Effects of Partial Suppression of Ribosomal Protein S6 on Organ Formation in Arabidopsis thaliana

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An expression library of Arabidopsis thaliana cDNAs was randomly introduced into A. thaliana. The transformant pool was used to obtain a line, c105, with reduced apical dominance and irregular positioning of leaves and flowers. The inserted DNA was a 3′-fragment of the ribosomal protein S6 gene with antisense orientation. The transcriptional level of the ribosomal protein S6 was lower in c105 than in the wild-type plant. Introduction of the same fragment into the wild-type plant gave phenotypes similar to those of c105, so the phenotypes of c105 were due to the S6 antisense expression. The phenotypes suggest selectively reduced function of specific proteins rather than an overall decrease in protein function caused by defective ribosomal biogenesis.

Key words: ribosomal protein S6; Arabidopsis thaliana; organ formation; ribosomal biogenesis

Ribosomal protein S6 (rpS6) is a component of the 40S ribosomal subunit of eukaryotic cells and is the major substrate for protein phosphorylation in the subunit. The phosphorylation level is associated with the protein synthesis rate, and increases in response to mitogens, so that the regulation of S6 phosphorylation is a key step in the control of cell proliferation. Phosphorylation of S6 caused the selective translation of mRNAs, called TOP mRNAs, that contain a 5′-terminal oligopyrimidine tract (5′-TOP). TOP mRNAs encode proteins associated with the translational apparatus such as ribosomal proteins, elongation factors, and a poly(A)-binding protein. Defective expression of the rpS6 gene seems to cause cell damage or death. Conditional deletion of the rpS6 gene in the liver of adult mice, however, gave unexpected results; the livers from starved animals deficient in S6 grow in response to nutrients, even though biogenesis of the 40S ribosome is abolished.1) It has also been reported that P-element-induced mutations of the Drosophila rpS6 gene results in overproliferation and abnormal development of hematopoietic cells.2) These reports suggest that the rpS6 gene might be involved in translational regulation in a more complex way than usually envisaged for a housekeeping gene.

We describe in this paper an Arabidopsis mutant screened from newly prepared lines expressing random Arabidopsis cDNAs. The expression level of the rpS6 gene in the mutant was lower because of overexpression of antisense RNA. The phenotypes cannot easily be explained by mere down-regulation of ribosomal biogenesis, and a possible mechanism for selective translational regulation by rpS6 in plants will be described below.

Materials and Methods

Generation of an Arabidopsis cDNA library. A binary vector was prepared for expression of cDNAs on the basis of pBI121.3) pBI121 was digested with HindIII and EcoRI, and the excised b-glucuronidase gene was replaced with a DNA fragment that had been prepared by the annealing of two oligo-DNAs, 5′-AGCTTAGGCTGCGCGGCGATCCCCGGGGCT-3′ and 5′-AATTAGCCCCGGGGATCCGCGGCCGCGAGCTCA-3′ (pBI121delGUS). The SphI fragment containing a polylinker site between the promoter and terminator of CaMV 35S RNA was excised from pRT105,4) and cloned into the HindIII site of pBI121delGUS after blunting the ends of both the vector and the insert, giving a binary vector with a polylinker site incorporating the EcoRI site (pBI-PL). pBI-PL was digested with EcoRI and dephosphorylated by calf intestine alkaline phosphatase treatment for ligation with cDNAs.

Total RNA was prepared from the aerial parts of 20-day-old Arabidopsis thaliana seedlings (ecotype Columbia) by the method of Logemann et al.5) Poly(A)⁺ RNA was purified from the total RNA with Dynabeads (Dynal) and used for the synthesis of cDNAs with EcoRI adapters, with use of a Marathon cDNA amplification kit (Clontech). These cDNAs were ligated into the dephosphorylated EcoRI site of pBI-PL, the resulting plasmids being used to transform...
E. coli cells (Electromax DH10B, Gibco BRL) by electroporation. About $2 \times 10^7$ independent clones were obtained after ligation with 0.2 µg of the vector plasmid. More than 70% of the clones had the cDNA insert in the vector.

The cDNA library constructed in pBI-PL was transferred from E. coli to Agrobacterium tumefaciens GV3010 (pMP90) by the triparental method. A. thaliana was transformed by vacuum infiltration. Transformants were selected on a Murashige-Skoog medium containing 100 mg/l kanamycin and 250 mg/l timenten (Smith-Kline Beecham AG, Switzerland) by incubation at 24°C under continuous light. The kanamycin-resistant plants were transplanted 20 days after germination to vermiculite and grown further for phenotype observation.

Isolation of c105 and sequence analysis of insert DNA. T1 plants were screened for morphological differences from the wild-type plant. T2 progeny from the putative mutants were examined for inheritance of the phenotypes. Genomic DNA was extracted from 100 mg of rosette leaves of the c105 line with a DNeasy plant kit (Qiagen), and PCR was done to amplify the cDNA insert with primers for the 35S promoter (5'-GACGCACAATCCCACTATCCT-TGG-3') and terminator (5'-TATGCTCAACACAT-GACGCCAACACAC-3'). The PCR fragment was cloned into a vector (pGEM-Teasy, Promega), giving pGEM-antiS6. The sequence of the insert cDNA was analyzed and compared with database sequences with the BLAST program.

Northern blotting. Total RNA was isolated from the kanamycin-resistant T2 progeny of c105 and wild-type plants with an RNeasy plant mini kit (Qiagen). Fifty micrograms and 10 µg of total RNA for detection of sense RNA and antisense RNA, respectively, were separated on a 1% agarose gel containing 1% formaldehyde, and transferred to a nylon membrane (Hybond-N⁺, Amersham Pharmacia). Antisense and sense RNA probes were synthesized with a DIG RNA labeling kit (Roche Molecular Biochemicals) with SalI-digested pGEM-antiS6 and NcoI-digested pGEM-antiS6 as the template DNAs, respectively. The RNA blots were hybridized with the probes and treated with an anti-digoxigenin-Fab alkaline phosphatase conjugate (Roche Molecular Biochemicals). An Attphos substrate set (Roche Molecular Biochemicals) was used as the substrate for detection of sense RNA, and fluorescent emission was detected with an FMBIO II fluorescence imaging analyzer (Hitachi). Antisense RNA was monitored by detection of the alkaline phosphatase activity as the coloration with nitroblue tetroxide chloride (100 mg/ml) and X-phosphate (50 mg/ml) (Boehringer).

Inverse PCR. The genomic DNA (500 ng) of c105 was digested with EcoRI, treated with phenol-chloroform, and recovered by ethanol precipitation. The resulting pellet was dissolved in 20 µl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and ligated to form circular DNAs. The reaction mixture was treated with phenol-chloroform, precipitated with ethanol, and dissolved in 10 µl of TE to give template DNA for inverse PCR. Nested PCR was done with two sets of primers designed for the sequence between the 35S terminator and T- DNA left border sequence: 5'-CGGCCAACATGTGTTATTAATTG-3' and 5'-CGAGAAGGAGGGAAGAAGCG-3' for the first PCR, and 5'-GCGTCAATTTGTATT-ACACCAC-3' and 5'-CGCCATCGGCTGGCGAAACCC-3' for the second PCR. The amplified DNA fragment was cloned into a vector (pGEM-Teasy, Promega) and the genomic DNA flanking the T-DNA left border was sequenced.

Reintroduction of the rpS6 antisense gene into wild-type Arabidopsis. The cDNA insert was excised from pGEM-antiS6 with EcoRI and cloned into the EcoRI site of pBI-PL with antisense orientation, the resulting vector being used to prepare Arabidopsis transformants as described above. The phenotypes of T2 progeny from about 20 transformant lines were observed.

Results

Screening and Phenotypes of c105

Some 700 transformants were obtained as kanamycin-resistant plants. PCR amplification of insert cDNAs of 24 randomly selected plants showed that they were derived from independent transformation events (not shown). The repression by the transgene integration of the mRNA level of endogenous genes was confirmed by results of northern blotting of 10 transgenic lines. The extent of repression varied from line to line, but all 10 lines had decreased amounts of transcript (not shown). Among about 700 lines, c105 was the sole line that showed heritable phenotypes. We found some other lines that could not generate progeny.

The c105 line was found as a putative mutant with a bushy appearance (Fig. 1). Detailed observation of the T2 progeny showed unusual positioning of the cauline leaves and flowers, in addition to the extensive growth of axillary shoots. Only one cauline leaf is formed at each position of inflorescence in wild-type A. thaliana, c105 generated two or three leaves close together. Two leaves at the base of the axillary shoot close to the leaf on the primary shoot frequently were observed, giving the impression of three leaves at one site (Fig. 2A). When three leaves formed close together on the primary shoot and two on each axillary shoot close to them, nine leaves were
seen in close proximity (Fig. 2B). This unusual positioning was also found with flowers (Fig. 2C), a flower and one leaf or more sometimes being observed in close proximity (Fig. 2D). Although the mutant phenotype had regularity in that the multiple organs were formed close together, there was some irregularity in phenotypes such as no petioles in a flower (Fig. 2E) or irregular phyllotaxy (Fig. 2F).

The T3 plants from individual T2 plants did not segregate for both the phenotype and kanamycin resistance, indicating that the T1 plant was homozygous for both the cause of the phenotype and the transferred-DNA (T-DNA) insertion. The presence of the same T-DNA in all T2 progeny was further confirmed by PCR with primers designed for the 35S primer and terminator, which amplified a DNA fragment containing a cDNA. The results showed amplification of DNA fragments all of identical size (not shown). The extent of the phenotypes was not identical among the progeny from each T2 plant, the T3 progeny from one T2 plant showing severer phenotypes than those from the other T2 plant. The phenotypes were variable even among T3 plants from a single T2 plant.

Identification of cDNA integrated in c105 and examination of its expression

As already mentioned, PCR amplified only one DNA fragment, suggesting only one T-DNA integration in c105. A homology search of the insert DNA showed that the 3′-half of the rpS6 gene (At5g10360, accession no. ATY14052) was cloned in the T-DNA in the antisense orientation (Fig. 3A).

As a first step to confirm that the mutant phenotypes were caused by the expression of antisense RNA, and to examine the effects of the antisense RNA in reducing the amount of mRNA of the internal S6 gene, northern blotting with either the sense or antisense RNA probe was done. Northern blotting with the sense RNA probe showed a substantial amount of antisense RNA of the partial S6 gene in c105 (Fig. 3B). The amount of sense RNA of the in-
ternal rpS6 gene was lower in c105 than in the wild-type plant (Fig. 3C).

**T-DNA insertion site**

Genomic DNA flanking the T-DNA left border was recovered by inverse PCR. The sequence of this genomic DNA showed that T-DNA had been inserted in an intergenic region between the genes encoding a laccase-like protein and a putative protein kinase at 17721 nt on the BAC clone F7J8 (accession no. AL137189). This result shows that the T-DNA did not disrupt any putative gene in c105.

**Generation of additional antisense S6 lines and their phenotypes**

The amount of mRNA of the rpS6 gene was less in c105 and the T-DNA had been inserted in an intergenic region, so the lower level of rpS6 mRNA might account for the altered phenotypes. To examine the possibility, the antisense rpS6 DNA fragment was introduced into wild-type Arabidopsis plants and we examined the phenotypes of the new antisense lines. Generally speaking, the major characteristics of the c105 line were found also in the transformants, such as the extensive growth of axillary shoots (Fig. 4A) and the formation of multiple leaves and flowers in close proximity (Figs. 4B–4C). Several transformant lines had the abnormal phyllotaxy (Fig. 4D) sometimes found in the c105 line. The extent of the phenotypes was segregated in most transformants, which showed 3-to-1 segregation for kanamycin resistance. This segregation in the progeny from some lines was initially apparent in very young seedlings as a difference in plant size (Fig. 4E). Segregation analysis of kanamycin resistance of the next generation showed that the smallest plants were homozygous, those of intermediate size were heterozygous, and those of normal size were wild-type plants. Some of the smallest homozygous plants grew only 2–3 cm tall even at later stages of growth (Fig. 4F), without normal indeterminate inflorescences, and with growth terminating when flowers formed. These phenotypes were not seen in the c105 line. The heterozygous plants had phenotypes similar to those of c105. These results indicate that the phenotypes seen in the c105 line occurred when there was expression of antisense RNA of rpS6 at a certain dose.

**Discussion**

New methods for functional genomics have been developed for the generation of new kinds of mutants in the postgenomic era of plant research.8,9 We regard the random expression of cDNAs in A. thaliana as one such method of much promise. The cDNAs are cloned nondirectionally in the vector, so
Partial Suppression of rpS6 Disturbing Organ Formation

Two possible mechanisms for the production of phenotypes are up-regulation of a gene function by overexpression of the sense RNA, and down-regulation of a gene function by expression of the antisense RNA or aberrant-sense RNA. LeClere and Bartel recently reported a similar kind of library in Arabidopsis. In their independent research, they did directional cloning of cDNAs to achieve cosuppression of internal mRNAs, a form of post-transcriptional gene silencing. The idea of random expression of cDNAs under the 35S promoter is described in their report, and our results further support the usefulness of this system.

Library screening gave a transformant line designated c105 with unusual phenotypes. The results of the northern blotting and re-introduction of T-DNA into wild-type plants showed that the phenotypes resulted from the reduced level of rpS6 gene expression due to the antisense expression of its C-terminal half. rpS6 is a component of the 40S ribosomal subunit of eukaryotic cells, and is involved in the formation of the ternary complex (eIF2, GTP, and met-tRNA) and the subsequent complexing with the mRNA-loaded 40S subunit that is an essential step in protein translation. rpS6 is therefore generally categorized as being a housekeeping gene, and complete disruption of the S6 function should be lethal. Two cytoplasmic rpS6 genes have been identified from the complete genomic sequence of Arabidopsis, AL353995 and AL031004, the former corresponding to the DNA fragment recovered from c105. The corresponding EST clones, ATY14052 (At5g10360) and AF034217 (At4g31700), respectively, are present, so both genes are expressed. These rpS6 genes have 84.0% nucleotide identity, and it is likely that they have redundant functions. The antisense system could simultaneously reduce the mRNA level of both rpS6 genes to an appropriate level that could not be achieved by knocking out of only one of the redundant genes, by complete suppression of both genes by RNAi, or by disruption of both genes. This is one practical advantage of the antisense method.

The characteristic phenotype of c105 involved the formation of multiple leaves and flowers in close proximity, which has, as far as we know, not previously been found in mutants. This phenotype cannot simply be explained by the reduced translational activity caused by a ribosomal defect, because evenly repressed protein function should cause overall damage to a plant by inhibiting all growth and developmental processes such as germination and stem and root elongation, as well as organ formation. The finding of this phenotype therefore suggests that the partial repression of rpS6 influenced the function of certain specific proteins. On the other hand, the
phenotype of reduced stature observed for some of the new transformants could be explained by an overall decrease in protein synthesis. The *Arabidopsis* gene *PINHEAD* is similar to the rabbit translation initiation factor eIF2C. This factor promotes the aforementioned complex formation (eIF2, GTP, and met-tRNA with the mRNA-loaded 40S ribosomal subunit). On the basis of the phenotypes of the *pinhead* mutant, it has been suggested that the *PINHEAD* gene product could promote the translation of a specific subset of mRNAs, although eIF2 must have a general role in the translation of all mRNAs. c105 and *pinhead* mutants have other similarities; the phenotypes of both mutants are variable, and both genes might be involved with positional information. There might be a mechanism characteristic of components of the translational apparatus to account for the similarities.

The synthesis of mammalian proteins associated with the translational apparatus encoded by TOP mRNA is selectively regulated by mitogenic and nutritional stimuli at the translational level. This regulation is mediated by the phosphorylation of rpS6 via the activation of rpS6 kinase upon a proliferative stimulus. Organ formation is an event involving cell proliferation, and might therefore be controlled by the selective translational regulation of some genes that have a characteristic structure in mRNAs like 5’-TOP, which is common to TOP mRNAs. The rpS6 expression in the c105 mutant might be reduced to a level not high enough to be lethal but is enough to repress the translation of certain genes that need high rpS6 activity. It is possible also that translation was evenly repressed, but the protein function was differently affected, since the translational level needed depends on the protein species. Antisense expression might not cause an overall translational defect, because the CaMV 35S promoter is not necessarily active in all tissues.

Some reports suggest a more complex role for the rpS6 protein. Volarevic et al. examined the effects of deletion of the S6 gene in mice, and showed that the liver from starved mice grows in response to nutrients even with abolished biogenesis of the 40S ribosomes. Their results showed that S6-deficient liver cells can synthesize proteins and grow. On the other hand, the liver cells failed to proliferate or induce cyclin E expression after partial hepatectomy, despite the formation of active cyclin D-CDK4 complexes. They argued, on the basis of the result that the formation of 40S ribosome biogenesis may have effects of deletion of the S6 gene in mice, and showed that the synthesis of mammalian proteins associated with the translational apparatus encoded by TOP mRNA is selectively regulated by mitogenic and nutritional stimuli at the translational level. This regulation is mediated by the phosphorylation of rpS6 via the activation of rpS6 kinase upon a proliferative stimulus. Organ formation is an event involving cell proliferation, and might therefore be controlled by the selective translational regulation of some genes that have a characteristic structure in mRNAs like 5’-TOP, which is common to TOP mRNAs. The rpS6 expression in the c105 mutant might be reduced to a level not high enough to be lethal but is enough to repress the translation of certain genes that need high rpS6 activity. It is possible also that translation was evenly repressed, but the protein function was differently affected, since the translational level needed depends on the protein species. Antisense expression might not cause an overall translational defect, because the CaMV 35S promoter is not necessarily active in all tissues.

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The phenotype characteristic of formation of multiple organs in close proximity suggests abnormal regulation of the length of the plastochron in c105. In normal *Arabidopsis* plants, when the shoot apical meristem has generated a leaf primordium in its peripheral zone, the next primordium is not immediately formed, and newly generated cells participate in stem formation. There may be a mechanism that suppresses the formation of the next organ for a certain time after an organ has been formed. This hypothesis presumes that the repressor of organ formation becomes functional upon organ formation, but is activated only after multiple organs have been formed in c105. Alternatively, there may be a factor that keeps stem formation going, but is inactivated at the time of organ formation, and is reactivated after the formation of an organ in wild-type *A. thaliana*, although such re-activation does not take place until the formation of more than one organ in c105. The rice mutants *pla1* and *sho1* have an altered plastochron and produce an increased number of leaves because of the reduced length of the plastochron. The phenotype of c105 is different from the phenotypes of these rice mutants in that the length of the plastochron is continuously reduced in those mutants, but not in c105; after almost simultaneously forming more than one leaf or flower, the normal time was needed until the next organ was formed. The reduced level of rpS6 may influence the hypothetical factors that control the timing of organ formation. The rpS6 antisense lines might therefore be good materials to study the mechanism for the temporal regulation of leaf and flower differentiation.

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**References**


