Supporting Information

Smith et al. 10.1073/pnas.0812729106

SI Materials and Methods

Genetic Stocks and Growth Conditions. Seeds were planted on Metromix 200 (Scotts) or Fafard #2 soil and stored at 4 °C for 2 days before transfer to growth chambers. Double mutants between *sqn* and *ago1–45*, *ago1–46*, *ago1–27*, and *ago1–47* were grown on 1/4× MS salts (GIBCO-BRL) with 0.8% agar (pH 5.7) but were otherwise treated the same as soil-grown plants. Double mutants were identified in segregating families by PCR, using allele-specific primers (Table S1).

For phenotypic analysis, plants were grown in 96-well flats with a photoperiod of 16 h light and 8 h dark under a 2:1 mix of cool white and wide-spectrum fluorescent lights (120 μE/min/m^2) at 23 °C. Abaxial trichomes were scored with a stereomicroscope 2 to 3 weeks after planting. After flowering, leaves were removed, attached to cardboard with double-sided tape, and then scanned in a digital scanner.

To test the effect of mutations on *L1* silencing, F2 plants from crosses of the *L1* transgene to *sqn*, *ago1–45*, and *ago1–27* were allowed to self-pollinate, and the resulting F3 families were screened on kanamycin to identify families homozygous for *L1* (kanR/kanR) and segregating for the relevant mutation. Mutant families were stained for GUS activity, according to Donnelly et al. (1). Analysis of the expression of *35S::GUS-SPL3* was conducted as previously described (2).

Micoarray Analysis and RNA Blots. Total RNA was isolated using TRIzol (Invitrogen) from 10-day-old seedlings grown on 1/4× MS salts and 0.8% agar under long-day conditions (16 h light, 8 h dark) or from floral buds of plants grown in soil under the same light conditions. For microarray analysis, the RNA was further purified with RNAeasy (Quiagen) columns, and then labeled, hybridized to the Affymetrix 8K array, and scanned by the University of Pennsylvania microarray facility. For the determination of mRNA levels, total RNA was run on an agarose gel, blotted on Hybond N$^+$ nylon membranes, and hybridized with ^{32}P-dCTP–labeled randomly primed probes (Prime-it II Random Primer Labeling Kit; Stratagene). Hybridization was carried out at 68 °C using PerfectHyb Plus buffer (Sigma). Blots were washed once in 2× SSC and 0.1% SDS for 5 min at room temperature, twice in 0.5× SSC and 0.1% SDS for 20 min at 68 °C, and once in 0.1× SSC 0.1% SDS for 20 min at 68 °C. miRNA levels were measured using 10–15 μg of low-molecular-weight RNA separated on 8 M urea/15% denaturing polyacrylamide gels and electrically transferred to Hybond N$^+$ nylon membranes, according to Dalmay et al. (3). Blots were hybridized with a complementary miRNA oligonucleotide (Table S1) labeled with ^{32}P-Y-ATP using T4 polynucleotide kinase (New England Biolabs) at 40 °C in ULTRAhyb-oligo hybridization buffer (Ambion), and were washed twice at 40 °C in 2× SSC and 0.5% SDS for 30 min. Blots were imaged on a Typhoon phosphoimager (Molecular Dynamics) after an overnight exposure; contrast was adjusted with Photoshop 7 (Adobe Systems). Band intensity was measured using Image J software (National Institutes of Health).

Quantitative Real-Time PCR. Total RNA was isolated from 12- or 18-day-old seedlings grown on 1/2× MS salts and 0.8% agar under long-day conditions at 23 °C. Quantitative real-time PCR was performed with primers that flank the miRNA target site (Table S1) on DNase-treated RNA in a 20-μL reaction using the QuantiTect SYBR Green PCR Kit (Qiagen). Thermal cycling conditions were as follows: 15 min at 95 °C, 44 cycles of 15 sec at 94 °C, 30 sec at 54 °C, and 30 sec at 72 °C, followed by a melting curve analysis. Values were normalized to the level of actin-2 or eukaryotic translation initiation factor 4A.

SQN-GFP Fusion Construct. RNA was isolated using TRIzol and reverse transcribed using the SuperScript First Strand kit (Invitrogen). The SQN cDNA was amplified with primers (Table S1) that were modified to include a 5′ NcoI site and a 3′ NcoI site and 8-aa linker, and the resulting product was ligated into the pGEM-T Easy vector (Promega) and transformed into bacteria. The cDNA was subsequently excised with NcoI and cloned into pCAMBIA 3300 to generate a C-terminal eGFP fusion transcript under the regulation of the CaMV 35S promoter. This construct was transformed into *Agrobacterium* GV3101 and transferred to plants (Col) by floral dipping.

Western Blots and Co-immunoprecipitation. To assay the effect of *sqn* on AGO1 proteins levels, ≈2 mL/1 g of extraction buffer [50 mM Tris HCL (pH 8.0), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1 mM DTT, 0.05% Nonidet P-40, and 1% Sigma plant protease inhibitor mixture] was added to wild-type and *sqn* floral buds ground in liquid nitrogen. Total protein extracts were run on an SDS-PAGE gel, transferred overnight at 30 V at 4 °C, and probed with antibody to AGO1 provided by Y. Qi (National Institute of Biological Sciences, Beijing, China).

For co-immunoprecipitation and mass spectrometry, 5 g of 5-day-old SQN-GFP seedlings were ground in a mortar with liquid nitrogen. The powder was allowed to defrost and then homogenized in extraction buffer [50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, and protease inhibitors mix mixture (Roche, 1 tablet per 50 mL)]. After grinding, the protein extract was sonicated 3 × 10 sec with a probe sonicator (MSE) at half-maximal power and incubated on ice for 30 min. The Nonidet P-40 in the protein extract was then diluted to 0.33%, followed by 10 min centrifugation at maximum speed at 4 °C. The supernatant was precleared with 50 μL CNBr-activated Sepharose 4B beads (GE Healthcare) with Tris-blocked active sites, for 30 min at 4 °C with rotation. The extract was centrifuged for 2 min at 1,000 rpm and the supernatant incubated for 1.5 h with rotation with 50 μL Sepharose 4B beads coupled to purified custom-made anti-YFP antibody (Eurogentec). The beads were collected by centrifugation (2 min, 1,000 rpm) and washed twice with extraction buffer with 0.1% Triton and 3 times with 50 mM ammonium carbonate. From this point the sample was prepared for Western blot for the co-immunoprecipitation or for MS measurement.

For MS measurements, 1 μL 50 mM DTT in 50 mM NH_4HCO_3 was added to the washed beads and incubated at 37 °C. After 1 h, 1 μL 100 mM iodoacetamide in 50 mM NH_4HCO_3 was added and incubated 1 h at room temperature in the dark. Subsequently, 1 μL 200 mM cysteine in 50 mM NH_4HCO_3 and 1 μL trypsin sequencing grade (0.5 μg/μL in 1 mM HCl) were added, and the beads were incubated overnight at 20 °C while shaking. The following day 1.2 μL trifluoroacetic acid was added to adjust to approximately pH 3, and the beads were centrifuged 3 min at maximum speed. The supernatant was subjected to nLC-MS/MS analysis using a nanoLC (Proxeon) and LTQ-Orbitrap (Thermo Electron). For Western blots, the beads were resuspended in 20–50 μL sample buffer, boiled, and run on an SDS-PAGE gel. Protein was

transferred overnight at 30 V at 4 °C. Blots were probed with an antibody to Hsp90 (Santa Cruz Biotechnology, sc-33755), AGO1 (provided by Y. Qi, National Institute of Biological Sciences, Beijing, China) or actin (Sigma, A0480).

1. Donnelly PM, Bonetta D, Tsukaya H, Dengler RE, Dengler NG (1999) Cell cycling and cell enlargement in developing leaves of *Arabidopsis. Dev Biol* 215:407–419.

2. Wu G, Poethig RS (2006) Temporal regulation of shoot development in *Arabidopsis thaliana* by miR156 and its target SPL3. *Development* 133:3539–3547.

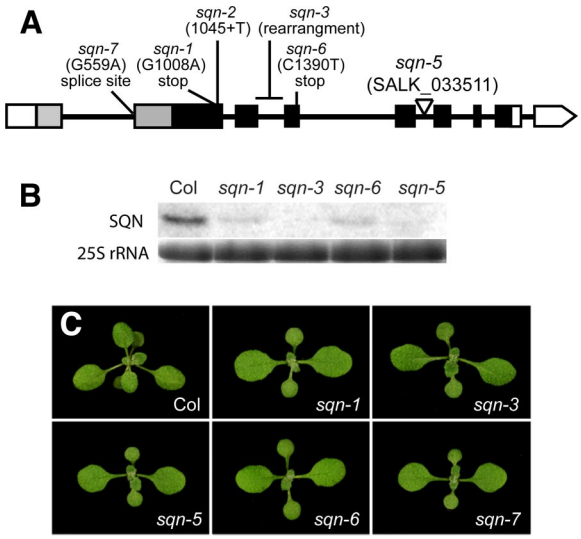

Fig. S1. Mutant alleles of *SQN*. (*A*) The position (relative to the translation start site) and nature of the molecular lesions in *sqn* alleles. The position of *sqn-3* was defined by the locations of primers that did not produce a PCR product. (*B*) Northern blot hybridized with a probe to *SQN* reveals that several *sqn* mutations dramatically reduce the mRNA from this locus. (*C*) Two week-old seedlings. Different alleles of *sqn* have the same phenotype.

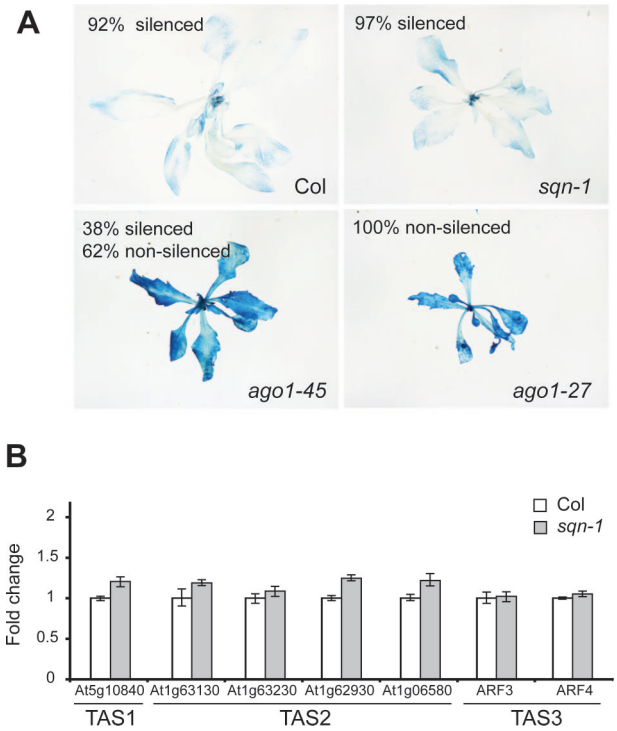

Fig. S2. The effect of *sqn-1*, *ago1–45*, and *ago1–27* on the posttranscriptional silencing of the L1 (35S:GUS) transgene, and the targets of transacting siRNAs. (*A*) Mature rosettes of Col, *sqn-1*, *ago1–45*, and *ago1–27* plants homozygous for L1, stained for GUS activity. Two phenotypes were observed: pale blue = silenced and dark blue = non-silenced. The frequency of these classes is given ($n \geq 25$). (*B*) Quantitative RT-PCR analysis of transcripts targeted by transacting siRNAs (±SEM).

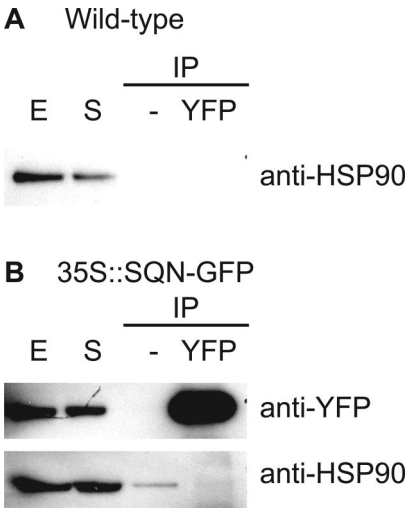

Fig. S3. Western blot of immunoprecipitate from 35S::SQN-GFP seedlings. Protein was extracted from 5 g of wild-type (*A*) or 35S::SQN-GFP (*B*) seedlings, and samples were taken for Western blot before and after centrifugation (E, crude extract; S, supernatant after centrifugation). Before immunoprecipitation, the supernatant was precleared with Sepharose beads that had Tris immobilized on the surface. The supernatant after centrifugation of the preclear beads was incubated with an anti-YFP antibody immobilized on Sepharose beads. Aliquots for Western blot were taken after the preclear (-) and after the immunoprecipitation (YFP). The membranes were incubated with either an anti-YFP antibody or an antibody against HSP90. SQN is efficiently pulled down by the anti-YFP antibody. The co-immunoprecipitation with HSP90 shows that SQN is not associated with HSP90 in this experiment.

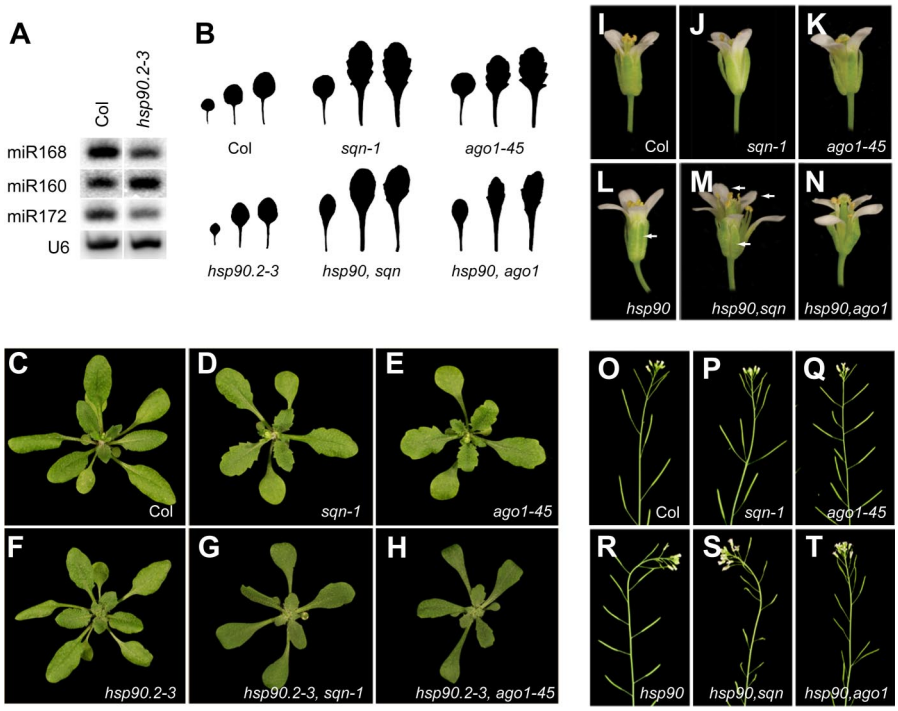

Fig. S4. The phenotype of *hsp90.2–3* and its interactions with *sqn-1* and *ago1–45*. (*A*) Northern blot of low-molecular-weight RNA from flower buds sequentially hybridized with oligonucleotides complementary to the functional strand of the indicated miRNAs. U6 was used as a loading control. (*B*) Scans of fully expanded leaves 1, 3, and 4. (*C–H*) Mature rosettes of single and double mutants. *hsp90.2–3 sqn-1* and *hsp90.2–3 ago1–45* are 1 week older than the single mutants. (*I–N*) Flowers of single and double mutants. Arrows indicate extra sepals and petals. (*O–T*) Inflorescences of single and double mutants.

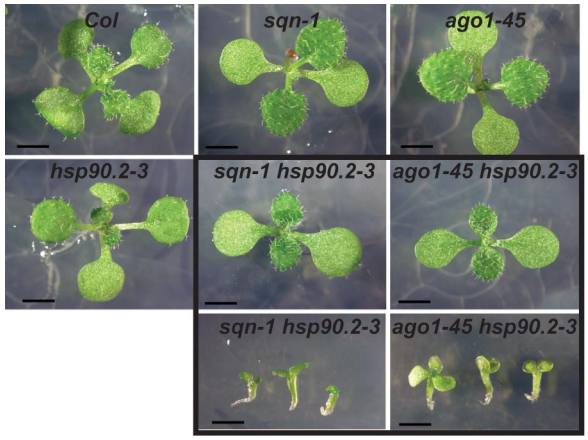

Fig. S5. *hsp90.2–3* interacts with *sqn* and *ago1–45* to produce abnormal seedlings. The frequency of the morphologic classes illustrated in this figure is provided in Table 1 in the main text. (Scale bar, 3 *mm*.)

Table S1. PCR primers used in this study

Genotyping	
sqn-1.dCAP.BslI R	TCTGAGAGTAAATCAAGGTCAAA
sqn-1.dCAP.BslI F	GAAAGCCCAGCTGCCTTATCTTG
ago1-45.dCAP.Sph1.F	TGAGCCATGGTCTCGGATGTTTCA
ago1-45.dCAP.Sph1.R	GAGACTATGCCGAGTTCAGTCTCACGCATG
ago1-46.dCAP.Nhe1.R	TGATGTCTCTGGCTCCATGTAGAAGCTAG
ago1-46.dCAP.Nhe1.F	TGCAAGATGCACACGCTCAGTTTC
ago1-27.dCAP.BannII.F	TGCAAGATGCACACGCTCAGTTTC
ago1-27.dCAP.BannII.R	ACTCAGCAGTAGAACATGACACGC
hen1-6.F salk_090960	AGCAATTCCTCAAAAAGGTCC
hen1-6.R salk_090960	TCGTGCATTCCGAGATTTTAC
hyl1-2.F salk_064863	AGTTCTCCCAGCGCTAATCTC
hyl1-2.R salk_064863	TTCTTGGAAATTGGATTGCAG
Quantitative RT-PCR primers	
At1 g01040/DCL1.F.rt	GATCCATTCCTAAGCGAAGTTTCAGAG
At1 g01040/DCL1.R.rt	GCCCGAGCAACATAAAGATCCATAG
At1 g30490/PHV.F.rt	AGACCTTGGCGGAGTTCCTTTG
At1 g30490/PHV.R.rt	GTTGCGTGAAACAGCTACGATACC
At1 g42970/GAPDH.F.rt	TCTTTCCCTGCTCAATGCTCCTC
At1 g42970/GAPDH.R.rt	TTTCGCCACTGTCTCTCCTCTAAC
At3 g11440/MYB65.F.rt	GATGGTTCCTGATAGCCATACAGTTAC
At3 g11440/MYB65.R.rt	TAGGCATCAACAGAGTCAAGGAGATC
At5 g37020/ARF8.F.rt	AGATGTTTGCTATCGAAGGGTTGTTG
At5 g37020/ARF8.R.rt	CCATGGGTCATCACCAAGGAGAAG
At1 g77850/ARF17.F.rt	AGCACCTGATCCAAGTCCTTCTATG
At1 g77850/ARF17.R.rt	TGGTGAATAGCTGGGGAGGATTTC
At1 g06580/PPR.F.rt	GTCTCTGCTCGAATACTGAGTGATATG
At1 g06580/PPR.R.rt	AAGCTGCCCCTCTTTCCCATAC
At5 g53950/CUC2-3.F.rt	GCACCAACACAACCGTCACAG
At5 g53950/CUC2-3.R.rt	GAATGAGTTAACGTCTAAGCCCAAGG
At5 g37020/ARF8.F.rt	AGATGTTTGCTATCGAAGGGTTGTTG
At5 g37020/ARF8.R.rt	CCATGGGTCATCACCAAGGAGAAG
At1 g48410/AGO1.F.rt	AAGGAGGTCGAGGAGGGTATGG
At1 g48410/AGO1.R.rt	CAAATTGCTGAGCCAGAACAGTAGG
At3 g60630/SCL6-III.F.rt	ACCAAGACCAGTCAGCGGTAATC
At3 g60630/SCL6-III.R.rt	AGTGTCGTCGTTGTTGTTGTTAAGG
At5 g43270/SPL2.F	ACGGGTTGGAGGTTGCTTGAGG
At5 g43270/SPL2.R	TTTCCGATACCGAGCACAATAG
At2 g33810/SPL3.F	CTTAGCTGGACACAACGAGAGAAGGC
At2 g33810/SPL3.R	GAGAAACAGACAGAGACACAGAGGA
At1 g53160/SPL4.F	TCAAGGGTAGAGATGACACTTCCTATGC
At1 g53160/SPL4.R	TCTCTCATCATAGCAAGTGATGGACCCTG
At3 g15270/SPL5.F	CCAGACTCAAGAAAGAAACAGGGTAGACAG
At3 g15270/SPL5.R	TCCGTGTAGGATTTAATACCATGACC
At1 g69170/SPL6.F	CCACCGTACAAGTAGACTCGTGAG
At1 g69170/SPL6.R	GAGATTTTGGTTGGGTTGGGTGA
At2 g42200/SPL9.F	CAAGGTTCAGTTGGTGGAGGA
At2 g42200/SPL9.R	TGAAGAAGCTCGCCATGTATTG
At1 g27370/SPL10.F	GTGTGGGAGAATGCTCAGGAGG
At1 g27370/SPL10.R	ACGGGAGTGTGTTTGATCCCTTGTG
At1 g27370/SPL10.F.rt	GTGGGAGAATGCTCAGGAGGC
At1 g27370/SPL10.R.rt	GAGTGTGTTTGATCCCTTGTGAATCC
At1 g27360/SPL11.F	AGTCCAAGTTTCAACTTCATGGCG
At1 g27360/SPL11.R	GAACAGAGTAGAGAAAATGGCTGCAC
At5 g50570/SPL13.R	GAAGCAAATGAGGGACTGACGACG
At5 g50570/SPL13.F	CCAATCTCTTCTTCTCCAAACAGTACCAGAAGC
At3 g57920/SPL15.F	GAATGTTTTATCACATGGAAGCTC
At3 g57920/SPL15.R	TCATCGAGTCGAAACCAGAAGATG
actin2.F	GCACCCTGTTCTTCTTACCG
actin2.R	AACCCTCGTAGATTGGCACA
EIF4a.F	AAACTCAATGAAGTACTTGAGGGAC
EIF4a.R	TCTCAAAACCATAAGCATAAATACCC
Northern probes	
miR168	TTCCCGACCTGCACCAAGCGA
miR164	TGCACGTGCCCTGCTTCTCCA

Genotyping	
miR159	TAGAGCTCCCTTCAATCCAAA
miR173	GTGATTTCTCTCTGCAAGCGAA
miR172	ATGCAGCATCATCAAGATTCT
miR390	GGCGCTATCCCTCCTGAGCTT
miR162	CTGGATGCAGAGGTTTATCGA
miR165	UCGGACCAGGCUUCAUCCCCC
miR156	GTGCTCACTCTCTTCTGTCA
miR169	TCGGCAAGTCATCCTTGGCTG
miR167	TAGATCATGCTGGCAGCTTCA
U6	AGGGGCCATGCTAATCTTCTC
SQN-GFP cloning	
SQN 3′Nco + linker	CACCATGGACCTGATAGCGGCGTTTACGAACATTTTGCGGTACTGC
SQN 5′NcoI	GCGTACCATGGGTAGGTCAAAGTGTTTCATGGAC