An Endogenous F-Box Protein Regulates ARGONAUTE1 in Arabidopsis thaliana

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Recommended Citation
Earley, K., Smith, M. R., Weber, R., Gregory, B. D., & Poethig, R. S. (2010). An Endogenous F-Box Protein Regulates ARGONAUTE1 in Arabidopsis thaliana. Silence, 1 http://dx.doi.org/10.1186/1758-907X-1-15

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Research An endogenous F-box protein regulates ARGONAUTE1 in Arabidopsis thaliana

K Earley, MR Smith, R Weber, BD Gregory and RS Poethig*

Abstract

ARGONAUTE1 (AGO1) mediates microRNA- and small interfering RNA-directed posttranscriptional gene silencing in Arabidopsis thaliana. Mutant alleles of SQUINT (SQN) slightly reduce AGO1 activity and have weak effects on shoot morphology. A screen for mutations that suppress the sqn phenotype produced loss-of-function mutations in the F-box gene FBW2. Mutations in FBW2 not only suppress sqn but also suppress many of the developmental phenotypes of weak, but not null, alleles of AGO1 by increasing AGO1 protein levels. Conversely, over-expression of FBW2 decreases the abundance of the AGO1 protein but not AGO1 messenger RNA, further indicating that FBW2 regulates AGO1 protein levels. fbw2 mutants have no obvious morphological phenotype, but display a reduced sensitivity to abscisic acid (ABA) that can be attributed to increased AGO1 activity. Our results indicate that FBW2 is a novel negative regulator of AGO1 and suggest that it plays a role in ABA signalling and/or response.

Background

Argonaute proteins are core components of the RNA-induced silencing complex (RISC) [1-3]. These proteins use microRNAs (miRNAs) and/or small interfering RNAs (siRNAs) as guides to direct RISC to a specific site in target mRNAs, resulting in the cleavage or translational repression of these target mRNAs. Some Argonaute proteins also promote transcriptional repression through their effect on chromatin structure [2-4].

ARGONAUTE1 (AGO1) is one of 10 Argonaute proteins in Arabidopsis thaliana [2,5]. Genetic analyses [6-9], as well as the identification of the small RNAs that co-purify with AGO1 [10,11], indicate that AGO1 plays a central role in both miRNA and siRNA-mediated RNA silencing. Arabidopsis is exquisitely sensitive to the level of AGO1 activity, as evident from the broad range of phenotypes displayed by hypomorphic mutations of this gene [5,6,8,12]. In wild-type plants, the expression of AGO1 is maintained at a constant level by a negative feedback loop involving miR168. AGO1 is a target of miR168 and negatively regulates its own activity by promoting the activity and stability of miR168 [9,13] and by promoting the activity of siRNAs derived from the AGO1 transcript [14]. AGO1 activity is negatively regulated by PNH/ZLL/AGO10 [15] and positively regulated by SQUINT (SQN), the Arabidopsis orthologue of the protein chaperone, Cyclophilin-40 [12].

Null alleles of SQN have a morphological phenotype that is nearly identical to the phenotype of weak loss-of-function alleles of AGO1 [12]. In order to identify genes involved in AGO1-mediated processes, we screened for mutations that suppress the phenotype of sqn-1. This screen yielded several alleles of the F-box gene FBW2. Here we show FBW2 is a negative regulator of AGO1 and controls the sensitivity of plants to the hormone abscisic acid.

Results

Mutations in FBW2 rescue the sqn phenotype

Previously we found that SQN directly or indirectly promotes AGO1 activity [12]. In particular, we showed that the phenotype of loss-of-function alleles of SQN can be largely, if not entirely, explained by a reduction in the activity of AGO1.

In order to study the mechanism of this regulation, we screened for ethyl methanesulfate (EMS)-induced mutations that suppress the phenotype of the null allele, sqn-1. sqn-1 transiently delays leaf initiation, accelerates the juvenile-to-adult transition and produces aberrant spacing of flowers in the inflorescence and an increase in carpel number [16] (Figure 1A and 1B). Three allelic mutations that partially suppress all of these phenotypes
were identified in this screen (Figure 1A, B and 1D). In addition to their effect on the morphological phenotype of sqn-1, these alleles decreased the expression of several miRNA-targeted genes (SPL3, SPL5, SPL9, AGO1, CUC2) previously shown to be over-expressed in sqn-1 [12] (Figure 1C). All three mutations had no obvious effect on shoot morphology or gene expression in the absence of sqn-1 (Figure 1A, B and 1C).

Using a map-based approach, we determined that this suppressor corresponds to FBW2 (F-BOX WITH WD-40) [17]. All three alleles change conserved residues in the predicted FBW2 protein (Figure 1D). Furthermore, a ~4,700-bp genomic construct expressing FBW2 under its native promoter (pFBW2::FBW2-FLAG) restored the original sqn-1 phenotype when transformed into sqn-1 fbw2-1 (Figure 1A). Two additional mutations of FBW2 were identified in the SALK collection of T-DNA insertions [18], SALK_144548 (fbw2-4) and SALK_071588C (fbw2-5; Figure 1D). Like the point mutations recovered in our screen, both of these mutations had no obvious morphological phenotype but nearly completely suppressed the phenotype of sqn-1 in double mutants (Figure 1A). A real time polymerase chain reaction (RT-PCR) of the FBW2 transcript in sqn-1 fbw2 double mutants revealed that fbw2-2 and fbw2-3 have no effect on the abundance of this transcript, fbw2-1 and fbw2-5 reduce, but do not eliminate, the transcript, and that fbw2-4 has no detectable FBW2 mRNA (Additional File 1: Figure S1A). We conclude that loss-of-function mutations of FBW2 suppress the phenotype of sqn-1.

FBW2 is predicted to encode a 317-amino acid protein with an N-terminal F-box domain [17] (Figure 1D). Although FBW2 was originally described as having a WD-40 domain [17], we found no evidence for the presence of a canonical WD-40 domain in this protein. A number of other predicted proteins in the Arabidopsis genome have sequence similarity to FBW2, but the similarity between these proteins is quite low (less than 32% identical), which suggests that FBW2 may be functionally unique (Additional File 1: Figure S1B). FBW2 is highly conserved in flowering plants (Additional File 1: Figure S1C), but is absent in the alga, Chlamydomonas reinhardtii. Interestingly, FBW2 has no strong sequence similarity to the Polerovirus P0 protein, an F-box protein that suppresses posttranscriptional gene silencing by destabilizing AGO1 [19,20].

Loss of FBW2 rescues hypomorphic ago mutations

Previous work from our laboratory suggested that the sole function of SQN is to promote the activity of AGO1 [12]. Given that fbw2 rescues null mutations of SQN, we recognized that FBW2 cannot function through SQN. A reasonable alternative hypothesis is that fbw2 mutations suppress sqn-1 by increasing the activity of AGO1. A pre-
The observed decrease in miRNA-regulated transcripts in sqn fbw2 and ago1 fbw2 compared with sqn and ago1 (Figure 1C and Figure 2D) could be explained by an increase in the accumulation of the miRNAs that target these transcripts for degradation. We did not favour this hypothesis because our genetic evidence suggests that FBW2 acts through AGO1, and ago1 hypomorphic alleles have limited effects on miRNA levels [9,12] (Figure 2E). Nevertheless, we compared the level of several miRNAs in sqn fbw2 and ago1 fbw2 double mutants with sqn and ago1 (Figure 2E). As predicted, sqn-1 and two hypomorphic ago1 alleles had weak or no effect on miRNA levels and there was no significant difference between the miRNA levels in these single mutants and sqn-1 fbw2 and ago1 fbw2 double mutants (Figure 2E). Individually, fbw2 mutations also had no effect on miRNA levels (Figure 2E). Thus, the reduction in the abundance of miRNA-regulated transcripts in sqn fbw2 and ago1 fbw2 double mutants is not the result of an increase in miRNA expression.

fbw2 mutations increase the abundance of AGO1

We reasoned that if fbw2 mutations suppress sqn-1 by increasing the activity of AGO1, it should be possible to replicate this effect by simply increasing the dose of AGO1. In order to test this hypothesis, we transformed a pAGO1::FLAG-AGO1 construct into sqn-1. This construct expresses a FLAG-AGO1 translational fusion under the regulation of the endogenous AGO1 promoter. Numerous sqn-1 plants expressing this construct had near-WT phenotypes (Figure 3A). Indeed, the phenotypes of several of these sqn-1 pAGO1::FLAG-AGO1 lines were essentially identical to sqn-1 fbw2 double mutants (Figure 3A). Western blots demonstrated that, as expected, sqn-1 pAGO1::FLAG-AGO1 plants had slightly more AGO1 protein than sqn-1 (Figure 3B). This result provides additional support for the conclusion that the phenotype of sqn mutations is a consequence of a reduction in AGO1 activity—a conclusion that was originally based entirely on the phenotypic similarity between sqn and ago1 mutations and the genetic interaction between these mutations [12]. It also supports the hypothesis that fbw2 suppresses sqn-1 by increasing the activity of AGO1. As a direct test of this hypothesis, we compared AGO1 protein levels in wild-type, sqn-1, ago1-25, ago1-45, and combinations of these mutations with fbw2. We found that sqn-1 produced a small but reproducible decrease in AGO1 in both 14-day old leaf tissue and floral tissue, and that fbw2-1 and fbw2-4 suppressed this effect; sqn-1 fbw2 double mutants had approximately the same amount of AGO1 as wild-type plants (Figure 3B and Figure 4C). Similar results were obtained with hypomorphic alleles of AGO1: ago1-25 and ago1-45 had reduced amounts of AGO1 protein compared to wild-type plants, and fbw2-1 and fbw2-4 corrected this defect (Figure 3C). We conclude that fbw2 suppresses the phenotype of sqn-1, ago1-25 and ago1-45 by increasing the abundance of AGO1.

We also examined the genetic interaction between fbw2 and several mutations that interfere with the biogenesis or stability of miRNAs, specifically se-1 (Figure 5A and 5B), hst-3 (Figure 5C and 5D), hyll-2 (Figure 5E and 5F), and hen1-6 (Figure 5G and 5H). In every case, double mutants had stronger vegetative phenotypes than the single mutants. Although we were initially surprised by this result, we recognized that the phenotypes of these double mutants are remarkably similar to the phenotype of plants transformed with a miR168-resistant version of AGO1, which results in the over-expression of AGO1 (Figure 5K) [9,13]. In order to determine if this was a reasonable explanation for the effect of fbw2, we introduced pAGO1::FLAG-AGO1 into se-1 and hst-3 (Figure 5I and 5J) and also assayed AGO1 proteins levels in single and double mutant plants (Figure 5L). Many hst-3 pAGO1::FLAG-AGO1 and se-1 pAGO1::FLAG-AGO1 primary transformants had phenotypes that were almost identical to hst-3 fbw2 and se-1 fbw2, and strongly resembled plants containing miR168-resistant AGO1 mRNA constructs (Figure 5H - I). Consistent with this observation, western blots revealed increased levels of AGO1 in se-1 fbw2, hen1-2 fbw2 and hst-3 fbw2 double mutants compared to the single mutants (Figure 5L). These observations support the conclusion that fbw2 enhances the phenotypes of se-1, hst-3, hyll-2 and hen1-6 by increasing AGO1 protein levels.

Over-expression of FBW2 phenocopies ago1 mutants

F-box proteins are part of the E3 ubiquitin ligase complex, a protein complex that targets substrates for ubiqui-
uitin-mediated proteolysis through the 26S proteasome [22,23]. F-box proteins bind to unique substrates and thus provide specificity to the complex. Evidence that FBW2 (SKIP18) is a component of a E3 ubiquitin ligase complex is provided by the observation that it interacts with several different components of this complex in yeast two-hybrid assays [24]. We examined the effect of overexpressing FBW2 in transgenic plants, using the constitutively expressed Cauliflower Mosaic Virus 35S promoter (35S::FBW2; hereafter FBW2ox). This approach was suggested by the observation that over-expressing F-box proteins typically enhances the degradation of their protein targets [22,25-27]. The vast majority of the primary transformants we obtained in this experiment had developmental phenotypes strikingly similar to those of hypomorphic ago1 alleles, consistent with the hypothesis that FBW2 represses the activity of AGO1 (Figure 4A). In contrast, plants over-expressing three genes closely related to FBW2--FBL9, FBL20, and SKIP1 (Additional File 1: Figure S1B)--have no noticeable phenotype (data not shown), further suggesting that FBW2 is functionally unique.

Figure 2 Mutations in FBW2 suppress hypomorphic ago1 mutations. (A) Eighteen-day-old rosettes of ago1 mutants and ago1 fbw2-1 double mutants. The shapes of leaves 1-4 are also illustrated. (B) The number of juvenile, adult and cauline leaves in ago1 and ago1 fbw2-1 double mutants (± standard deviation). (C) 14-day-old fbw2-1, ago1-36 and ago1-36/fbw2-1 double mutants grown on MS media. (D) Relative abundance of microRNA (miRNA) targets in various genotypes as measured by quantitative-real time polymerase chain reaction in 14-day-old (top) or 20-day-old (bottom) rosettes. Target genes were normalized to EIF4. Actin was used as a non-target control (± standard deviation). (E) Small RNA blots of low-molecular-weight RNA isolated from 14-day-old or 20-day-old rosettes probed with oligonucleotides complementary to specific miRNAs. Methionyl transfer RNA was used as a loading control.
In addition to promoting miRNA-mediated silencing, AGO1 is required for at least some forms of siRNA-mediated gene silencing, including the silencing of the 35S::GUS transgene present in the L1 line [7]. In order to determine if FBW2 affects this aspect of AGO1 function, we transformed FBW2ox into L1 plants and assayed for GUS activity in families that were homozygous for the L1 transgene and segregating FBW2ox. In contrast to L1 plants—which had low levels of GUS activity—plants containing both L1 and FBW2ox had high levels of GUS activity (Figure 4B), like L1 ago1 mutants [7]. These results suggest that FBW2 affects both the miRNA- and siRNA-dependent activities of AGO1.

We tested to see if FBW2 promotes the degradation of AGO1 by examining the level of AGO1 protein in siRNA-dependent activities of AGO1. The increase in the transcript levels of these miRNA-regulated genes is consistent with the decreased level of AGO1 protein in FBW2ox plants; AGO1 promotes miRNA-mediated gene silencing and, thus, a decrease in the abundance of this protein should lead to an increase in the level of miRNA-regulated transcripts. In contrast to its effect on the AGO1 protein, FBW2ox had no effect on the abundance of the YFP-ZLL (AGO10) fusion protein (Additional File 2: Figure S2A)[28].
Interestingly, destabilization of AGO1 by the viral F-box protein P0 is also insensitive to MG132 [19]. Although fbw2 produces an observable increase in the amount of AGO1 protein in genetic backgrounds in which miRNA activity is compromised (Figures 3B and 3C, 4C and 5L), we were unable to detect a significant increase in AGO1 protein in fbw2 single mutants (Figure 4C). We suspect this is because the miR168-dependent feedback mechanism that regulates AGO1 expression [13] partially corrects for slight increases in the level of this protein in fbw2 mutants. Mutations that interfere with the activity of miR168 (for example, sqn, ago1 and hen1) disrupt this feedback mechanism, thereby making AGO1 more susceptible to other factors that regulate its expression.

**fbw2 is hyposensitive to abscisic acid (ABA)**

Although we were unable to observe an increase in AGO1 protein in fbw2 single mutants, these mutants have a phenotype that is indicative of an increase in AGO1. Mutations that interfere with miRNA biogenesis—including hst, se, hyl1, dcl1 and hen1—confer hypersensitivity to the phytohormone ABA [30,31]. These mutations inhibit both seed germination and root elongation in the presence of low levels of ABA, probably due to the misregulation of the miR159 targets MYB101 and MYB33 [32]. We reasoned that if fbw2 mutations increase AGO1 levels this should result in increased miRNA activity and produce the opposite phenotype, namely, hyposensitivity to ABA. Indeed, this is what we found.

Seeds of various genotypes were grown on varying concentrations of ABA and scored for germination after 5 days. ago1-25 and FBW2ox were hyposensitive to ABA, with ago1-25 showing the greatest response. In the presence of ABA, both of these AGO1-deficient genotypes produced a significant decrease (P < 0.001 at 0.75 μM ABA) in seed germination relative to wild-type plants (Figure 6A); FBW2ox also displayed a slightly enhanced sensitivity to ABA in a root elongation assay (Figure 6B). Thus, AGO1 is required for a normal ABA response. In contrast, fbw2 mutants displayed a significantly increased rate of germination (P < 0.01 at 0.75 μM ABA) and increased rate of root elongation in the presence ABA (P < 0.03; Figure 6A and 6B). This result provides additional support for the conclusion that FBW2 normally represses the activity of AGO1 and reveals a physiological function for FBW2.

**Discussion**

The results presented here demonstrate that FBW2 is a negative regulator of AGO1. We show that loss of FBW2
suppresses the phenotype of mutations that reduce the activity of AGO1 and that this effect is associated with an increase in AGO1 protein levels. Conversely, overexpressing FBW2 produces an ago1 loss-of-function phenotype and leads to a decrease in the level of AGO1. Although a loss of FBW2 does not produce a major change in the abundance of AGO1 in an otherwise wild-type background, the reduced sensitivity of fbw2 mutants to ABA is consistent with a slight increase in AGO1 activity. Previous studies have demonstrated that Arabidopsis is hypersensitive to changes in AGO1 activity [8,9,12] so it would not be surprising if even a minor change in the abundance of this protein is biologically significant. Over-expressing FBW2 did not produce an observable decrease in the Argonaute protein AGO10/ZWL/PNH, suggesting that FBW2 may act specifically on AGO1.

The simplest and most direct way in which FBW2 could regulate AGO1 is by binding directly to this protein, thereby directing it to a proteasome-independent degradation pathway. However, we have been unable to detect a direct interaction between FBW2 and AGO1 (data not shown). Consequently, we cannot eliminate the possibility that FBW2 acts indirectly, through its effect on a protein required for AGO1 stability. This protein cannot be SQN because fbw2 mutations rescue the phenotype of the null allele, sqn-1. Furthermore, over expressing FBW2 produces a much more severe phenotype than that of sqn null alleles. Recent work suggests that the viral F-box protein, P0, acts by targeting an unknown component of the AGO1 RISC complex, leading to the destabilization and degradation of AGO1 [33]. It may be that FBW2 regulates this same protein or another protein within the RISC complex. Loss-of-function mutations in this hypothetical FBW2-regulated factor would be expected to have the same phenotype as FBW2ox plants, such as an ago1 loss-of-function phenotype. Extensive screens for genes required for miRNA and siRNA biogenesis and activity by us and others have produced many loss-of-function alleles of AGO1 but, with the exception of sqn, have yet to reveal other ago1-like mutants. Determining the identity of this unknown protein (if it exists) may require a biochemical approach.

The activity of AGO1 in Arabidopsis is regulated by a variety of different mechanisms that act together to maintain the expression of this protein at a constant level [9-11,13,15]. This is critical because both an increase and a decrease in the abundance of AGO1 have significant effects on plant development. An important component of this homeostatic mechanism is the negative regulation of AGO1 by miR168 [9,13]. miR168 represses AGO1 in an AGO1-dependent fashion: a decrease in the activity of AGO1 leads to a decrease in miR168 activity and a subsequent increase in AGO1 expression, whereas an increase in AGO1 activity has the opposite effect. We believe this feedback loop is responsible for the observation that fbw2 mutations individually have no major effect on AGO1 because these same mutations elevate AGO1 protein levels in combination with mutations that interfere with miRNA biogenesis or activity.

Such finely tuned posttranscriptional regulation of an Argonaute protein is not unique to AGO1. The stability and sub-cellular localization of the mammalian protein Ago2 are influenced by hydroxylation and phosphorylation [34,35], while the turnover of Ago2 is controlled by an E3 ubiquitin ligase [36]--a mechanism that may be quite similar to the mechanism we propose here. Furthermore, Ago2 is post-translationally controlled by a variety of environmental and developmental cues, which operate via well-defined pathways [34-36]. It would not be surprising if environmental and developmental signals also play important roles in the regulation of AGO1. The observation that fbw2 has little, or no effect, on plant morphology, but decreases the sensitivity of plants to ABA, is relevant in this case. Among other things, ABA regulates the response to water stress. Therefore, the effect of fbw2 on ABA sensitivity suggests that changes in AGO1 activity may underlie the response to this and other environmental signals. The potential involvement
of FBW2 in such regulatory pathways is an interesting subject for future studies.

**Conclusion**

Our results demonstrate that FBW2 is a negative regulator of AGO1 and acts by destabilizing this protein. Although we are unable to determine whether FBW2 destabilizes AGO1 directly or via an effect on an as yet unknown protein, these results add yet another layer of control to the already complex mechanism responsible for AGO1 homeostasis. Loss of FBW2 affects the sensitivity of plants to the growth regulator ABA, suggesting a possible role for FBW2 in hormone response pathways.

**Methods**

**Genetic stocks and growth conditions**

Unless otherwise noted, all mutations described in this paper are in the Columbia background. fbw2-1, fbw2-2 and fbw2-3 were identified in M2 families of EMS-mutagenized sqn-1 plants. Primers for genotyping can be found in Additional File 3: Table S1. Plants containing pGreen0229 AGO1::FLAG-AGO1 were provided by D Baulcombe (University of Cambridge, Cambridge, UK) [21]. ago1-25, ago1-27, 2m-AGO1 lines and L1 lines were provided by H Vaucheret (Institut National de la Recherche Agronomique, Versailles, France) [7,8]. Plants containing YFP-AGO10 (pZLL::YFP-ZLL) in the Ler background were obtained from T Laux [28]. fbw2-4 (SALK_144548), fbw2-5 (SALK_071588), hyl1-2 (SALK_090960), hyll-2 (SALK_064863) and se-1 were obtained from the Arabidopsis Biological Resource Center http://abrc.osu.edu/ [18]. Unless otherwise noted, plants were grown on Farfard soil at 23°C under 16 h fluorescent illumination. Wild-type or fbw2 plants segregating ago1-36 were grown on Murashige and Skoog (MS) plates (0.5% MS, 1% sucrose, 0.8% agar pH 5.7). GUS assays were performed as previously described [37].

**RT-PCR**

Total RNA was isolated using TRIzol (Invitrogen, CA, USA) from total above-ground tissue of 14-day-old seedlings. RNA was immobilized on RNeasy Plant Columns (Qiagen, CA, USA) and treated with DNase (Qiagen). cDNA was amplified from 1-4 μg of RNA using SuperScript II (Invitrogen). Quantitative RT PCR was performed using Sybr Green PCR Master Mix (Applied Biosystems, CA, USA) on a StepOnePlus™ RT-PCR System (Applied Biosystems). FBW2 RT Primers are described in Additional File 3: Table S1. Primers for RT-PCR of miRNA targets were previously described [12]. Transcript levels were normalized against EIF4 levels in all experiments.

**RNA blots**

RNA blots were generated and analysed as previously described [12,38]. Briefly, total RNA was isolated using TRIzol (Invitrogen) from total above-ground tissue of 14-day-old seedlings. High molecular weight RNA was removed by precipitating with PEG-8000 (5%) and NaCl (500 mM). Low molecular weight (LMW) RNA was subsequently precipitated with 300 mM NaOAc and 2 vol 100% EtOH and washed with 70% EtOH. LMW RNA was separated on an 8 M urea/15% denaturing polyacrylamide gel and was transferred to a Hybond N membrane (Amersham Pharmacia, NJ, USA). Oligonucleotide probes were labelled using T4 polynucleotide kinase (New England Biolabs, MA, USA) with [γ -32P]-ATP at 40°C in ULTRAhyb-oligo hybridization buffer (Ambion, CA, USA). Membranes were hybridized with oligonucleotide probes complementary to specific miRNA targets.

**Western blot and immunoprecipitation experiments**

Fourteen-day-old, 20-day-old seedlings, or floral buds were grown using liquid nitrogen and resuspended in 1:3 w/v extraction buffer [20 mM Tris pH 7.5, 300 mM NaCl, 5 mM MgCl2, 1× Protease Inhibitor Cocktail (Sigma, MO, USA) 1 mM PMSF, 1 mM DTT]. Equal amounts of soluble protein were separated on an 8.5% SDS-PAGE gel, transferred to a nitrocellulose membrane and membranes were blocked [tris buffered saline with tween (TBS-T) with 5% milk]. Anti-AGO1 (1:500; antibody provided by Y Qi and Xiaofeng Cao, National Institute of Biological Sciences, Beijing, China), anti-FLAG monoclonal (1:2000) (Sigma - F1804), anti-FLAG HRP conjugated (1:2000; Sigma - A8592), anti-GFP (Invitrogen - A-6455), anti-cMyc rabbit (1:2000; Sigma - C3956), were incubated overnight in TBS-T + 5% milk at 4°C. Anti-actin (1:10,000) (Sigma - A0480) was incubated at room temperature for 2 h.

**Constructs**

The FBW2 coding or FBW2 genomic region was amplified by PCR using the FBW2 primers (Additional File 3: Table S1). PCR products were TOPO cloned into pENTR-D TOPO (Invitrogen). The FBW2 coding and the 4701-base pair genomic FBW2 genomic region were recombined into pEG100 (35S) and pEG302 (FLAG), respectively [39]. For AGO1::FLAG-AGO1 constructs, genomic AGO1::FLAG-AGO1 was amplified from the pGreen0229 FLAG-AGO1 construct using AGO1 F and R primers (Additional File 3: Table S1) [21]. PCR products were cloned into pENTR-D TOPO vector and recombined into the Cambia 3301 vector containing a Gateway cassette. All constructs were transformed using the Floral Dip technique [40].
ABA experiments
Sterilized seeds were plated on 1% sucrose LS plates containing 0, 0.5, 0.75, 1.0, 1.5 or 2.0 μM ABA, imbibed for 3 days in the dark at 4°C and grown for 5 days under long day conditions (16 h light, 8 h dark). Plants were scored for greening of the cotyledons. Experiments were repeated at least three times. In order to determine the effect of ABA on root growth, sterilized seeds were plated vertically on 1% sucrose LS plates, imbibed for 3 days, and grown for 5 days under long day conditions (16 h light, 8 h dark). Seedlings of equal size were then transplanted to vertical plates containing either 0 μM or 2.5 μM ABA, and grown for 5 days or more under long day conditions (16 h light, 8 h dark). Primary root length was measured for each plant and root inhibition was determined as the average root length of plants grown at 2.5 μM ABA relative to the average root length of plants grown at 0 μM ABA.

Additional material

Additional file 1 Figure S1. (A) Gene structure of FBW2 showing the location of primers used in real time polymerase chain reaction and the abundance of FBW2 messenger RNA in sns-1 and mutants doubly mutant for sns-1 and different alleles of fbw2. All target genes were normalized to EF14. (B) Alignments of the Arabidopsis F-box genes most closely related to At4g08990 (FBW2): At4g05497 (FBW9), At4g09546 (FBL20), At5g57900 (SKIP1). (C) Alignments of the amino acid sequences of FBW2 and its predicted orthologs in other flowering plants. All alignments were made using the following assembled contigs from PlantGDB unless otherwise noted: Citrus sinensis (PUT-157a-Citrus_sinensis-6728447), Gossypium raimondii (PUT-157a-Gossypium_raimondii-11427), Glycine max (PUT-161a-Glycine_max-76896), Oryza sativa Japonica (GenBank AK070008), Asparagus officinalis (GenBank CV288647), Vitis vinifera (PUT-157a-Vitis_vinifera-16230), Nicotiana tabacum (PUT-163a-Nicotiana_tabacum-5592838), Lactuca sativa (PUT-157a-Lactuca_sativa-30499), Pinus taeda (PUT-157a-Pinus_taeda-7928250).

Additional file 2 Figure S2. (A) Western blot comparing levels of ARGONAUTE1 (AGO1) and yellow fluorescent protein-AGO1 in plants with and without FBW2(or constructs. Anti-AGO1 and anti-GFP antibodies were used to probe each blot. Poncereau staining was used as a loading control. (B) Levels of AGO1-FLAG do not decrease in plants treated with the protease inhibitor MG132. Blots were probed with anti-FLAG monoclonal antibody or anti-ubiquitin antibody. The anti-ubiquitin antibody demonstrates an overall increase in ubiquitination in MG132 treated plants, as expected from a decrease in proteasome activity.

Additional file 3 Table S1. Polymerase chain reaction primers used in this study.

Abbreviations
ABA: abscisic acid; AGO1: ARGONAUTE1; EMS: ethyl methane sulfonate; mRNA: messenger RNA; miRNA: microRNA; RISC: RNA-induced silencing complex; RT-PCR: real time polymerase chain reaction; siRNA: small interfering RNA; SQN: SQUINT; TBS-T: tris buffered saline tween.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
KWE performed most of the work described in this paper and co-wrote the manuscript with RSP. MRS performed the sns suppressor screen. MRS and KWE mapped FBW2. RW performed the ABA experiments under the guidance of BDG.

Acknowledgements
We are grateful to Matthew Willmann and Stewart Gillmor for their comments on this manuscript. This work is supported by NIH Grant RO1GM081893 to RSP. KWE is supported by an NIH NRSA postdoctoral fellowship (F32GM084591) from the NIGMS. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or the National Institutes of Health.

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Received: 23 March 2010 Accepted: 12 July 2010

Published: 12 July 2010

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doi: 10.1186/1758-907X-1-15
Cite this article as: Earley et al., An endogenous F-box protein regulates ARGONAUTE1 in Arabidopsis thaliana. Silence 2010, 1:15