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Report

The Polerovirus F Box Protein P0 Targets ARGONAUTE1 to Suppress RNA Silencing

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Summary

Plants employ post-transcriptional gene silencing (PTGS) as an antiviral defense response [1]. In this mechanism, viral-derived small RNAs are incorporated into the RNA-induced silencing complex (RISC) to guide degradation of the corresponding viral RNAs [2]. ARGONAUTE1 (AGO1) is a key component of RISC: it carries the RNA slicer activity [3]. As a counter-defense, viruses have evolved various proteins that suppress PTGS [4]. Recently, we showed that the Polerovirus P0 protein carries an F box motif required to form an SCF-like complex, which is also essential for P0's silencing suppressor function [5]. Here, we investigate the molecular mechanism by which P0 impairs PTGS. First we show that P0's expression does not affect the biogenesis of primary siRNAs in an inverted repeat-PTGS assay, but it does affect their activity. Moreover, P0's expression in transformed *Arabidopsis* plants leads to various developmental abnormalities reminiscent of mutants affected in miRNA pathways, which is accompanied by enhanced levels of several miRNA-target transcripts, suggesting that P0 acts at the level of RISC. Interestingly, ectopic expression of P0 triggered AGO1 protein decay in planta. Finally, we provide evidence that P0 physically interacts with AGO1. Based on these results, we propose that P0 hijacks the host SCF machinery to modulate gene silencing by destabilizing AGO1.

Results and Discussion

P0 Suppresses RNA Silencing Triggered by dsRNA

The P0s of Beet western yellows virus (BWYV, $P0^{BW}$) and Cucurbit aphid-borne yellows virus (CABYV, $P0^{CA}$), two Poleroviruses, were identified as silencing suppressor proteins (SSPs) in a sense-PTGS assay [6] and assumed to interfere with the production and/or action of siRNAs. To identify the level of action of P0 in the silencing pathway, we analyzed the effect of P0 on production of this RNA species in an inverted repeat (IR)-PTGS assay. Leaves of *N. benthamiana* were infiltrated with a mixture of *Agrobacterium tumefaciens* cultures harboring binary vectors encoding a double-stranded (ds)RNA inducer "GFFG" deriving from the GFP gene [7] plus either $P0^{BW}$, $P0^{CA}$, P38 (P38 is the SSP of Turnip crinkle virus [8]), or the empty vector pBin61. Primary siRNAs, which are directly produced through the processing of the IR stem by an RNase III-type Dicer-like (DCL) protein, are detected at a similar level in the absence or presence of $P0^{BW}$ or $P0^{CA}$ (Figure 1B, GF probe, lanes 1–3), indicating that both proteins interfere with a step in the PTGS pathway downstream of primary siRNA production by DCL. Conversely, P38 provoked a strong reduction of these siRNAs (Figure 1B, lane 4), consistent with its known effect on DCL4 and DCL2 in *Arabidopsis* [9]. The presence of the SSPs was confirmed by western blot with specific antibodies (Figure 1C).

To analyze the effect of the SSPs on secondary siRNAs, we repeated the agroinfiltration assays in the presence of a construct expressing GFP. Secondary siRNAs whose production involves a host RNA-dependant RNA polymerase (RDR6) are visualized by a probe (P probe) targeting a region outside of the inverted GFFG repeat. In the absence of the GFFG RNA, GFP accumulation is already significantly diminished via the S-PTGS pathway (Figures 1A–1C, lane 5), but the GFP silencing was much stronger in the presence of the GFFG RNA (Figures 1A–1C, lane 6), as expected from the transitivity triggered by the primary siRNAs derived from this construct. The patches infiltrated with $P0^{BW}$, $P0^{CA}$, or P38 displayed bright-green fluorescence, indicating that the SSPs were functional, which was confirmed by the presence of GFP mRNA and protein (Figures 1A–1C, lanes 7–9). As found previously, the levels of primary siRNAs produced in the presence of $P0^{BW}$ or $P0^{CA}$ (lanes 7 and 8) were similar to that observed in the control (lane 6), whereas those produced in the presence of P38 were barely detectable (lane 9). Interestingly, both P0 proteins diminished the production of secondary siRNA (P probe; lanes 7 and 8). We conclude that P0 inhibits a step in the PTGS pathway downstream of DCL-mediated primary siRNA production. The interference of P0 with secondary but not primary siRNA production resembles the behavior of AGO1 mutants [10] and thus suggests that P0 acts at the level of RISC (see below).

Expression of $P0^{BW}$ Produces Developmental Aberrations in Transgenic *Arabidopsis*

To further investigate the mechanism underlying P0 suppressor activity, we generated *A. thaliana* transgenic plants expressing P0. Transformed seedlings expressing $P0^{BW}$ under control of the strong constitutive CaMV 35S promoter were unable to proceed much beyond embryonic development (see Figure S1 in the Supplemental Data available with this article online). P0 expression in 4-week-old 35S-P0 seedlings was examined by reverse transcription followed by real-time quantitative PCR (RT-qPCR), and P0 mRNA level was normalized to Col-0 seedlings (Figure 2B). The mRNA level present in these seedlings was extremely high, more than 140,000-fold compared to a nontransformed plant.

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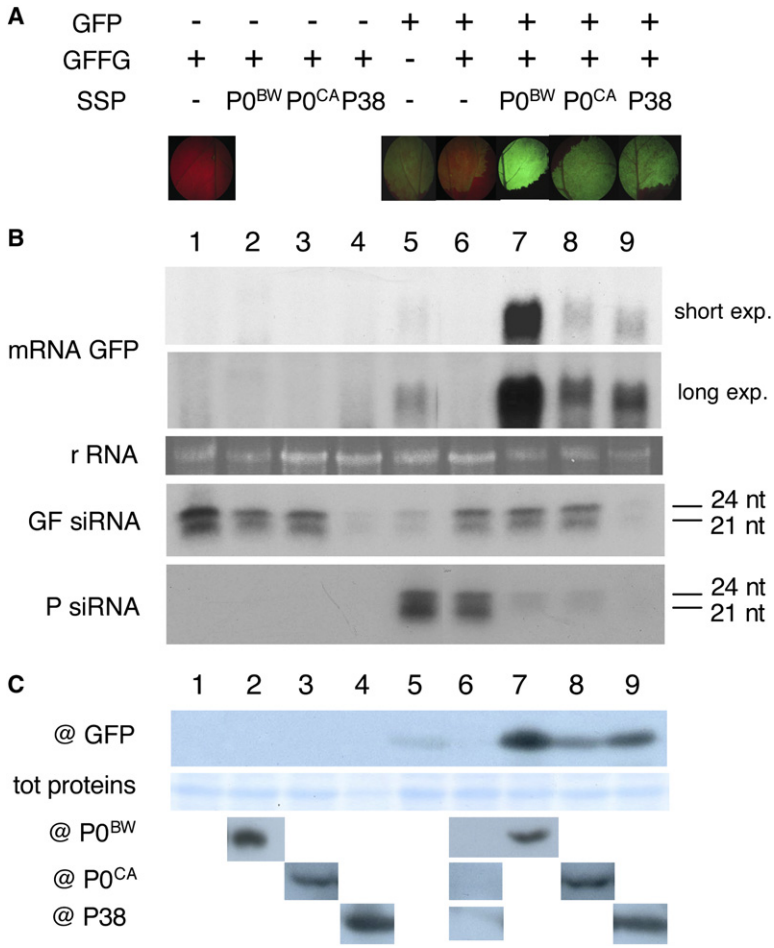

Figure 1. P0 Suppresses Silencing Triggered by an Inverted-Repeat Sequence

Leaves of *N. benthamiana* were infiltrated with a mixture of Agrobacteria containing the binary vectors encoding no SSP (–), $P0^{BW}$, $P0^{CA}$, or P38 in addition to those for GFP and/or GFFG as indicated above each lane.

(A) GFP fluorescence was visualized 5 days postinfiltration under UV light.

(B) RNA were extracted and analyzed for the presence of GFP mRNA by a GFP cDNA probe. Small RNA were hybridized to specific probes for the GF or P part of the GFP [7].

(C) Total proteins were extracted from the same leaves and analyzed by western blot for the presence of GFP. Samples coinfiltrated with a SSP were immunoassayed for the presence of the corresponding protein via specific antisera. Loading controls (rRNA and total proteins) are provided.

This result infers that expression of $P0^{BW}$ targets a step essential for early development in *Arabidopsis*.

To overcome this difficulty, we chose to express $P0^{BW}$ in a conditional manner by using the XVE vector system, in which transgene transcription can be tightly regulated by treatment with estradiol [11]. Eighteen independent XVE-$P0^{BW}$ transformants were characterized. In the absence of estradiol, plant development was indistinguishable from that of wild-type *Arabidopsis* (Figure 2A, a). When transferred to medium containing the chemical inducer, half of the T2 lines showed abnormalities on developing leaves after 5 to 7 days of treatment. A high fraction of the progeny of these plants also developed the altered phenotype upon estradiol induction (~75% and 90%, respectively, for lines L18 and L21). The plants showed upwards curling and severe crumpling of newly developing leaves (Figure 2A, b, c, e). A reduction of the main root length and an increase in number of lateral roots was also noted (Figure 2A, f). When the induced plants were transferred to soil, most of them continued to show leaf abnormalities and reduced growth although no further estradiol treatment was applied. In some cases, the flowering stems showed a tendency to twist and curl and eventually became fasciated (Figure 2A, g; Figure S1). Inflorescence exhibited an abnormal phyllotaxy pattern and reduced fertility was noticed. Untreated plants showed a normal phenotype and produced fertile seeds.

Transgenic lines L18 and L21 were further characterized for P0 expression by RT-qPCR. RNA was extracted from newly developing leaves of in vitro P0 plantlets 3–14 days after addition of estradiol or DMSO, as a control. Quantification of P0 mRNA from induced plants was normalized to that of noninduced plants. The relative level of P0 mRNA expression was highest (~53,000) at 3 days postinduction (pi, the earliest time measured) and gradually decreased to ~400 at 14 days pi (Figure 2B). L18 expressed lower P0 mRNA levels than did L21, presumably because of partial silencing resulting from a higher copy number of the P0 transgene in L18 (see Figure S2 for details). Antibodies against P0 [6] detected the protein in estradiol-induced plants at 3 days pi, but not in untreated plants (Figure 2C).

$P0^{BW}$ Expression Affects Accumulation Levels of Several miRNA-Targeted mRNAs

The abnormal phenotypes displayed by the P0-expressing plants have similarities to those reported for transgenic *Arabidopsis* expressing other viral SSPs [12–14], including altered leaf morphology (elongated, curled, and/or serrated leaves) and flower abnormalities with reduced fertility. These earlier studies established a correlation between the morphological defects and perturbation of the endogenous miRNA pathway. Our IR-PTGS assay demonstrated that primary siRNA accumulation was unaltered by P0 expression, implying that P0 interferes

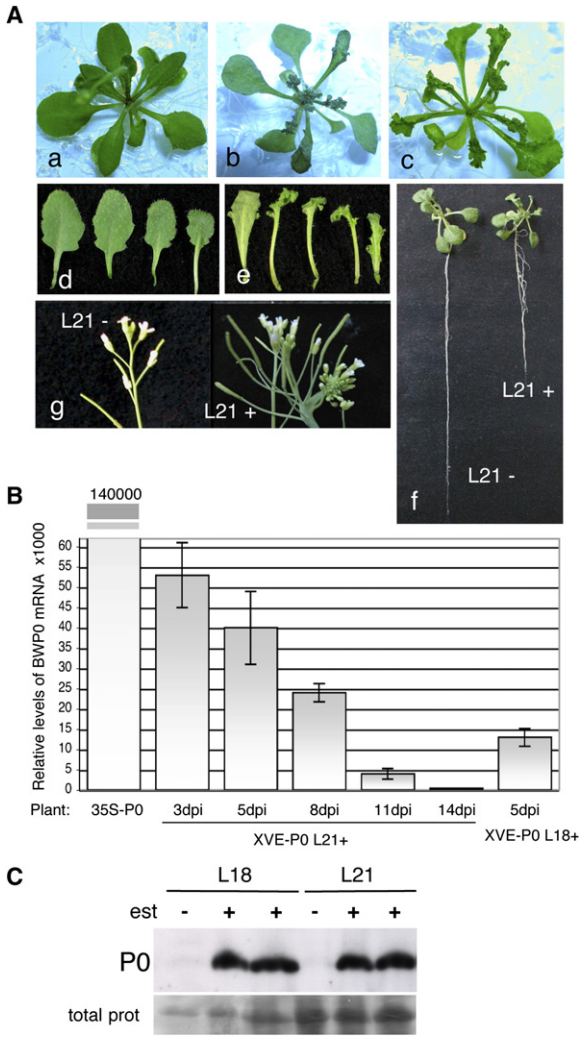

Figure 2. BWYV-Encoded P0 Protein Induces Developmental Defects in *Arabidopsis*

(A) Phenotypic effects in XVE-P0^{BW} L21 transgenic line upon induced expression of P0^{BW}. A 3-week-old noninduced XVE-P0^{BW} plant (a) compared to estradiol-induced XVE-P0^{BW} plants 7 days after treatment (b, c). Leaves of a noninduced XVE-P0^{BW} plant (d) were compared to leaves of an estradiol-induced XVE-P0^{BW} plant (e). Comparison of the root system (f) and flowers (g) of a noninduced (L21−) and an induced (L21+) XVE-P0^{BW} plant.

(B) P0 transcript analysis by real-time quantitative PCR in transgenic plants. 4-week-old 35S-P0^{BW} seedlings were pooled and tested 4 weeks after germination (35S-P0^{BW}). For XVE-P0^{BW} L21, a time course of P0 transcript accumulation was performed after estradiol induction on 10-day-old seedlings from 3 to 14 days pi (XVE-P0^{BW} L21+). XVE-P0^{BW} L18 was tested 5 days after treatment (L18+). Quantification of P0 mRNA was normalized to that of *ACTIN2,* then either to the value of Col-0 seedlings (for 35S-P0^{BW} plants) or to that of noninduced plants (for XVE-P0^{BW}-induced plants), the standard values being arbitrarily fixed to 1. Error bars represent the standard deviation from three replicates.

(C) P0 protein detection in noninduced (−) and induced (+) XVE-P0^{BW} L18 and L21 plants 3 days after estradiol (est) treatment. A loading control is provided.

with a step downstream of DCL-mediated siRNA production. An essential component at the crossroad between the miRNA and PTGS pathways is the RISC effector complex [2, 15]. RISC containing AGO1 has been shown to be active in both siRNA- and miRNA-mediated cleavage of transcripts [3, 14, 16, 17], so any step involving AGO1 is a potential target site for P0 inhibition. We therefore investigated the effect of P0 on the accumulation level of miRNA-targeted mRNAs.

We selected 12 known miRNA-target genes and analyzed their mRNA accumulation by RT-qPCR 5 dpi in induced versus noninduced XVE-P0^{BW} L21 plants. We hypothesized that P0 acts at the level of RISC, so we also included in our analysis a strong hypomorphic allele of AGO1, *ago1-11* [18], which shows enhanced accumulation of some miRNA-targeted transcripts [19]. The tested genes can be divided into two groups with respect to their response to induction of P0 expression. Six of the twelve genes analyzed showed a strong upregulation of transcript accumulation (by at least 3-fold) after P0 induction: *SPL10, AGO1, HAP2C, ARF17, MYB65,* and *DCL1* (Figure 3A). The *SPL10* and *HAP2C* transcripts, in particular, accumulated to very high levels in both induced P0 lines and *ago1-11* plants (up to 10-fold). The *AGO1* and *MYB65* mRNA, on the other hand, accumulated to considerably higher levels in the P0-induced plants than in the *ago1-11* mutant. Ronemus et al. [19] obtained similar results by microarray analysis of *ago1-11* plants (strong upregulation of *SPL* and *HAP2C* transcripts and moderate or no modification for several of the other genes tested). The second group of genes showed little (*CUC2, TIR1,* and *SCL6 III*) or no (*TCP10, AP2*, and *SCL6 IV*) difference at the transcript levels after P0 induction (Figure 3B). Similar behavior of these transcripts was recorded with line L18 of XVE-P0^{BW} (data not shown). miRNA-regulated genes play crucial steps in plant development (reviewed in [20, 21]), so the effect of P0 expression on such target genes can explain the pleiomorphic developmental defects observed for estradiol-induced XVE-P0^{BW} plants. Examination of miRNA levels revealed, at most, only small reductions for some of the miRNA tested (see Figure S3). In conclusion, the effect of P0 on accumulation of the various miRNA-target genes generally parallels the effect of a hypomorphic *ago1* mutant on these transcripts, further supporting the hypothesis that AGO1 is a target of P0.

P0^{BW} Provokes AGO1 Decay In Planta

If P0 targets AGO1, the most straightforward scenario would be that P0 acts as an F box protein in an SCF complex that recognizes AGO1 and promotes its degradation by the proteasome. Indeed, Baulcombe et al. [22] have recently reported that, among several SSPs tested in a transient expression assay, P0 was the only SSP that had a destabilizing effect on AGO1. We therefore sought to determine the effect of P0 induction on AGO1 stability in the XVE-P0^{BW} plants. We introgressed the transgenic XVE-P0^{BW} L21 line into the *FLAG-AGO1/ago1-36* mutant background in which a functional epitope-tagged version of AGO1, FLAG-AGO1, is expressed under control of the *AGO1* promoter [3]. Double transgenic plants were selected and further analyzed. Strikingly, P0 induction leads to a massive drop of FLAG-AGO1 protein levels after estradiol induction (Figure 4A). The diminished AGO1 protein levels are not the consequence of a reduction of *AGO1* transcription, because *AGO1* transcripts are strongly upregulated in induced XVE-P0^{BW} plants (Figure 3A).

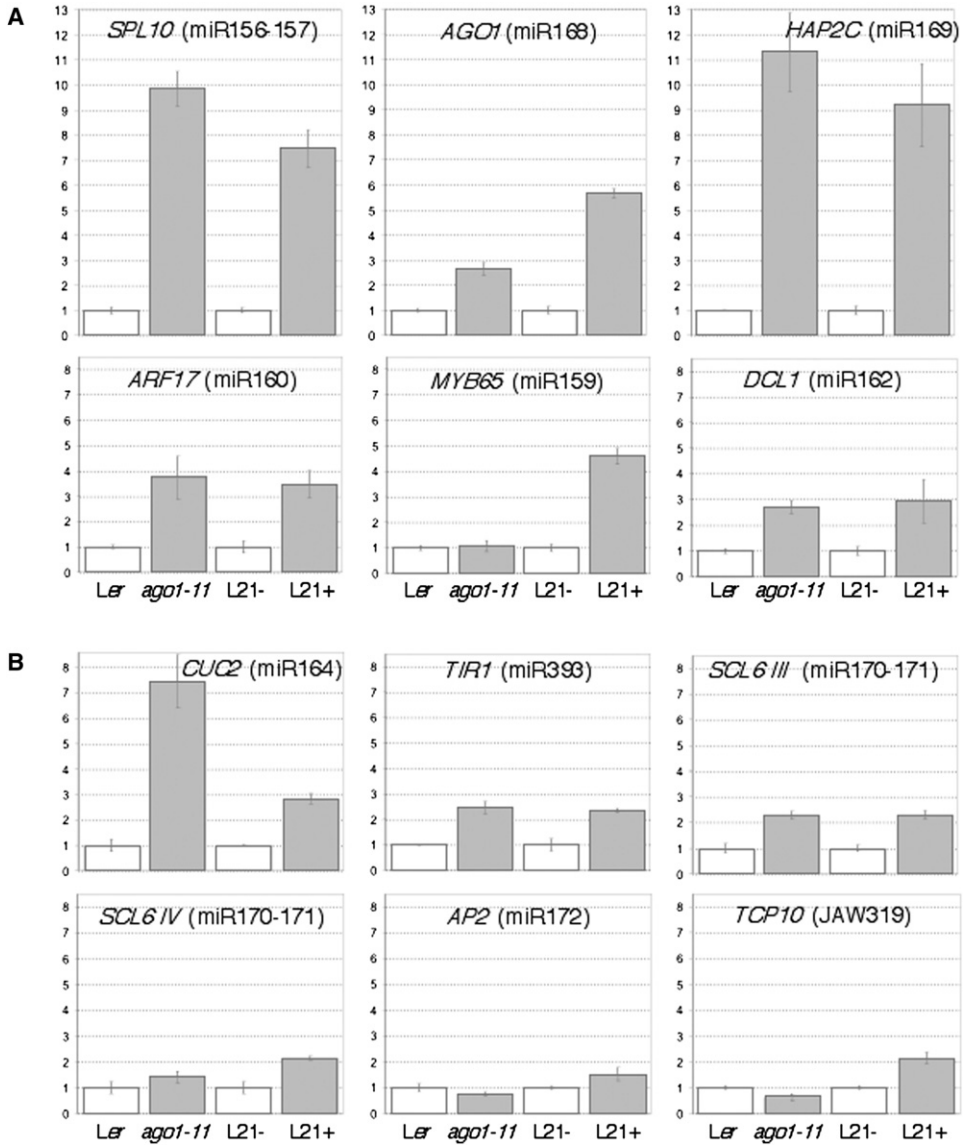

Figure 3. Levels of miRNA-Targeted mRNAs in P0-Expressing Plants

P0-expressing plants show increased levels of some miRNA-targeted mRNAs (A), whereas others are only slightly or even not at all affected (B). Relative levels of miRNA-targeted mRNAs were obtained by real-time quantitative PCR. RNA were extracted 5 days after chemical treatment from the younger developing leaves showing aberrant phenotype or the corresponding leaves from nontreated plants. The same target transcripts were analyzed for seedlings from an *ago1-11* allele. Quantifications were first normalized by comparison to *ACTIN2* and then to the values of L*er* control plants (for *ago1-11*) or to those of noninduced plants (L21−). Because transcription level of untreated Col0, L*er*, and L21 plants showed no significant variation (see Figure S4), the controls were arbitrarily fixed to 1. Error bars represent the standard deviation from three replicates, and similar results were obtained in three independent biological repeats. Control tests performed in order to evaluate the influence of estradiol on nontransgenic Col0 plants proved the innocuity of the chemical treatment on the expression of the genes analyzed (see Figure S4).

P0 Interacts with AGO1 In Vitro and In Vivo

Based on the above findings, we propose that P0, as part of an SCF complex, targets AGO1 for degradation and, as a consequence, disarms the RNA silencing machinery. At this stage, however, we cannot rule out the possibility that P0 expression indirectly affects AGO1 stability by targeting, for example, another protein that stabilizes AGO1. To test for the existence of a direct physical interaction, we first carried out yeast two-hybrid experiments, with binding domain and activation domain fusions of both P0 and AGO1 being tested. No interaction between these two proteins was observed, although the fusion proteins were expressed (data not shown). This lack of interaction is, however, not unexpected because most SCF targets require post-translational modifications such as phosphorylation or N-glycosylation as a prerequisite for their recognition by F box proteins [23, 24].

We next used in vitro pull-down assays, with AGO1 produced in planta, to test for an interaction. $P0^{CA}$ and $P0^{BW}$ were produced in *Escherichia coli* as translational fusion proteins with GST and incubated with immunoprecipitated Flag-AGO1 protein extracted from *Flag-AGO1/ago1-36* transgenic *Arabidopsis* plants [3]. The

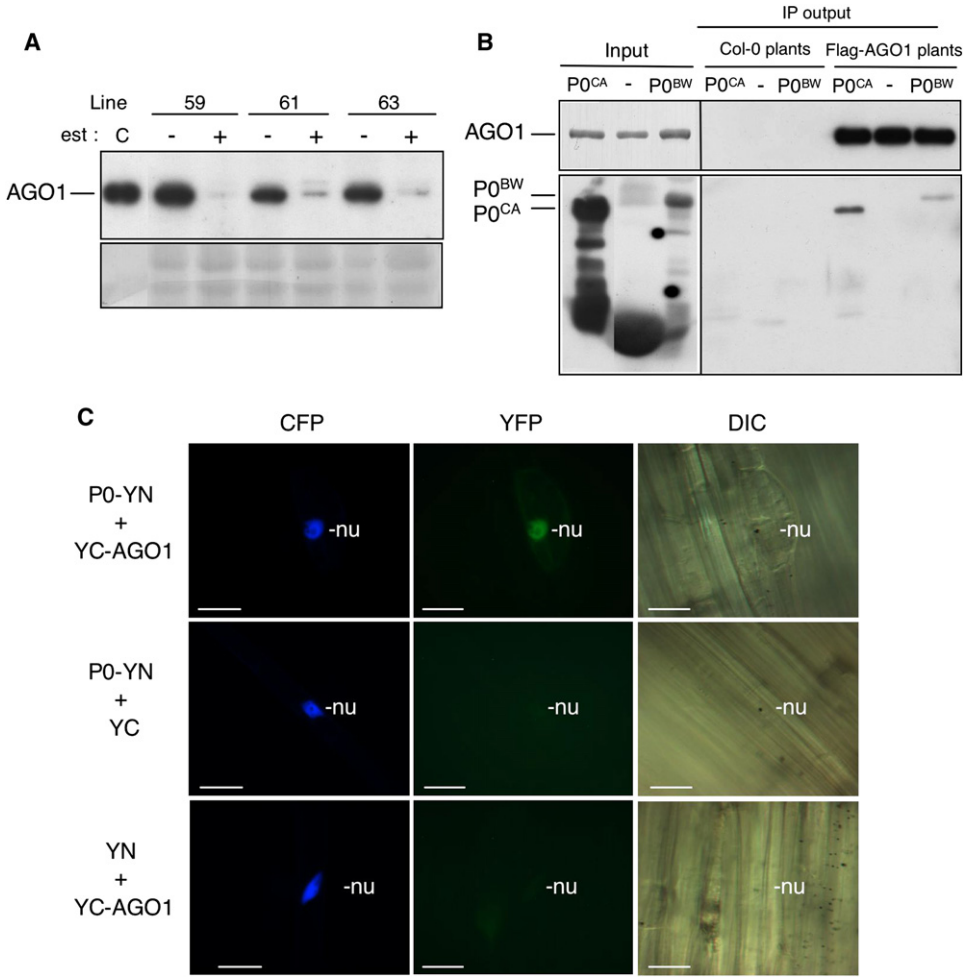

Figure 4. P0 Interacts with AGO1 and Mediates Its Degradation

(A) AGO1 is destabilized in FLAG-AGO1/XVE-P0^{BW}-induced plants. Total protein extract was prepared from a pool of in vitro plantlets after 7 days of treatment with estradiol (+) or with DMSO (−). Three independent lines were tested for the presence of AGO1 by western blot with an anti-FLAG antiserum (top gel). C refers to control proteins from an immunoprecipitated sample from FLAG-AGO1 plant. est, estradiol. A loading control is provided in the bottom gel.

(B) P0 and AGO1 interact in vitro. GST-P0^{CA} (P0^{CA}) and GST-P0^{BW} (P0^{BW}) fusion proteins and single GST protein (−) were produced, purified with glutathione sepharose beads and eluted before being mixed with anti-FLAG-immunoprecipitated proteins from either Col-0 plants or FLAG-AGO1 transgenic plants. The blotted membrane was cut in two, the upper part was probed with FLAG-HRP-conjugated antibodies, and the lower part was probed with a cocktail of antisera specific for P0^{CA} and GST-P0^{BW}. A fraction (1/12) of the purified GST-P0^{CA}, GST-P0^{BW}, GST, and AGO1 proteins was loaded on the left panel (input). Because of high background in the first lane (P0^{CA}), a shorter exposure was shown. Coimmunoprecipitated proteins on anti-FLAG affinity beads (IP output) from Col-0 or FLAG-AGO1 plants are shown in the middle panel and right panel, respectively.

(C) Interaction between P0^{CA} and AGO1 by means of bimolecular fluorescence complementation (BIFC). Different combinations of plasmid containing the indicated YN- and YC-fusion proteins were bombarded on dark-grown mustard seedlings. A transfection control CPRF2 expressing a fused CFP targeted to the nucleus (nu) [26] was systematically included to identify the transformed cells. Images were recorded 5 hr after bombardment via CFP- and YFP-specific filters. A differential interference contrast image is shown (DIC). All images are at the same magnification; scale bar represents 40 μm.

purified GST-P0^{CA} and GST-P0^{BW} cosedimented with Flag-AGO1, unlike the GST protein control, as shown when the blot was probed with a mixture of antibodies specific for P0^{BW}, P0^{CA}, and GST (Figure 4B). Importantly, no pull-down product was obtained when extracts from nontransgenic Col-0 plants were tested.

To provide further evidence for a physical interaction between P0 and AGO1 in vivo, we carried out bimolecular fluorescence complementation (BiFC) experiments [25, 26]. Plasmids P0^{CA}-YN and YC-AGO1 were cobombarded into etiolated mustard hypocotyls. A strong YFP signal was observed in the nucleus of 78% of the cells examined (45/58; Figure 4C), similar to cells transfected with the positive control P0^{CA}-YN + YC-ASK2 [5]. Only weak fluorescence in a few cells was observed after bombardment with the following plasmid control combinations: P0^{CA}-YN + YC or YN + YC-AGO1 (8/51 and 11/48 cells, respectively; Figure 4C). The interaction between P0 and AGO1 in the nucleus is consistent with the subcellular localization of ASK1/2 and AtCUL1 and the notion that SCF ubiquitylation occurs primarily in this cellular compartment [27, 28]. Taken together, our

data support the hypothesis that AGO1 could be a direct target of the viral F box protein P0.

Proteolysis as a Novel Strategy to Suppress RNA Silencing

In this report, we propose a model in which P0 triggers the degradation of AGO1 to suppress gene silencing. However, the present data do not exclude the possibility that P0 might also have other cellular targets that could contribute to its silencing suppressor activity. Future work will address this issue. Interestingly, another SSP has recently been reported to directly interact with AGO1, *Cucumber mosaic virus* 2b, which inhibits AGO1's cleavage activity in RISC [14]. Thus, AGO1 represents a common target of at least two different SSPs, a strategy that impedes its participation in normal RISC activity. Strikingly, both CMV 2b and P0 were found to colocalize with AGO1 in the nucleus ([14] and this work).

Although the ubiquitin-dependant proteolysis machinery is widely exploited by viruses to manipulate host cell pathways (reviewed in [29]), this is to our knowledge the first example of a viral-encoded F box protein for which a cellular host target protein has been identified. This finding raises a number of interesting questions. Does recognition of AGO1 by P0 require post-translational modification of AGO1 and, more generally, what role if any do such modifications play in regulation of AGO1 activity? In view of the fact that AGO1 is a member of a multigene family, are some of the nine other AGO1-related proteins also targeted by P0? It will also be interesting to learn whether there is tissue specificity in the expression pattern of the *AGO* family members. This question is of considerable importance, because it is not yet known whether AGO1 is the major player in virus-induced silencing in all tissue types. Furthermore, such tissue specificity could at least partly explain the phloem restriction of Poleroviruses [30]. Finally, it will be interesting to investigate whether AGO proteins and/or other proteins of the RNA silencing machinery, is (are) target(s) of endogenous ubiquitin E3 ligase(s) in a nonviral context, raising the provocative possibility that protein degradation could exert a considerable measure of control over the small RNA-mediated degradation pathway.

Experimental Procedures

Plasmids, Plant Transformation, and Induction Conditions

BWYV P0 and CABYV P0 clones were described previously [6]. BWYV P0 construct was introduced into the XhoI-SpeI sites of pER8 [11] to produce pXVE-P0^{BW} and mobilized into *Agrobacterium tumefaciens* strain GV3101. Plant transformation and induction conditions are indicated in Supplemental Experimental Procedures.

RNA and Protein Analysis from *A. thaliana*

RNA and protein analysis are described in Supplemental Experimental Procedures.

Bimolecular Fluorescent Complementation

BiFC was performed as described [5]. The *AGO1* coding sequence was isolated as a 3147-nucleotide restriction fragment and cloned into pENTR3C (Invitrogen). The resulting vector was used to introduce the *AGO1* sequence into the split YFP destination vectors by Gateway Scientific (St. Louis) technology to obtain YC-AGO1. The other vectors were already described [5].

Supplemental Data

Four figures and Experimental Procedures are available at http://www.current-biology.com/cgi/content/full/17/18/1615/DC1/.

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