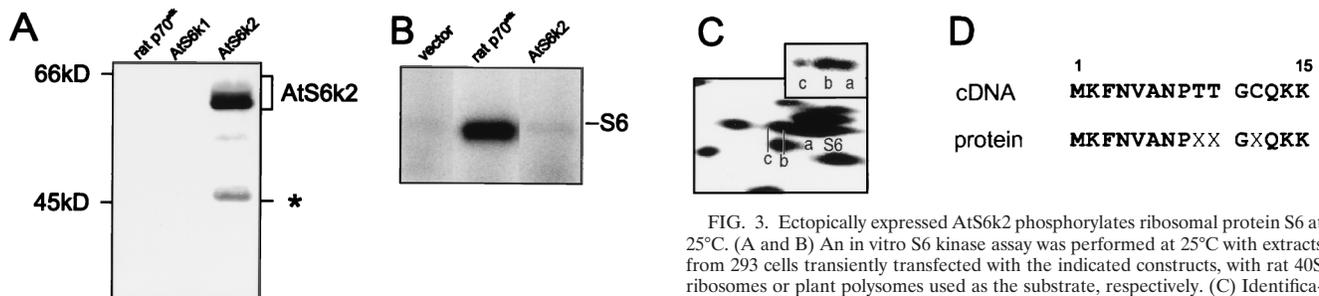


FIG. 2. Ectopic expression of *AtS6k2* in 293 cells. (A) Detection of ectopically expressed *Arabidopsis* AtS6k2 by Western blotting in extracts (20  $\mu$ g of total protein) derived from human 293 cells transiently transfected with either rat p70<sup>s6k</sup>, AtS6k1, or AtS6k2. Differentially phosphorylated forms of AtS6k2 (in brackets) and a putative degradation product (\*) are indicated. (B) Ectopically expressed AtS6k2 does not phosphorylate mammalian S6 when rat 40S ribosomes are employed as the substrate at 37°C.

FIG. 3. Ectopically expressed AtS6k2 phosphorylates ribosomal protein S6 at 25°C. (A and B) An in vitro S6 kinase assay was performed at 25°C with extracts from 293 cells transiently transfected with the indicated constructs, with rat 40S ribosomes or plant polysomes used as the substrate, respectively. (C) Identification of the phosphorylated plant protein as S6. After incubation with AtS6k2, plant ribosomal proteins were separated on two-dimensional polyacrylamide gels (18), and proteins corresponding to differentially phosphorylated forms of putative S6 (inset: autoradiography of a, b, and c) were microsequenced. (D) The obtained N-terminal sequence of the phosphorylated plant proteins and sequence of the corresponding *Arabidopsis* S6 cDNA (EMBL accession no. Y14052) clone are shown. The entire *Arabidopsis* S6 protein shows 64% amino acid identity to human S6 (3).

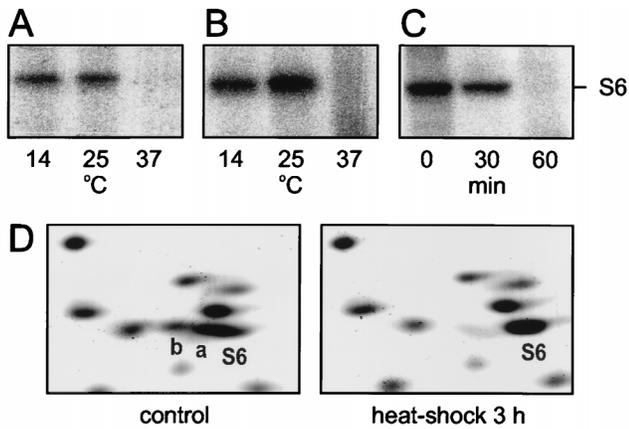


FIG. 4. Characterization of AtS6k activity in planta. (A and B) AtS6k activity isolated from *Arabidopsis* cells, grown in suspension at 25°C, and ectopically expressed AtS6k2, respectively, phosphorylate mammalian S6 in similar temperature-dependent fashions in vitro. Total protein extract from transfected 293 cells (20 µg) or plant extract (1 mg) was immunoprecipitated by addition of 3 µl of AtS6k-specific antibody and assayed for S6 kinase activity. (C) Exposure of *Arabidopsis* suspension culture cells to 37°C in vivo inactivates AtS6k. The duration of heat treatment of the plant cell culture prior to the isolation of AtS6k is indicated. Immunoprecipitated AtS6k activity was assayed at 25°C in vitro. (D) Endogenous S6 protein is dephosphorylated in correlation with inactivation of S6 during heat shock.

ylate S6 in ribosomes derived from plants. The results show that the ectopically expressed AtS6k2, but not mammalian p70<sup>s6k</sup>, was capable of phosphorylating a plant ribosomal protein with an  $M_r$  of 30K (Fig. 3B). To ensure that this protein was equivalent to plant 40S ribosomal protein S6, the corresponding protein spot was resolved on two-dimensional polyacrylamide gels (Fig. 3C), and the amino terminus of the protein was sequenced. The amino-terminal sequence of the isolated protein was identical to the *Arabidopsis* S6 protein sequence determined from a cDNA clone isolated by hybridization with an oligonucleotide corresponding to a conserved motif of S6 (Fig. 3D). Furthermore, S6 was the only protein phosphorylated by AtS6k2 when either 40S or 60S subunits were employed as the substrate (data not shown). Thus, ectopically expressed AtS6k2 is capable of employing S6 from both plant and animal cells as an in vitro substrate at physiological temperatures.

**Endogenous AtS6k is inactivated by heat shock in *Arabidopsis* suspension culture cells.** The data above imply that the loss of S6 phosphorylation in plants in response to heat shock could in part be explained by loss of endogenous AtS6k activity at high temperatures. To test this possibility, extracts were prepared from either *Arabidopsis* suspension culture cells grown at 25°C (Fig. 4A) or 293 cells ectopically expressing AtS6k2 (Fig. 4B). AtS6k proteins were immunoprecipitated with a polyclonal antibody raised against a conserved portion of the plant kinases, and the activity was assayed at increasing temperatures, with mammalian 40S ribosomes used as a substrate. The results demonstrate that plant-derived AtS6k (Fig. 4A), similarly to AtS6k2 ectopically expressed in mammalian cells (Fig. 4B), specifically employs S6 as a substrate in vitro at lower but not higher temperatures. Interestingly, if AtS6k2 derived from 293 cells is first incubated at 37°C, for as short a time as 1 min, and then assayed at 25°C, no activity is detected (data not shown). Consistent with this observation, incubation of *Arabidopsis* suspension cultures for increasing times at 37°C led to inactivation of the plant kinase when assayed in vitro at the permissive temperature of 25°C (Fig. 4C). In the same cul-

tures, dephosphorylation of endogenous S6 followed the inactivation of the kinase (Fig. 4D). Thus, in plant cells AtS6k is inactivated after exposure to heat shock (37°C), but in mammalian cells the activity is protected at the same temperature. This may indicate that the activity of the plant kinase is altered at higher temperatures but that a chaperonin system that facilitates folding of the plant kinase into an active conformation at 37°C exists in mammalian cells. The data above support the inability of others to detect AtS6k activity from ectopically expressed kinase (38) and are consistent with the effects heat shock has on S6 phosphorylation in plants (27) (Fig. 4D).

**Regulation of AtS6k2 activity in mammalian cells.** Mitogenic activation of p70<sup>s6k</sup> is associated with phosphorylation at three key residues, T229, S371, and T389 (24, 25, 34), all of which are conserved in AtS6k1 and AtS6k2. This observation, combined with the more slowly migrating forms of AtS6k2, suggested that it also may be regulated by phosphorylation. Indeed, the activity of immunoprecipitated AtS6k2, prepared from transiently transfected 293 cells, was reduced threefold by treatment with potato acid phosphatase, an effect which was blocked in the presence of the competitive inhibitor 4-nitrophenyl phosphate (Fig. 5A). These data further raised the possibility that AtS6k2 may be regulated by the same signalling pathway as p70<sup>s6k</sup>. To assess this possibility, the effects of serum as well as two upstream inhibitors of mitogen-induced p70<sup>s6k</sup> activation, rapamycin and wortmannin, were tested on AtS6k2 transiently expressed in 293 cells. The ectopically expressed kinases were immunoprecipitated, and S6 kinase activity was measured. Extracts from serum-stimulated cells transiently transfected with mammalian p70<sup>s6k</sup> exhibited increased S6 kinase activity in an immunocomplex assay, a response which was abolished by pretreatment with either rapamycin or wortmannin (Fig. 5B). Strikingly, the plant AtS6k2 also was activated by serum when expressed in human cells, though to a lower extent than mammalian p70<sup>s6k</sup>, an effect which may reflect its higher basal activity in quiescent mammalian cells. Like that of the mammalian kinase, activation of AtS6k2 was sensitive to wortmannin; however, it was resistant to rapamycin (Fig. 5B). Lack of rapamycin sensitivity is consistent with the fact that AtS6k2 contains no region homologous to the mammalian amino terminus (Fig. 1), which is required for rapamycin sensitivity (4, 5, 35). Collectively, these data indicate that AtS6k2 activation is mediated by the same signalling pathway as p70<sup>s6k</sup> in mammalian cells.

**AtS6k2 substitutes for p70<sup>s6k</sup> function in vivo.** Mammalian p70<sup>s6k</sup>/p85<sup>s6k</sup> is believed to be the only kinase responsible for modulating in vivo S6 phosphorylation, even though other kinases have been reported to phosphorylate S6 in vitro (7). Consistent with this hypothesis, recent studies have demonstrated that transient transfection of a rapamycin-resistant mutant of p70<sup>s6k</sup> can protect S6 from dephosphorylation by the macrolide (32). Given that AtS6k2 phosphorylates S6 in vitro and exhibits rapamycin resistance, it was reasoned that if the plant homolog is functional, it also should prevent rapamycin-induced dephosphorylation of S6. Since transfection efficiency in human 293 cells is high (70 to 80%), and rapamycin abolishes S6 phosphorylation (16), protection against rapamycin should be readily discernible by analyzing endogenous S6 phosphorylation. To test this possibility, cells were transfected with either mammalian p70<sup>s6k</sup> or AtS6k2, labelled with <sup>32</sup>P, quiesced, and serum stimulated in the absence or presence of rapamycin (Fig. 6A). The results show that mitogen stimulation of cells expressing mammalian p70<sup>s6k</sup> leads to increased <sup>32</sup>P incorporation into S6 (Fig. 6A) and that this increase is abolished by rapamycin. In contrast, transient expression of AtS6k2 raises basal levels of S6 phosphorylation (Fig. 6B),

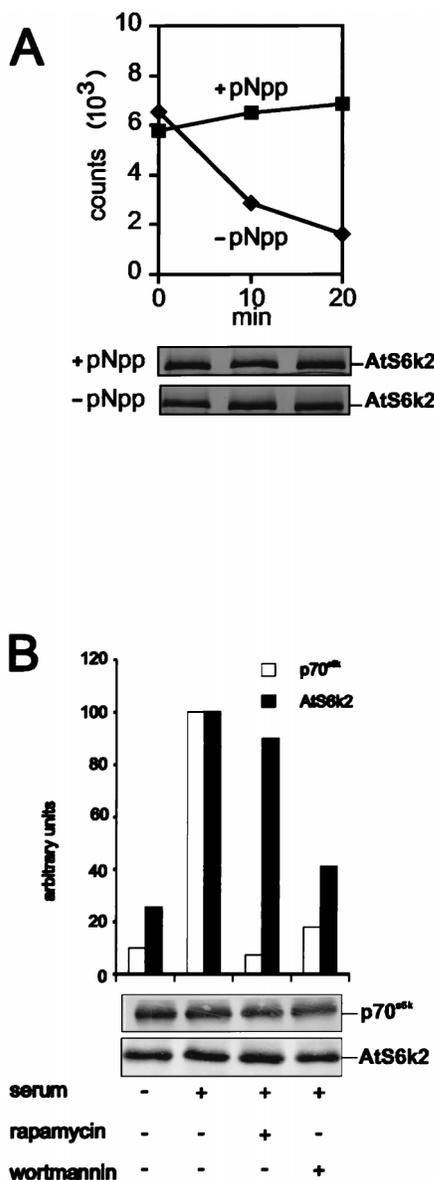


FIG. 5. Regulation of AtS6k2 by phosphorylation. (A) Immunoprecipitates of ectopically expressed AtS6k2 were incubated with potato acid phosphatase for 20 min at 20°C in the presence (+) or absence (-) of the competitive inhibitor pNpp (see Materials and Methods), and S6 kinase assays were performed with rat 40S ribosomes employed as the substrate. Data are from a representative assay that was repeated three times. The AtS6k2 immunoprecipitated in the assay was quantified by Western blotting with alkaline phosphatase (lower panels). (B) Human 293 cells were transiently transfected with rat p70<sup>S6k</sup> and *Arabidopsis AtS6k2* cDNAs. The next day, the cells were washed twice and then quiesced for 24 h in the absence of serum. The cells were then incubated with either rapamycin (20 nM) or wortmannin (200 nM) for 15 min. Serum was added to 10% and the cells were incubated for 30 min prior to extraction. The kinases were immunoprecipitated and assayed, with rat 40S ribosomes employed as the substrate. The intensity of radiolabelled bands was quantified with a Phosphor-Imager (Molecular Dynamics). Expression levels of p70<sup>S6k</sup>, as detected with M1-specific antibody (9), and of AtS6k2, detected with B2-specific antibody, are indicated. Data shown are representative of the results obtained from three independent experiments.

consistent with the higher basal activity of this kinase in the quiescent state (Fig. 5B). Addition of serum increases the extent of phosphorylation; however, more striking, this effect is largely protected in the presence of rapamycin (Fig. 6B). Tak-

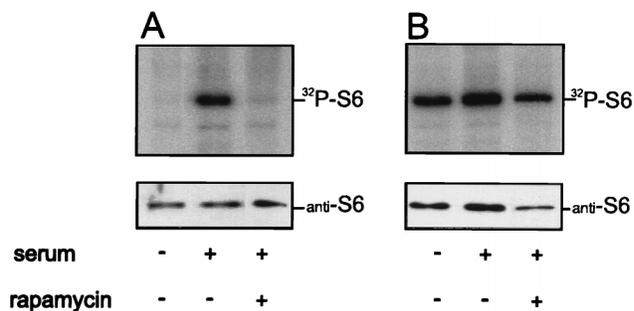


FIG. 6. *Arabidopsis AtS6k2* substitutes for mammalian p70<sup>S6k</sup> in vivo. Human 293 cells were transiently transfected with myc epitope-tagged p70<sup>S6k</sup> (A) or myc epitope-tagged AtS6k2 (B). Following 24 h of starvation in Dulbecco modified Eagle medium without serum and phosphate, cells were incubated in the presence of [<sup>32</sup>P]orthophosphate (0.4 mCi/5ml) for 1 h and then pretreated with rapamycin (20 nM) for 30 min prior to stimulation by the addition of 10% serum and incubation for 1 h. Total ribosomes were prepared, and equal amounts of ribosomes were extracted with acetic acid and analyzed by SDS-PAGE and Western blotting. The amount of <sup>32</sup>P-labelled S6 protein was detected by autoradiography (upper panels), and the total amount of S6 protein was evaluated by Western blotting using an S6-specific antibody and enhanced chemiluminescence detection. The position of the phosphorylated S6 is indicated.

ing advantage of the fact that the activity is resistant to rapamycin, the results demonstrate that AtS6k2 can substitute in vivo for the mammalian p70<sup>S6k</sup> in modulating S6 phosphorylation. These data establish AtS6k2 as a valid plant homolog of the mammalian enzyme.

## DISCUSSION

We demonstrate here that *A. thaliana* contains two genes, designated *AtS6k1* and *AtS6k2*, encoding two closely related proteins which display high homology with the mammalian p70<sup>S6k</sup>. Besides their high homology to p70<sup>S6k</sup>, three lines of evidence support the hypothesis that these two plant kinases represent homologs of the mammalian kinase. First, AtS6k2 selectively phosphorylates mammalian and plant ribosomal S6 protein in vitro. Second, if *A. thaliana* suspension culture cells are exposed to heat shock, the activity of immunoprecipitated endogenous kinase becomes rapidly inactivated, consistent with the observation that heat-shock abrogates S6 phosphorylation in plants (27). Finally, ectopically expressed AtS6k2 can substitute for mammalian p70<sup>S6k</sup> in vivo.

Previously, Zhang et al. (38) ectopically expressed recombinant baculovirus Atpk1, equivalent to AtS6k1, in Sf9 insect cells and demonstrated that instead of S6, this kinase selectively phosphorylated two small ribosomal proteins, thought to be equivalent to the small acidic 60S ribosomal proteins P1 and P2. Although the phosphorylation of an S6 homolog was not noted, examination of the results of their SDS-PAGE, under assay conditions where [ $\gamma$ -<sup>32</sup>P]ATP of high specific activity was employed, reveals a weakly labelled band migrating with an  $M_r$  of 30K, which could correspond to S6. In addition, these assays were conducted at 37°C, which in the case of AtS6k2 ablates in vitro kinase activity. Thus, on the basis of the findings presented here, it will be important to reassess the earlier observations obtained with AtS6k1 at temperatures conducive to plant growth. We have so far failed in our attempts to ectopically express AtS6k1 in human 293 cells and thus have not been able to test its activity against either plant or mammalian ribosomes. However, given the 87% identity at the amino acid level between AtS6k1 and AtS6k2 and the total conservation of intron-exon boundaries within the genomic sequences, the two

isoforms probably have originated from gene duplication of a common ancestor.

The activity of AtS6k2 is reduced below detection level in vitro at temperatures higher than 37°C (Fig. 2B and 4A and B); in addition, endogenous S6 kinase is rapidly inactivated when *A. thaliana* suspension culture cells are exposed to heat shock (Fig. 4C). On the other hand, the enzyme is obviously active in vivo in mammalian cells grown at this temperature (Fig. 6B). This finding may indicate that the activity of the plant kinase is altered at higher temperatures in vitro but that there exists a chaperonin system within the mammals that facilitates folding of the kinase into an active conformation at 37°C in vivo. Although the action of phosphatases cannot be totally excluded, it seems an unlikely explanation for the observed findings, since all experiments were carried out in the presence of phosphatase inhibitors and a corresponding effect on p70<sup>s6k</sup> was not detected (data not shown). Thus, it is tempting to speculate that either the lack of a similar chaperonin system or the sensitivity of this chaperonin system to high temperatures (37°C or higher) could be the reason for the inactivation of AtS6k2 in vivo during heat shock of plant cells.

The results presented here demonstrate that ectopically expressed AtS6k2 can respond to mitogenic stimulation and substitute for mammalian p70<sup>s6k</sup> in rapamycin-treated 293 cells. Recent studies have implicated increased S6 phosphorylation in the selective translational upregulation of a subset of essential mRNAs containing an oligopyrimidine tract at their transcriptional start site (16, 17, 20, 31). These messages encode many components of the translational apparatus, including ribosomal proteins and elongation factors. It could be that S6 phosphorylation and the AtS6k2 signalling pathway in plants are involved in a response similar to that in mammalian cells. Transcriptional start sites of mRNAs encoding plant ribosomal proteins have been mapped in only a few cases, yet polypyrimidine tracts are present in mRNAs coding for the S16 protein in rice (39) as well as the S11, S15, and S28 proteins in *A. thaliana* (GenBank). Earlier studies showed that cytokinin increases S6 protein phosphorylation in detached pumpkin cotyledons (36). With the tools developed here, it will be possible to elucidate the role of phytohormones in *Arabidopsis* S6 kinase regulation and subsequent S6 protein phosphorylation during plant cell growth. In parallel, the use of transgenic plants with altered levels of AtS6k2 expression will be particularly useful in determining the impact of the pathway during plant development.

The ectopically expressed AtS6k2 is activated by serum in mammalian cells and phosphorylates S6 (Fig. 6). The activation of mammalian p70<sup>s6k</sup> is associated with phosphorylation at multiple sites, and treatment with phosphatases in vitro or pretreatment with inhibitors in vivo, such as rapamycin, wortmannin, or the methylxanthine SQ20006, induces p70<sup>s6k</sup> inactivation (12). Similarly, treatment of immunoprecipitated AtS6k2 with potato acid phosphatase reduced the specific S6 kinase activity threefold, indicating that the activity of the plant kinase is also regulated by differential phosphorylation. As pointed out earlier, three sites critical for p70<sup>s6k</sup> activation, T229, S371, and T389, are conserved in AtS6k1 and AtS6k2. The importance of these sites for mammalian p70<sup>s6k</sup> activity has been established previously (5, 24, 25, 34); it will now be important to determine whether the homologous sites are also phosphorylated in AtS6k2 in plants. Furthermore, the structural elements in mammalian p70<sup>s6k</sup> required for activation and substrate recognition also appear to be conserved in the plant kinases. Indeed, the catalytic and linker domains, which include the key regulatory phosphorylation sites of the mammalian p70<sup>s6k</sup> listed above, are also conserved in the recently

described *Drosophila* p70<sup>s6k</sup> (30, 33). However, the plant enzyme, and to a lesser extent the *Drosophila* enzyme, differ from the mammalian homolog at their carboxy and amino termini, which are implicated in regulating the key phosphorylation sites associated with kinase activation in the mammalian cell. Thus, even though the plant kinase contains the conserved elements which classify it as a ribosomal protein S6 kinase, the structural motifs involved in the regulation of this activity appear distinct. It will now be important to identify the signalling components which operate on these structural motifs to bring about kinase activation in plants.

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