# Mutations in the GW-repeat protein SUO reveal a developmental function for microRNA-mediated translational repression in *Arabidopsis*

**Li Yang, Gang Wu, and R. Scott Poethig[1]**

Department of Biology, University of Pennsylvania, Philadelphia, PA 19104-6018

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**Plant microRNAs (miRNAs) typically mediate RNA cleavage, but examples of miRNA-mediated translational repression have also been reported. However, the functional significance of this latter process is unknown. We identified *SUO* in a screen for *Arabidopsis* mutations that increase the accumulation of the miR156-regulated gene *SPL3*. *suo* has a loss-of-function phenotype characteristic of plants with reduced Argonaute (AGO)1 activity. An analysis of RNA and protein levels in *suo* mutants demonstrated that this phenotype is a consequence of a defect in miRNA-mediated translational repression; the effect of *suo* on vegetative phase change is attributable to a reduction in miR156/miR157 activity. *SUO* encodes a large protein with N-terminal bromo-adjacent homology (BAH) and transcription elongation factor S-II (TFS2N) domains and two C-terminal GW (glycine and tryptophan) repeats. SUO is present in the nucleus, and colocalizes with the processing-body component DCP1 in the cytoplasm. Our results reveal that SOU is a component of the miRNA pathway in *Arabidopsis* and demonstrate that translational repression is a functionally important aspect of miRNA activity in plants.**

MicroRNAs (miRNAs) are important regulators of gene expression throughout eukaryotes. In animals, miRNAs repress gene expression by inhibiting translation and by promoting mRNA degradation (1). In plants, miRNAs primarily mediate RNA cleavage (2). Some plant miRNAs also promote translational repression, but the extent and functional significance of this process is still unknown.

Evidence that miRNAs repress translation in plants emerged soon after their discovery. One of the first miRNAs to be identified, miR172, targets the transcription factor APETALA2 (AP2). Aukerman and Sakai (3) found that overexpressing miR172 decreases the abundance of the AP2 protein without affecting the abundance of the AP2 mRNA, whereas Chen (4) reported that mutations that reduce miR172 levels increase the abundance of the AP2 protein without affecting AP2 mRNA. Subsequently, it was reported that overexpressing the miR156 target, *SPL3*, produced an increase in the SPL3 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE3) transcript without producing a corresponding increase in the SPL3 protein (5). Additional evidence for miRNA-mediated translational repression in plants came from the discovery that mutations in the microtubule-severing protein KATANIN (KTN) and the cap-binding protein VARICOSE (VCS) increase the amount of protein produced by some miRNA-regulated genes without causing a corresponding increase in the abundance of their mRNAs (6, 7). The observation that ARGONAUTE1 (AGO1) ribonucleoprotein complexes are associated with polysomes provides additional support for the conclusion that miRNAs repress translation in *Arabidopsis* (8).

However, the functional significance of miRNA-mediated translational repression in plants is unknown because miRNAs that mediate translational repression also cause transcript cleavage (7, 9–12), making it difficult to distinguish the relative importance of these processes. Furthermore, it is still unclear whether the morphological phenotypes of mutants that affect miRNA-mediated translational repression are attributable to a defect in this process, rather than to the other processes in which these genes are involved (13, 14). We identified loss-of-function mutations of *SUO* in a screen for mutations that affect the expression of a gene involved in vegetative phase change. In addition to accelerating the expression of adult traits, *suo* mutants have a variety of defects characteristic of genes required for miRNA biogenesis or function. Here, we show that *SUO* encodes a GW (glycine and tryptophan) repeat protein required for miRNA-mediated translational repression. The evidence that the mutant phenotype of *suo* can be largely, if not entirely, attributed to a defect in miRNA activity indicates that translational repression is a functionally important aspect of miRNA activity in *Arabidopsis*.

## Results

**Identification of *suo*.** miR156 and miR157 are highly expressed early in shoot development and promote the expression of juvenile traits by repressing the expression of 10 members of the SPL transcription factor family in *Arabidopsis* (5, 11, 15). Mutations that interfere with the biogenesis of miR156/miR157, or that reduce the activity of AGO1, cause an increase of SPL transcripts and accelerate the expression of adult vegetative traits (16–18).

To identify genes required for the expression and/or action of miR156/miR157, we screened for mutations that enhance the expression of the miR156/miR157-regulated reporter *pSPL3::eGFP-SPL3*. Plants transformed with *pSPL3::eGFP-SPL3* had no detectable GFP in leaves 1 and 2, but GFP was expressed increasingly brightly in the nuclei of subsequent leaf primordia and fully expanded leaves (19). *pSPL3::eGFP-SPL3* seeds were mutagenized with ethyl methanesulfonate and the M2 progeny of these plants were screened under a stereomicroscope for seedlings with elevated GFP expression. One mutant identified in this screen had elevated GFP expression in leaf 5 and also had more serrated leaves (Fig. 1*A*). We named this mutant *suo-1* (meaning "shuttle" in Chinese) to reflect its precocious phenotype. Several additional alleles of *SUO* were subsequently identified in our laboratory and in the SALK T-DNA insertion collection (see below).

As seedlings, *suo* mutants are most readily identifiable by their slow rate of development and their slightly enlarged first two leaves (Fig. 1 *B* and *C*). Under long day (LD) conditions, *suo-2* plants produced three to four leaves lacking abaxial trichomes ($3.2 \pm 0.6$, $n = 24$), whereas wild-type Columbia (Col) plants produced four or five ($4.3 \pm 0.4$, $n = 24$) (Fig. 1*C*). Consistent with their accelerated abaxial trichome production, the fully expanded rosette leaves of mutant plants were more serrated

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[1]To whom correspondence should be addressed. E-mail: spoethig@mail.sas.upenn.edu.

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than normal and displayed the elongated shape and short, thick petiole typical of adult leaves (Fig. 1*C*). The siliques of mutant plants were occasionally spaced irregularly (Fig. 1 *D* and *E*) or fused at the base (Fig. 1*F*). *suo* mutants were also more drought-resistant than wild-type plants: 96% of Col plants ($n$ = 25) withered after being exposed to drought stress for 2 wk, compared with only 12% of *suo-2* plants ($n$ = 20) (Fig. 1*G*). Because drought resistance is often associated with a change in abscisic acid (ABA) sensitivity, we tested the effect of ABA on seed germination. Consistent with their drought-resistant phenotype, *suo* mutants were hypersensitive to ABA. Eighty percent of Col seeds germinated in the presence of 1 μM ABA, compared with only 40% of *suo-2* and 0% of *suo-3* seeds ($n$ = 120 for each genotype) (Fig. 1*H*). *suo* mutants also produced significantly fewer rosette leaves (7.4 ± 0.7, $n$ = 24) than Col (10.1 ± 0.6, $n$ = 24), both because of their reduced rate of leaf initiation and because they stopped producing leaves earlier than normal (Fig. 1*I*). Thus, *SUO* is required for a wide range of biological processes. The observation that the effect of *suo* on abaxial trichome production and leaf shape is semidominant (Fig. S1) suggests that *SUO* is haploinsufficient.

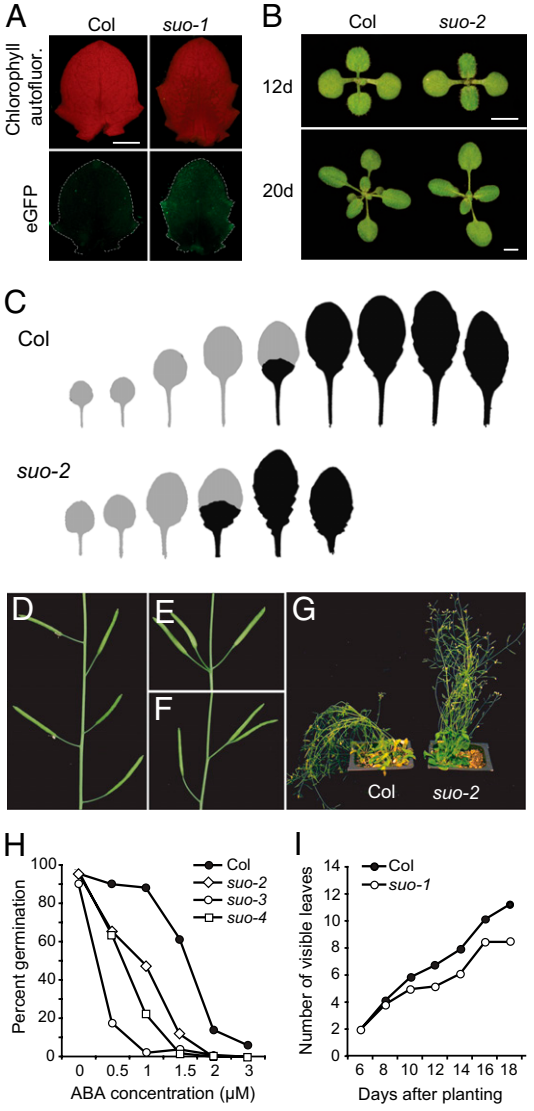


**Fig. 1.** *suo* has a pleiotropic phenotype. (*A*) The fifth leaf of *pSPL3::eGFP-SPL3* and *suo-1 pSPL3::eGFP-SPL3* plants. (Scale bar: 1 mm.) (*B*) 12- and 20-d-old wild-type Col and *suo* mutants. (Scale bar: 5 mm.) (*C*) Fully expanded rosette leaves of Col and *suo-2*. Gray indicates leaves lacking abaxial trichomes, and black indicates leaves with abaxial trichomes. (*D*) Siliques are spaced evenly in Col. In *suo,* siliques are sometimes clustered at one node (*E*) or fused at the base (*F*). (*G*) Col and *suo-2* plants after 2 wk without water. (*H*) *suo* mutants have a lower germination rate on ABA-containing plates than Col. (*I*) The rate of leaf initiation is reduced in *suo*.

**SUO Encodes a GW-Repeat.** Map-based cloning revealed that *suo-1* is a point mutation in At3g48050 that converts a glycine to an arginine at position 324 (Fig. 2*A*). *suo-2* is a 14-bp deletion in At3g48050 (nucleotides 2041–2054 of the coding sequence), which introduces a stop codon immediately downstream of the deletion (Fig. 2*A*). Four additional T-DNA insertion alleles (*suo-3* to *suo-6*) were obtained from the *Arabidopsis* Biological Resource Center (Fig. 2*A*) and were found to have a phenotype identical to *suo-1* and *suo-2*. Quantitative RT-PCR (qRT-PCR) revealed that *suo-2* and *suo-3* significantly reduce the abundance of the SUO transcript (Fig. S2*A*). This result, and the observation that all of these alleles have the same morphological phenotype, suggests that this phenotype represents the null, or nearly null, phenotype of At3g48050. A *35S::At3g48050* construct rescued the mutant phenotype of *suo-2*, confirming that this gene corresponds to *SUO* (Fig. S2*B*).

The primary transcript of At3g48050 contains four exons and encodes a 1,613-aa protein (Fig. 2 *A* and *B*) that is conserved throughout plants (Fig. S3) but has no close relative in animals. The most highly conserved part of the protein is its N-terminal end, which possesses a bromo-adjacent homology (BAH) domain and a region with similarity to the transcription elongation factor S-II (TFS2N). The central part of the protein has no recognizable domains and is poorly conserved between species. The C-terminal part of the protein contains two highly conserved GW-containing sequences, often found in proteins that interact with Argonaute, a key regulator of miRNA and siRNA-mediated gene silencing (20, 21). In addition, SUO contains five conserved L/FDLN sequences, which closely resembles the EAR/DLN transcriptional repressor motif L/FDLNL/F(x)P (22).

*SUO* has a closely related paralog (95% amino acid identity), At3g48060, located 3.6 kb from its 3′ end (Fig. S4*A*). Although qRT-PCR revealed that this gene is expressed at nearly the same level as *SUO*, an RNA-null mutation of At3g48060 (SALK_086029) had no homozygous phenotype (Fig. S4 *B* and *C*). Furthermore, the phenotype of plants heterozygous for SALK_086029 and *suo* was identical to that of plants heterozygous for *suo* (Fig. S3*D*). Given the exquisite sensitivity of plants to the dose of *SUO* (Fig. S1), these results either indicate that At3g48060 has very little activity or that it is functionally distinct from *SUO*.

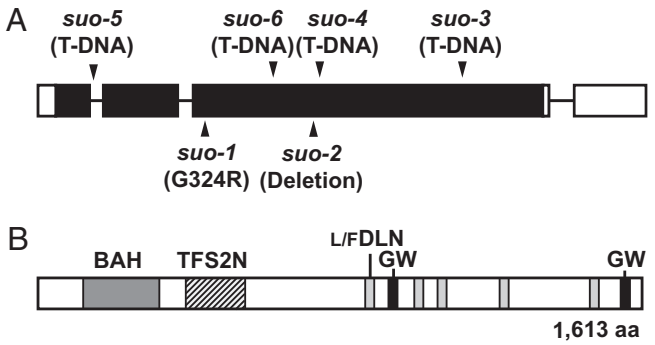


**Fig. 2.** *SUO* encodes a GW-repeat protein. (*A*) Genomic structure of At3g48050, and the location and nature of *suo* alleles. Box, exon; line, intron; open box, UTR. (*B*) The domain organization of the At3g48050 protein.

**Vegetative Phenotype of *suo* Is Attributable to a Defect in miR156.** The constellation of defects displayed by *suo* is characteristic of mutants that disrupt miRNA biogenesis or function (18, 23). To explore the possibility that *SUO* is involved in these processes, we generated double mutants between *suo* and mutations that affect miRNA biogenesis (*abh1, se*), miRNA export/stability (*hst*), and miRNA activity (*sqn*, *ago1*) (2) (Fig. 3). *suo-2* interacted relatively weakly with the AGO1-defective mutants (18) *ago1-45* and *sqn-1*. Under short day (SD) conditions, *suo-2 sqn-1* and *suo-2 ago1-45* double mutants produced one less leaf lacking abaxial trichomes and had a slightly stronger leaf shape phenotype than either single mutant (Fig. 3 *A–F*). *suo-2* interacted more strongly with *hst*, *abh1*, and *se*. Although double mutants had only a modestly more severe abaxial trichome phenotype, they were much smaller than the single mutants and displayed the up-curled leaf phenotype typical of mutants with severe defects in miRNA activity (Fig. 3 *G–L*). These results suggest that SUO acts in association with SQN and/or AGO1, and independently of ABH1, SE, or HST.

Constitutive overexpression of miR156 under the regulation of the CaMV 35S promoter delays vegetative phase change and flowering and accelerates leaf initiation (11, 15, 24, 25), which is the opposite of the *suo* phenotype. To determine whether *SOU* is required for the activity of miR156, we examined the effect of *suo-2* on the phenotype of *35S::MIR156A*. Double mutants (*35S::MIR156A suo-2*) had six fewer juvenile leaves (Fig. 4*A*) and a slower rate of leaf initiation (Fig. 4*B*) than *35S::MIR156A* transgenic plants. As an additional test of this hypothesis, we asked whether loss-of-function mutations in miR156-regulated genes suppress the *suo* phenotype. Indeed, the leaf morphology (Fig. 4*C*) and rate of leaf initiation (Fig. 4*D*) of *suo-2 spl9-4 spl15-1* mutants was intermediate between that of *suo-2* and *spl9-4 spl15-1*. This result supports the hypothesis that the phase change phenotype of *suo* is attributable to an increase in the expression of miR156-regulated SPL genes resulting from a decrease in the activity of miR156.

**SUO Is Not Required for miRNA Biogenesis.** To define the molecular function of SUO, we examined the effect of *suo* mutations on the abundance of mature miRNAs and their precursor transcripts. RNA blots of 14-d-old seedlings revealed that mature miRNAs were largely unaffected (miR156, miR161, miR164, miR398) or elevated (miR159, miR165/miR166, miR167, miR169, miR171, miR172) in *suo* mutants (Fig. 5*A*). *suo-3* and *suo-4* had a slightly stronger effect than *suo-2*. qRT-PCR revealed that the abundance of miRNA precursors was correlated with the abundance of the mature miRNA, suggesting that the increase in mature miRNA levels is a consequence of increased transcription of their precursors (Fig. 5*B*). With the exception of CUP-SHAPED COTYLEDON2 (CUC2), there was no significant difference in the abundance of miRNA-regulated transcripts in *suo* and Col plants (Fig. 5*C*). Thus, SUO is not required for miRNA biogenesis or stability or for the destabilization of miRNA-regulated transcripts.

**SUO Promotes miRNA-Mediated Translational Repression.** *suo-1* was originally identified because it increases the fluorescence of eGFP-SPL3 (Fig. 1*A*). qRT-PCR revealed no significant difference in the abundance of the eGFP-SPL3 transcript in *suo-1* vs. Col (Fig. 6*A*), but Western blots probed with an antibody to GFP confirmed that the eGFP-SPL3 protein is elevated in the third and fourth leaf of *suo-1* (Fig. 6*B*). Similar results were obtained for a *pSPL9::GUS-SPL9* reporter construct transformed into Col and *suo-1* plants: β-glucuronidase (GUS) activity was approximately twofold higher in *suo-1* compared with Col, and this increase was not associated with an increase in the level of the GUS-SPL9 transcript (Fig. 6*C*). These results suggest that SUO is required for the translational repression of both the SPL3 and SPL9 transcripts.

If SUO is specifically involved in miRNA-mediated translational repression, the increased expression of SPL3 and SPL9 in *suo* mutants should be dependent on the presence of a miR156 target site. To test this prediction, we examined the effect of *suo-2* on the expression of constructs in which the miR156 target site was absent (*pSPL3::GUS-rSPL3*) or mutated (*pSPL9::rSPL9-GUS*) (26) (Fig. 6 *D* and *E*). *suo-2* had no effect on the mRNA levels of these constructs, nor did it produce a significant increase in their GUS activity (Fig. 6 *D* and *E*). Thus, the elevated protein levels of SPL3 and SPL9 in *suo* is dependent on their sensitivity to miR156.

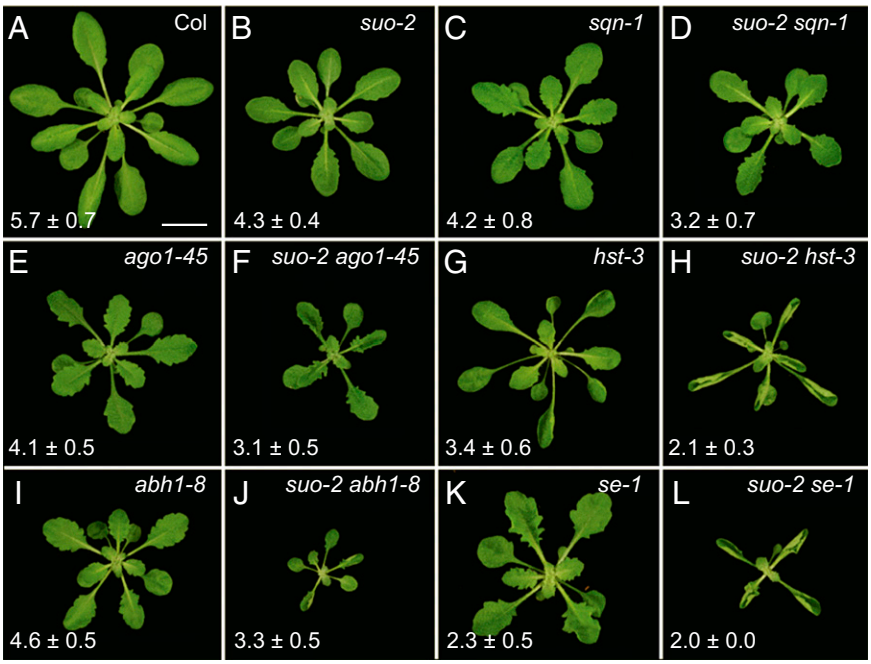


**Fig. 3.** *suo* interacts genetically with mutations in the miRNA pathway. The morphology of the rosettes of one-month-old Col (*A*) and mutant (*B–L*) plants grown in short days. *suo-2* interacts more strongly with *hst-3* (*G* and *H*), *abh1-8* (*I* and *J*), and *se-1* (*K* and *L*) than with *sqn-1* (*C* and *D*) and *ago1-45* (*E* and *F*). The numbers represent the number of juvenile leaves. (Scale bar: 1 cm.)

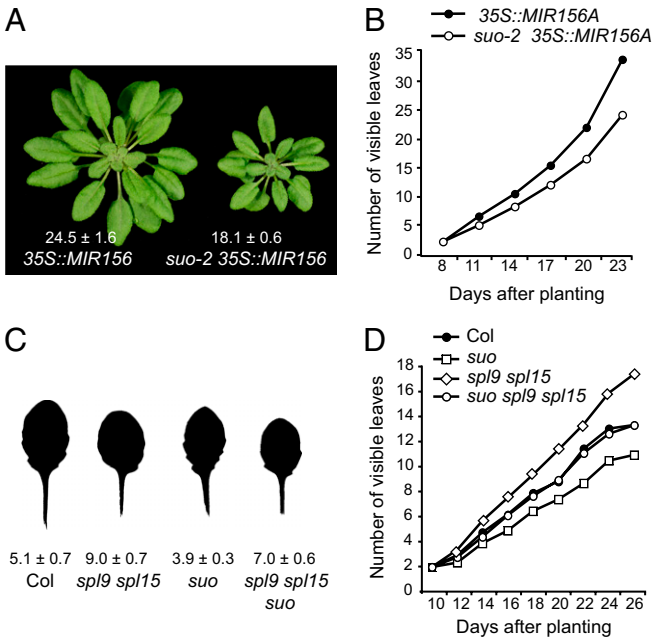


**Fig. 4.** *suo* interferes with the function of *miR156*. (*A*) The morphology of *35S::MIR156A* and *suo-2 35S::MIR156A* plants grown in long days (LD). The number of leaves lacking abaxial trichomes is indicated (± SD). (*B*) The rate of leaf initiation of *35S::MIR156A* and *suo-2 35S::MIR156A* plants grown in LD. (*C*) The morphology of the fifth leaf and the number of leaves lacking abaxial trichomes (± SD) in Col and *spl9-4 spl15-1*, *suo-2*, and *suo-2 spl9-4 spl15-1* plants. (*D*) The rate of leaf initiation in Col and *spl9-4 spl15-1*, *suo-2*, and *suo-2 spl9-4 spl15-1* plants grown in LD.

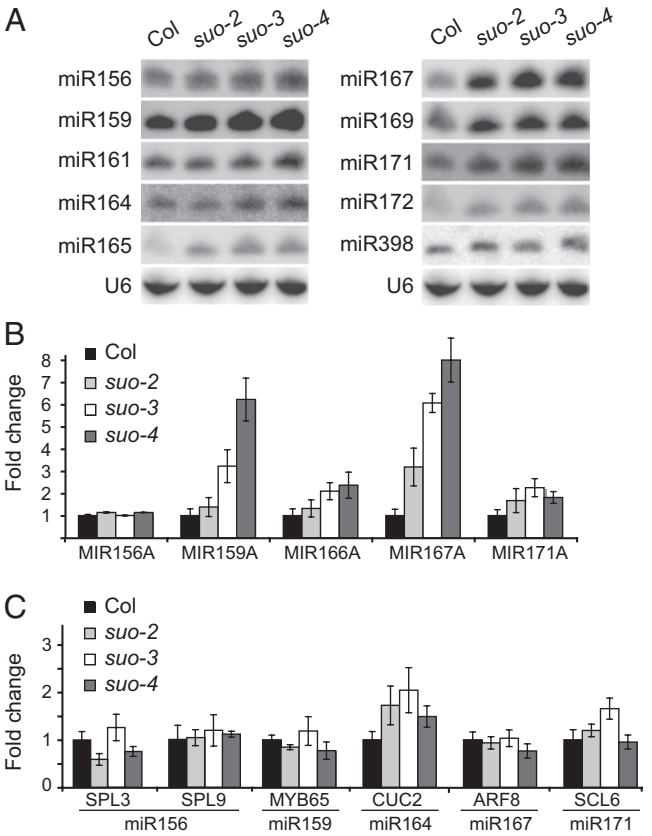


**Fig. 5.** Accumulation of miRNAs and their targets in *suo*. (*A*) RNA blot of small RNA from 14-d seedlings of Col and *suo* mutants, sequentially hybridized with probes for various miRNAs. (*B*) qRT-CR analysis of the abundance of the primary transcripts of miRNAs in 14-d seedlings of Col and *suo* mutants. (Bars: ± SD of 3 technical replicates.) (*C*) qRT-CR analysis of miRNA-regulated transcripts in 14-d-old seedlings of Col and *suo* mutants. The miRNA targeting each transcript is indicated.

We also examined the effect of *suo* on the expression of *CSD1* and *CSD2,* genes that are translationally repressed by miR398 under low copper conditions (6). Consistent with the results obtained for *SPL3* and *SPL9*, *suo* mutations had little or no effect on copper/zinc superoxide dismutase (CSD)1 and CSD2 mRNA (Fig. 6*F*) but increased the abundance of both proteins (Fig. 6*G*). *CSD2* mRNA was slightly elevated in *suo-4*, but this effect is unlikely to be significant because it was not observed in *suo-2* and *suo-3* and was much less than the observed increase in CSD2 protein. These results provide additional evidence that SUO promotes miRNA-mediated translational repression.

Processing bodies (P-bodies) are cytoplasmic structures that have been implicated in miRNA-mediated translational repression and mRNA degradation (1, 27). To determine the subcellular location of SUO, we produced transgenic *Arabidopsis* plants expressing *35S::SUO-eGFP*; this transgene rescued the mutant phenotype of *suo-2* (Fig. S2*B*), demonstrating that the SUO-eGFP protein is functional. SUO-eGFP was strongly expressed in the nucleus in *Arabidopsis* root cells (Fig. 7*A*) and in *Nicotiana benthamiana* epidermal cells (Fig. 7*B*) and was also present in cytoplasmic foci that resembled P-bodies in size and number. We also coinfiltrated the *35S::SUO-eGFP* construct into *Nicotiana benthamiana* leaves along with *pDCP1::DCP1-CFP*; DCP1 promotes the activity of the decapping enzyme DCP2, and is found exclusively in P-bodies (14). SUO-eGFP expression overlapped with DCP1-CFP expression in cytoplasmic foci in *N. benthamiana* cells (Fig. 7 *C–E*), indicating that they are indeed P-bodies. To determine whether SUO is required for P-body assembly, we transformed wild-type and *suo-2* plants with *pDCP1::DCP1-CFP*. There was no significant difference in the size or number of CFP-expressing bodies in these genotypes (Fig. 7 *F–H*), indicating that SUO is not essential for P-body formation.

## Discussion

The results presented here indicate that SUO is a component of the translation repression machinery in *Arabidopsis* and suggest that it is specifically required for miRNA-mediated translational repression. Evidence that SUO promotes translational repression is provided by the observation that *suo* mutations increase the SPL3, SPL9, CSD1 and CSD2 proteins without affecting the abundance of the mRNAs for these proteins, and by the presence of SUO in P-bodies: structures known to be involved in this process. We believe that SUO is likely to be specifically required for miRNA-mediated translational repression because of the phenotypic similarity between *suo* mutants and mutants with reduced AGO1 activity (*ago1-45* and *sqn-1*) and because the effects of *suo* on gene expression are dependent on a miRNA target site. It is also significant that *suo* interacts more strongly with mutations that affect miRNA biogenesis than with either *ago1* or *sqn* mutations. The simplest interpretation of this genetic result is that SUO operates independently of genes involved in miRNA biogenesis and in association with AGO1.

The presence of two conserved GW domains in SUO supports this conclusion. GW/WG motifs are commonly found in proteins that interact with Argonaute. In *Arabidopsis*, GW/WG-containing regions of the largest subunit of PolV, NRPE1 (20), and the transcription factor KTF1/RDM3/SPT5-like (28, 29), mediate the interaction of these proteins with AGO4. Similarly, the plant viral proteins, P1 and P38, contain two GW domains that mediate their interaction with AGO1 (30, 31). In mammals,

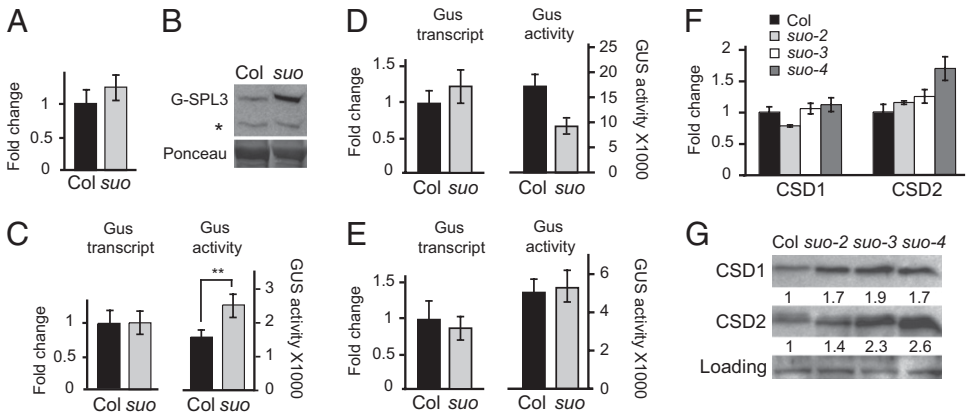


**Fig. 6.** SUO is required for miRNA-mediated translational repression. (*A*) qRT-PCR analysis of eGFP-SPL3 mRNA in 0.5-cm primordia of leaves 3 and 4 of *pSPL3::eGFP-SPL3* and *suo-1 pSPL3::eGFP-SPL3* plants. (*B*) Western blot of leaves 3 and 4 of Col and *suo-1* plants transformed with *pSPL3::eGFP-SPL3,* probed with an antibody to GFP. The asterisk indicates a nonspecific band that serves as a loading control. (*C*) qRT-PCR analysis of SPL9-GUS transcripts and MUG assays of GUS activity (pmol 4-MU/min × μg protein) in Col and *suo-2* plants transformed with *pSPL9::GUS-SPL9*. *suo-2* significantly increases GUS activity ($P < 0.05$). (*D*) qRT-PCR analysis of rSPL3-GUS transcripts and MUG assays of GUS activity in Col and *suo-2* plants transformed with *pSPL3::rSPL3-GUS*. (*E*) qRT-PCR analysis of GUS-rSPL9 transcripts and MUG assays of GUS activity in Col and *suo-2* plants transformed with *pSPL9::GUS-rSPL9*. (*F*) qRT-PCR analysis of the CSD1 and CSD2 mRNAs in *suo-2*, *suo-3*, and *suo-4* plants. (*G*) Western blot of Col and *suo* mutants probed with an antibody to CSD1 and CSD2. (Error bars: ± SD.)

miRNA-directed translational repression and transcript degradation requires GW182, a P-body localized protein that interacts with Ago2 via an N-terminal GW/WG domain (32). In *Caenorhabditis elegans*, the function of GW182 is provided by the GW proteins, AIN1 and AIN2, which are also located in P-bodies and promote miRNA-mediated translational repression and mRNA degradation via an interaction with Argonaute proteins (33, 34). SUO is structurally different from GW182/AIN1/AIN2, but is functionally similar in that it promotes translational repression by miRNAs and is located in P-bodes. These similarities suggest that SUO may be a functional analog of GW182.

Although *suo* has no apparent effect on the transcript levels of genes regulated by miRNAs, the primary transcripts of several miRNA genes (pri-miRNAs) and mature miRNAs derived from these transcripts were elevated in *suo* mutants. This observation is particularly interesting in light of the observation that SUO is present in the nucleus and contains sequence motifs found in proteins involved in DNA and histone methylation (BAH) (35) and transcriptional regulation (TFS2N, L/FDLN) (22). Proteins containing BAH or L/FDLN domains typically repress gene expression (22, 35), which is consistent with the observation that pri-mRNAs are elevated in *suo* mutants. If SUO regulates the transcription of miRNA genes, the question of how it is directed to these genes will need to be answered. It may be that there is a much closer physical interaction between factors involved in the transcription, processing, and functioning of miRNAs than is currently imagined. It is also intriguing that *suo* increases the abundance of some miRNAs without causing a decrease in the level of the transcripts targeted by these miRNAs. This observation suggests that, in *suo*, miRNAs become trapped in the translational repression machinery and are unable to mediate mRNA cleavage.

How important is translational repression for miRNA activity in plants? Null alleles of DCL1 (the dicer that produces miRNAs in *Arabidopsis*) are lethal very early in embryogenesis (36). By contrast, putative null alleles of *suo* have a relatively weak phenotype. We have no evidence that miRNA-mediated translational repression is completely absent in *suo* mutants, and this seems unlikely given the existence of the closely related *SUO* paralog At3g48060. On the other hand, it is significant that a null allele of At3g48060 has no obvious homozygous phenotype and does not interact genetically with *suo,* whereas *SUO* is haploinsufficient. These results indicate that SUO has significantly greater biological activity than At3g48060 and suggest that the phenotype of *suo* may indeed be an accurate reflection of the contribution of translation repression to miRNA activity in plants. If so, this relatively weak phenotype implies that translational repression is less important for miRNA activity in plants than transcript cleavage. An alternative possibility is that SUO is only required for the activity of some miRNAs. It will be important to determine whether SUO is generally required for miRNA activity and whether other GW proteins in *Arabidopsis* (37) also play a role in miRNA-mediated translational repression.

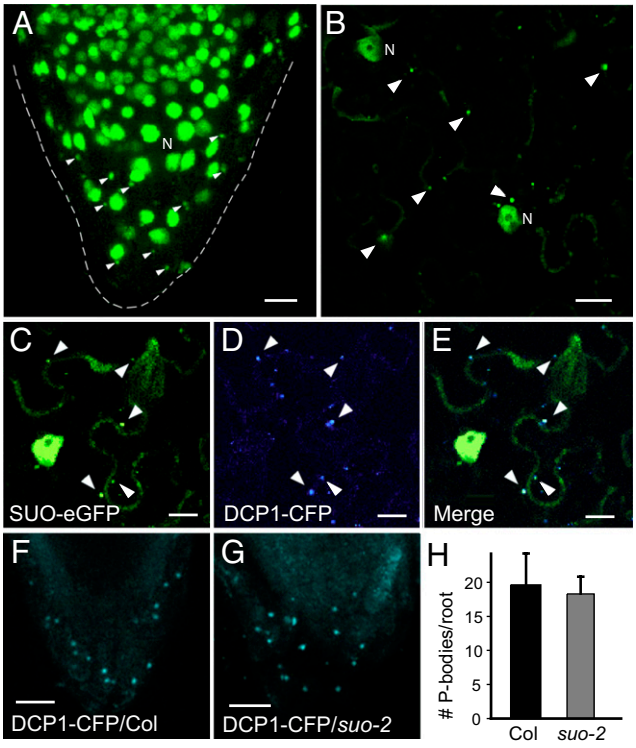


**Fig. 7.** SUO is present in the nucleus and in P-bodies. (*A* and *B*) Confocal images of eGFP fluorescence in the root apex of a 3-d-old *Arabidopsis* seedling (*A*) and an epidermal cell of a *N. benthamiana* leaf (*B*) transformed with *35S::SUO-eGFP*. n, nuclei; arrowheads, fluorescence in cytoplasmic foci. (*C–E*) Subcellular localization of SUO and DCP1 in tobacco epidermal cells cotransformed with *35S::SUO-eGFP* and *pDCP1::DCP1-CFP*. CFP fluorescence is pseudocolored in blue and eGFP in green. Arrows indicate some of the cytoplasmic foci in which SUO-eGFP and DCP1-CFP colocalize. (*F* and *G*) Root apical cells of wild-type and *suo-2* plants transformed with *pDCP1::DCP1-CFP*. (*H*) The number of CFP-expressing bodies in the root apex of wild-type and *suo-2* plants transformed with *pDCP1::DCP1-CFP*. (Scale bars: 10 μm in *A*, *F*, and *G*; 20 μm in *B–E*.)

## Materials and Methods

**Plant Material and Growth Conditions.** All of the genetic stocks used in this paper were in a Columbia background. *pSPL3::eGFP-SPL3* seeds were mutagenized according to (38), and M2 seeds were bulk harvested from groups of ~100 mutagenized plants. M2 seedlings were screened for elevated GFP expression 14 d after germination with a stereomicroscope. *suo-3* (SALK_074555), *suo-4* (SALK_020387), *suo-5* (SALK_060573), and *suo-6* (CS836050) were obtained from the *Arabidopsis* Biological Resource Center. *suo-2* was identified as a second-site mutation in a transgenic line. The effect of *suo* on leaf morphology was determined using plants grown in short days (10 h light:14 h dark; 23 °C), under a 3:1 combination of cool white (F032/841/Eco; Sylvania) and wide spectrum (Gro Lite WS; Interlectric Corp.) fluorescent lights, at a light intensity of 300 μmol/$m^2$ per sec.

**Plasmid Construction and Microscopy.** The *pSPL3::eGFP-SPL3* line has been described previously (39). To generate the *35S:SUO-eGFP* construct, the *SUO* coding region was inserted into the NcoI site in P3301-GUS, and the GUS sequence in P3301-GUS was replaced by eGFP at the NcoI and BstEII sites. The primers used to generate this construct are listed in Table S1. The *pDCP1::DCP1-CFP* construct was a gift from Nam Hai Chua (Rockefeller University). Constructs were transformed into *Agrobacterium tumefaciens* GV3101 and coinfiltrated into *Nicotiana benthamiana* leaves.

**RNA and Protein Analysis.** Total RNA was isolated using TRIzol (Invitrogen), purified with QIAGEN RNeasy, treated with RNase-free DNase, and processed for small RNA blots as described in Ref. 11. qRT-PCR was performed using SuperScript II reverse transcriptase and Power SYBR Green PCR master mix (Applied Biosystems) and normalized to ACTIN2. To induce miR398 expression, Col and *suo* were grown in Fafard #2 soil without added fertilizer. Probe and primer sequences are listed in Table S1. Western blots were processed according to Ref. 40 and were incubated with anti-GFP (G1544; Sigma) or anti-CSD1/CSD2 (a gift from Dan Kliebenstein, University of California–Davis) at room temperature for 2 h.

**MUG Assay.** Fifteen primary transformants of miR156-sensitive or -resistant GUS reporters for SPL3 and SPL9 (*pSPL3::GUS-SPL3*, *pSPL3::rSPL3-GUS*, *pSPL9::SPL9-GUS*, and *pSPL9::rSPL9-GUS*) were harvested for expression analysis. The GUS protein level in each transgenic plant was measured by the MUG assay according to Ref. 11), and the GUS transcript level in these same plants was determined by qRT-PCR.

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1. Fabian MR, Sonenberg N, Filipowicz W (2010) Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem* 79:351–379.
2. Voinnet O (2009) Origin, biogenesis, and activity of plant microRNAs. *Cell* 136: 669–687.
3. Aukerman MJ, Sakai H (2003) Regulation of flowering time and floral organ identity by a microRNA and its *APETALA2*-like target genes. *Plant Cell* 15:2730–2741.
4. Chen X (2004) A microRNA as a translational repressor of *APETALA2* in *Arabidopsis* flower development. *Science* 303:2022–2025.
5. Gandikota M, et al. (2007) The miRNA156/157 recognition element in the 3′ UTR of the *Arabidopsis* SBP box gene *SPL3* prevents early flowering by translational inhibition in seedlings. *Plant J* 49:683–693.
6. Brodersen P, et al. (2008) Widespread translational inhibition by plant miRNAs and siRNAs. *Science* 320:1185–1190.
7. Beauclair L, Yu A, Bouché N (2010) microRNA-directed cleavage and translational repression of the copper chaperone for superoxide dismutase mRNA in *Arabidopsis*. *Plant J* 62:454–462.
8. Lanet E, et al. (2009) Biochemical evidence for translational repression by *Arabidopsis* microRNAs. *Plant Cell* 21:1762–1768.
9. Llave C, Xie Z, Kasschau KD, Carrington JC (2002) Cleavage of *Scarecrow*-like mRNA targets directed by a class of *Arabidopsis* miRNA. *Science* 297:2053–2056.
10. Kasschau KD, et al. (2003) P1/HC-Pro, a viral suppressor of RNA silencing, interferes with Arabidopsis development and miRNA function. *Dev Cell* 4:205–217.
11. Wu G, Poethig RS (2006) Temporal regulation of shoot development in *Arabidopsis thaliana* by miR156 and its target *SPL3*. *Development* 133:3539–3547.
12. Sunkar R, Kapoor A, Zhu JK (2006) Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in *Arabidopsis* is mediated by downregulation of miR398 and important for oxidative stress tolerance. *Plant Cell* 18:2051–2065.
13. Burk DH, Liu B, Zhong R, Morrison WH, Ye ZH (2001) A katanin-like protein regulates normal cell wall biosynthesis and cell elongation. *Plant Cell* 13:807–827.
14. Xu J, Yang JY, Niu QW, Chua NH (2006) *Arabidopsis* DCP2, DCP1, and VARICOSE form a decapping complex required for postembryonic development. *Plant Cell* 18: 3386–3398.
15. Schwab R, et al. (2005) Specific effects of microRNAs on the plant transcriptome. *Dev Cell* 8:517–527.
16. Vaucheret H, Vazquez F, Crété P, Bartel DP (2004) The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev* 18:1187–1197.
17. Park MY, Wu G, Gonzalez-Sulser A, Vaucheret H, Poethig RS (2005) Nuclear processing and export of microRNAs in *Arabidopsis*. *Proc Natl Acad Sci USA* 102: 3691–3696.
18. Smith MR, et al. (2009) Cyclophilin 40 is required for microRNA activity in *Arabidopsis*. *Proc Natl Acad Sci USA* 106:5424–5429.
19. Cardon GH, Höhmann S, Nettesheim K, Saedler H, Huijser P (1997) Functional analysis of the *Arabidopsis thaliana* SBP-box gene *SPL3*: A novel gene involved in the floral transition. *Plant J* 12:367–377.
20. El-Shami M, et al. (2007) Reiterated WG/GW motifs form functionally and evolutionarily conserved ARGONAUTE-binding platforms in RNAi-related components. *Genes Dev* 21:2539–2544.
21. Ding L, Han M (2007) GW182 family proteins are crucial for microRNA-mediated gene silencing. *Trends Cell Biol* 17:411–416.
22. Ohta M, Matsui K, Hiratsu K, Shinshi H, Ohme-Takagi M (2001) Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. *Plant Cell* 13:1959–1968.
23. Zhang J-F, et al. (2008) The disturbance of small RNA pathways enhanced abscisic acid response and multiple stress responses in Arabidopsis. *Plant Cell Environ* 31:562–574.
24. Wang JW, Schwab R, Czech B, Mica E, Weigel D (2008) Dual effects of miR156-targeted *SPL* genes and *CYP78A5/KLUH* on plastochron length and organ size in *Arabidopsis thaliana*. *Plant Cell* 20:1231–1243.
25. Wu G, et al. (2009) The sequential action of miR156 and miR172 regulates developmental timing in *Arabidopsis*. *Cell* 138:750–759.
26. Yang L, Conway SR, Poethig RS (2011) Vegetative phase change is mediated by a leaf-derived signal that represses the transcription of miR156. *Development* 138:245–249.
27. Xu J, Chua NH (2011) Processing bodies and plant development. *Curr Opin Plant Biol* 14:88–93.
28. Bies-Etheve N, et al. (2009) RNA-directed DNA methylation requires an AGO4-interacting member of the SPT5 elongation factor family. *EMBO Rep* 10:649–654.
29. He XJ, et al. (2009) An effector of RNA-directed DNA methylation in *Arabidopsis* is an ARGONAUTE 4- and RNA-binding protein. *Cell* 137:498–508.
30. Giner A, Lakatos L, García-Chapa M, López-Moya JJ, Burgyán J (2010) Viral protein inhibits RISC activity by argonaute binding through conserved WG/GW motifs. *PLoS Pathog* 6:e1000996.
31. Azevedo J, et al. (2010) Argonaute quenching and global changes in Dicer homeostasis caused by a pathogen-encoded GW repeat protein. *Genes Dev* 24:904–915.
32. Eulalio A, Tritschler F, Izaurralde E (2009) The GW182 protein family in animal cells: New insights into domains required for miRNA-mediated gene silencing. *RNA* 15: 1433–1442.
33. Ding L, Spencer A, Morita K, Han M (2005) The developmental timing regulator AIN-1 interacts with miRISCs and may target the argonaute protein ALG-1 to cytoplasmic P bodies in *C. elegans*. *Mol Cell* 19:437–447.
34. Ding XC, Grosshans H (2009) Repression of *C. elegans* microRNA targets at the initiation level of translation requires GW182 proteins. *EMBO J* 28:213–222.
35. Callebaut I, Courvalin JC, Mornon JP (1999) The BAH (bromo-adjacent homology) domain: A link between DNA methylation, replication and transcriptional regulation. *FEBS Lett* 446:189–193.
36. Schauer SE, Jacobsen SE, Meinke DW, Ray A (2002) *DICER-LIKE1*: Blind men and elephants in *Arabidopsis* development. *Trends Plant Sci* 7:487–491.
37. Karlowski WM, et al. (2010) Genome-wide computational identification of WG/GW Argonaute-binding proteins in Arabidopsis. *Nucleic Acids Res* 38:4231–4245.
38. Hunter C, et al. (2006) Trans-acting siRNA-mediated repression of *ETTIN* and *ARF4* regulates heteroblasty in *Arabidopsis*. *Development* 133:2973–2981.
39. Yamaguchi A, et al. (2009) The microRNA-regulated SBP-Box transcription factor SPL3 is a direct upstream activator of *LEAFY, FRUITFULL*, and *APETALA1*. *Dev Cell* 17: 268–278.
40. Earley K, Smith M, Weber R, Gregory B, Poethig R (2010) An endogenous F-box protein regulates ARGONAUTE1 in *Arabidopsis thaliana*. *Silence* 1:15.