Brassinosteroids inhibit miRNA-mediated translational repression by decreasing AGO1 on the endoplasmic reticulum

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ABSTRACT

Translational repression is a conserved mechanism in microRNA (miRNA)-guided gene silencing. In Arabidopsis, ARGONAUTE1 (AGO1), the major miRNA effector, localizes in the cytoplasm for mRNA cleavage and at the endoplasmic reticulum (ER) for translational repression of target genes. However, the mechanism underlying miRNA-mediated translational repression is poorly understood. In particular, how the subcellular partitioning of AGO1 is regulated is largely unexplored. Here, we show that the plant hormone brassinosteroids (BRs) inhibit miRNA-mediated translational repression by negatively regulating the distribution of AGO1 at the ER in Arabidopsis thaliana. We show that the protein levels rather than the transcript levels of miRNA target genes were reduced in BR-deficient mutants but increased under BR treatments. The localization of AGO1 at the ER was significantly decreased under BR treatments while it was increased in the BR-deficient mutants. Moreover, ROTUNDIFOLIA3 (ROT3), an enzyme involved in BR biosynthesis, co-localizes with AGO1 at the ER and interacts with AGO1 in a GW motif-dependent manner. Complementation analysis showed that the AGO1–ROT3 interaction is necessary for the function of ROT3. Our findings provide new clues to understand how miRNA-mediated gene silencing is regulated by plant endogenous hormones.

Keywords: AGO1, Arabidopsis thaliana, brassinosteroids, endoplasmic reticulum, miRNA, translational repression

INTRODUCTION

MicroRNAs (miRNAs) influence biological processes by negatively regulating gene expression post-transcriptionally (Kim, 2009; Rogers and Chen, 2013). During miRNA-mediated gene silencing, miRNA molecules function as guides by base pairing with their target miRNAs. The ARGONAUTE proteins (AGOs) function as effectors to induce mRNA cleavage, mRNA deadenylation, and mRNA decay, as well as translational repression (Kim, 2009; Rogers and Chen, 2013). In plants, miRNA-mediated gene silencing was originally presumed to occur predominantly at the transcript level, but increasing evidence has suggested that miRNA-guided translational repression is widespread in plants (Aukerman and Sakai, 2003; Chen, 2004; Gandikota et al., 2007; Brodersen et al., 2008). ALTERED MERISTEM PROGRAM1 (AMP1), an integral membrane protein, promotes miRNA-mediated translational repression at the endoplasmic...
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reticulum (ER) by associating with ARGONAUTE1 (AGO1) (Li et al., 2013). A recent study also demonstrated that cytoplasmic HYPOSTATIC LEAVES1 (HYL1), a core component of the miRNA processing machinery, localizes to the ER and associates with AGO1 and AMP1 to promote translational repression (Yang et al., 2021). MiRNA-mediated translational repression may be cell type-dependent, as it was enhanced in Arabidopsis thaliana sperm cells (Grant-Downton et al., 2013). In addition, the efficiency of miRNA-mediated translational repression might be regulated by environmental (i.e., temperature) and developmental (i.e., age) factors in plants (Born et al., 2018). However, how AGO1 is regulated during miRNA-mediated translational repression remains unclear.

Although miRNAs and AGO1, the major miRNA effector in Arabidopsis (Baumberger and Baulcombe, 2005), are associated with polysomes during translational repression (Lanet et al., 2009), the subcellular location where miRNAs repress the translation of their target miRNAs remained elusive until 2015. Then, the ER was identified as the site of miRNA-mediated translational repression in plants (Li et al., 2016a) and mammals (Barman and Bhattacharyya, 2015; Axtell, 2017), and AGO1 was determined to localize at the ER based on transient expression of GFP-AGO1 in Nicotiana benthamiana leaves (Li et al., 2013; Michaeli et al., 2019). The findings that AGO1 was evenly distributed in the cytoplasm (Wang et al., 2011; Bologna et al., 2018; Trolet et al., 2019) but showed faint signals in the nucleus (Bologna et al., 2018; Liu et al., 2018; Bajczyk et al., 2019; Dalmadi et al., 2019) indicate that the subcellular partitioning of AGO1 might determine the mode of miRNA-guided gene silencing. However, the mechanism underlying the subcellular partitioning of AGO1 remains largely unknown.

The endogenous plant hormone brassinosteroids (BRs) play essential roles in plant growth, development, and responses to environmental cues. The pathway of BR biosynthesis involves several enzymes, including DE-ETIOLATED2 (DET2) (Li et al., 1996), CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM (CPD) (Szekerés et al., 1996), ROTUNDIFOLIA3 (ROT3) (Kim et al., 1998), and DWARF4 (DWF4) (Choe et al., 1998). The expression levels of those key genes are usually controlled by BRs via a negative feedback loop (Zhao and Li, 2012). Brassinosteroids are perceived by the plasma membrane receptor BRASSINOSTEROID INSENSITIVE1 (BRI1) (Li and Chory, 1997; He et al., 2000) and its co-receptors to activate a series of phosphorylation events that regulate the expression of downstream genes for various BR responses. Loss-of-function mutants, deficient in either BR biosynthesis or perception, display dwarf phenotypes of reduced cell elongation, crinkly leaves, short petioles, and reduced male fertility (Zhao and Li, 2012), which are partially reminiscent of the miRNA activity-deficient mutants (Voinnet, 2009; Rogers and Chen, 2013). These findings suggest that the BR pathway and the miRNA pathway might have active crosstalk in various biological processes.

In this study, we identified ROT3, an above-mentioned gene required for BR biogenesis, as a negative regulator of miRNA-mediated translational repression in a forward genetic screen. Further analysis showed that BRs significantly inhibit miRNA-mediated translational repression, since BR treatments altered the protein levels rather than the mRNA levels of miRNA target genes. The degree of localization of AGO1 at the ER was decreased under BR treatments but enhanced in the BR-deficient mutants. Moreover, the GW motif-dependent interaction of ROT3 with AGO1 conversely promotes the function of ROT3. Our findings demonstrate that BRs regulate the subcellular distribution of AGO1 at the ER to inhibit miRNA-mediated translational repression and that AGO1 facilitates the function of ROT3, thus revealing a regulatory feedback mechanism between the miRNA pathway and the BR pathway.

RESULTS

ROT3 inhibits miRNA-mediated translational repression

We previously conducted an ethyl methanesulfonate mutagenesis screen to isolate new genes involved in regulating miRNA activity using the dcl1-14 mutant, which has reduced miRNA levels (Zheng et al., 2011). We were interested in mutants with pleiotropic phenotypes and either compromised miRNA accumulation or de-regulated miRNA or protein levels of miRNA target genes. Using this strategy, we successfully identified several genes involved in miRNA biogenesis (Zheng et al., 2009; Li et al., 2016b; Su et al., 2017). In this study, we isolated a mutant showing a pleiotropic phenotype, particularly crinkly and round leaves with extremely short petioles, which was reminiscent of mutants defective in miRNA activity (i.e., the fry1-6 mutant) (Gy et al., 2007; Moro et al., 2018; You et al., 2019) (Figure 1A). Map-based cloning identified that the mutant contained a point mutation in At4g36380 (ROT3) (Figure 1B), which encodes a cytochrome P-450 that is required for BR biosynthesis by converting typhasterol into castasterone. This rot3 allele, which we refer to as rot3-5, harbors a C-to-T mutation in the last exon causing the conversion of the 508th arginine to a stop codon (Figure 1B). We crossed the rot3-5 mutant with the rot3-2 mutant, which is a null allele with a missense mutation (G80E) resulting in dysfunctional ROT3 (Kim et al., 1998), and observed that the F1 progenies displayed the rot3-2 mutant phenotype (Figure 1C). This confirmed that ROT3 is responsible for the phenotypes observed in the rot3-5 mutant.

To examine which step of the miRNA pathway was affected in the rot3-5 mutant, we first examined the miRNA levels by northern blots. The levels of mir156, mir159, mir164, mir165, mir167, mir168, mir172, and mir398 in the rot3-5 mutant were comparable to those of the wild-type (WT) Col-0 (Figure 2A). Consistent with those results, reverse-transcription quantitative PCR (RT-qPCR) analysis confirmed no altered levels of mature miRNAs in the mutant (Figure 2B). Next, we examined the transcript levels of eight genes targeted by seven of these miRNAs in Col-0 and the rot3-5 mutant by RT-qPCR. There were no significant differences between Col-0 and the mutant (Figure 2C). These results indicated that ROT3 is not required for miRNA biogenesis or miRNA-guided mRNA cleavage of target genes.
Next, we detected the protein levels of miRNA target genes to investigate whether ROT3 is required for miRNA-mediated translational repression. Since miR398 is strictly induced by Cu^{2+}-limited conditions to silence Cu/Zn SUPEROXIDE DISMUTASE2 (CSD2) at both the mRNA cleavage level and the protein translation level (Sunkar et al., 2006), CSD2 is thus a widely acceptable marker for evaluating miRNA-mediated translational repression in Arabidopsis (Brodersen et al., 2008; Li et al., 2013). Therefore, we introduced the Col-0 plants expressing CSD2-HA and CSD2-HA with mutated miR398 binding sites (mCSD2-HA) under the control of the native CSD2 promoter (CSD2p::CSD2-HA and CSD2p::mCSD2-HA) into the rot3-5 mutant by genetic crosses and examined the accumulation of the transgenic CSD2 proteins using the anti-HA antibody under Cu^{2+}-depleted conditions (i.e., miR398 present). Much lower CSD2-HA protein levels were detected in the rot3-5 mutant background compared to the Col-0 background (Figure 2D). By contrast, the mutation of miR398 binding sites rescued the accumulation of CSD2-HA in the rot3-5 mutant background to the same level as in the Col-0 background (Figure 2D). RT-qPCR analysis confirmed that neither CSD2 mRNA levels (Figure 2H) nor miR398 levels (Figure 2I) were altered when the transgenes were introduced into the rot3-5 mutant. Therefore, we concluded that the activity of miR398 to repress CSD2 at the translational level was enhanced in the rot3-5 mutant.

Figure 1. The phenotypes of rot3-5 and microRNAs (miRNA) deficient mutants
(A) Morphology of three-week-old Col-0, dcl1-14, fry1-6, amp1-30, ago1-27, rot3-5, det2-1, bni1-301, rot3-5 dcl1-14, rot3-5 fry1-6, rot3-5 amp1-30, and rot3-5 ago1-27 plants. (B) Diagram of the three rot3 alleles. Black rectangles represent exons, and deletion regions are highlighted in red. Lines indicate intronic regions. (C) Analysis of rot3-5 and rot3-2 alleles. Three-week-old F1 progeny are shown. Scale bars in both (A) and (C) are 1 cm.
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To further determine whether ROT3 generally functions in miRNA-mediated translational repression, we generated the rot3-5 mutants containing the following transgenes: TOE1p::TOE1-Myc (targeted by miR172), TOE1p::mTOE1-Myc (with mutated miR172 binding sites); CUC1p::CUC1-GFP (targeted by miR164), and CUC1p::mCUC1-GFP (with mutated miR164 binding sites), miR172-mediated repression of APETALA2 (AP2)-type transcription factors (such as AP2 and TOE1) is
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Based on the resemblance of leaf morphology between the BR-deficient mutants and the fyy1-6 mutant (Figure 1A), in which either miRNA degradation or miRNA processing was affected (Gy et al., 2007; Moro et al., 2018; You et al., 2019), we next investigated whether miRNA-mediated translational repression is generally inhibited by BRs. We therefore examined the protein levels of miRNA target genes in several other BR-deficient mutants, including det2-1 (Li et al., 1996) and bri1-301 (Li and Chory, 1997; He et al., 2000). Under the Cu^2+ -depleted condition (i.e., miR398 present), the levels of endogenous CSD2 were much lower in the mutants deficient in both BR biosynthesis (rot3-2 and det2-1) and BR signaling (bri1-301) (Figure 3A), whereas the miRNA levels of the previously mentioned miRNA target genes were not altered in all the mutants (Figure S1A). In contrast, under the Cu^2+ -enriched condition (i.e., miR398 absent), no obvious differences of the CSD2 protein levels were observed between Col-0 and the BR-deficient mutants (Figure 3A), except for a slight decrease of CSD2 in the det2-1 mutant compared to Col-0 (Figure 3A), indicating that the regulation of CSD2 protein levels by BRs is specifically mediated by miR398. Similarly, the protein levels of endogenous AP2 were also remarkably reduced in the mutants deficient in the BR pathway (Figure 3B), while the mRNA levels of miRNA-target genes remained unchanged (Figure S1A). To further assess whether the reduction of the protein levels of miRNA target genes in the BR-deficient mutants is miRNA-dependent, we compared the protein levels of TOE1-Myc and mTOE1-Myc in the Col-0 and det2-1 backgrounds. TOE1-Myc was obviously reduced in the det2-1 mutant background compared to the Col-0 background (Figure 3C). However, the level of mTOE1-Myc was similar between the det2-1 and Col-0 backgrounds (Figure 3C), indicating that the translational regulation of TOE1 by BRs is miR172-dependent. Of note, the levels of both TOE1 mRNA and miR172 were unchanged after the TOE1-Myc and mTOE1-Myc transgenes were introduced into the det2 mutant (Figure 2H, I).

To conclusively explore whether the deficiency in BR function was the cause of enhanced efficiency of miRNA-mediated translational repression, we then applied 2, 4-epi-brassinolide (epiBL), a bioactive BR, to two-week-old Col-0 plants to test whether the mRNA level or the protein level is regulated by exogenous BRs. RT-qPCR analysis showed that the mRNA levels of miRNA target genes were not altered by BR treatments (Figure S1B). CPD and DWF4, as the positive controls, were downregulated by BR treatments, which is consistent with previous studies (Szekeres et al., 1996; Choe et al., 1998; He et al., 2000), indicating that the BR treatments were effective. By contrast, the protein levels of AP2 (targeted by miR172) and CSD2 (targeted by miR398) were significantly induced by BR treatments (Figure 3D). Notably, AGO1, although it is targeted by miR168, was slightly decreased by BR treatments (Figure 3D), indicating that the regulation of AGO1 may be complicated. To further investigate if the repression role of BRs in the regulation of miRNA target genes is miRNA-dependent, we compared the accumulation of the fusion proteins with or without BR treatments in CSD2p::CSD2-HA, CSD2p::mCSD2-HA, TOE1p::TOE1-Myc, and TOE1p::mTOE1-Myc transgenic plants. The translation of only the wild-type CSD2-HA and TOE1-Myc proteins but not the mCSD2-HA and mTOE1-Myc proteins with mutated miRNA binding sites were induced by BR treatments (Figure 3E). Northern blot analysis further confirmed that their corresponding miRNA levels were unaffected in the BR-deficient mutants or by BR treatments (Figure S1C). We conclude that miRNA-mediated translational repression is generally inhibited by BRs.

BR11 is required for the BR-mediated inhibition of miRNA-mediated translational repression

To further investigate whether BR biosynthesis or BR signaling is necessary for miRNA-mediated translational repression, we applied epiBL to mutants deficient in either BR biosynthesis (rot3-2, rot3-5, and det2-1) or BR signaling (bri1-301 and bri1-5), and then investigated which mutants were not responsive to the BR-mediated induction of the protein levels of miRNA targets. As shown in Figure 4A and 4B, the levels of the endogenous AP2 and CSD2 proteins were decreased in all five mutants under the control growth condition, which is consistent with our above results (Figures 2, 3). However, in the rot3 and det2 mutants the levels of both AP2 and CSD2 were restored to those in Col-0 after BR treatments (Figure 4A, B), indicating that BR biosynthesis-deficient mutants (rot3 and det2) are hypersensitive to the BR-mediated inhibition of miRNA-target genes. By contrast, the levels of AP2 and CSD2 remained unchanged under BR treatments in the BR receptor-defective mutants (two bri1 alleles) (Figure 4A, B). Statistical analysis further confirmed that the rot3 and det2 mutants were hypersensitive but the bri1 mutants were insensitive to BR-mediated enhancement of miRNA-mediated translational repression (Figure 4A, B), suggesting that BR11 is necessary for the BR-mediated inhibition of miRNA-mediated translational repression.

To further assess whether the restoration of the protein levels of miRNA target genes in the BR biosynthesis-deficient mutants by exogenous BR treatments is miRNA-dependent,
we compared the protein levels of CSD2-HA and mCSD2-HA in the Col-0 and rot3-5 backgrounds with or without BR treatments. Similar to endogenous CSD2 (Figure 4A, B), the protein level of CSD2-HA in the rot3-5 background was significantly increased compared to that in Col-0 by BR treatments (Figure 4C). By contrast, no further enhancement of the level of mCSD2-HA was detected in the rot3-5 background under BR treatments (Figure 4D), indicating that the restoration of CSD2 by BR treatments in BR biosynthesis-deficient mutants is miR398-dependent. Based on all of these results, we conclude that BR signaling is necessary for the inhibition of miRNA-mediated translational repression.

As AMP1 was reported to promote miRNA-mediated translational repression via interaction with AGO1 (Li et al., 2013), we investigated whether BR-mediated inhibition of miRNA-mediated translational repression is related to the function of AMP1. The morphology of the rot3-5 amp1-30 double mutant was similar to that of the rot3-5 single mutant (Figure 1A), and the protein levels of CSD2 were also comparable between the rot3-5 and rot3-5 amp1-30 mutants
By contrast, the double mutant ago1-27 rot3-5 was morphologically similar to the ago1-27 mutant (Figure 1A), suggesting that miRNA-mediated translational repression is epistatic to the BR function. The genetic interaction between ROT3 and AMP1 thus indicates that the regulatory role of BRs on miRNA-mediated translational repression might be independent of AMP1.

Brassinosteroid treatments decreased localization of AGO1 at the ER

Since miRNA-mediated translational repression occurs at the ER, and AGO1 shuttles among the cytosol, nucleus, and ER, we hypothesized that BRs might interfere with the localization of AGO1 at the ER, thereby negatively regulating miRNA-mediated translational repression. To test this, we first validated the localization of AGO1 at the ER using the stable transgenic plants expressing AGO1p::GFP-AGO1 (Figure 5A, BR−). Importantly, the
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GFP-AGO1 signal co-localized with that of ER-mCherry, with a correlation coefficient of 0.42 ± 0.04 (Figure 5B). These results demonstrate that AGO1 localizes at the ER in Arabidopsis, consistent with previous findings (Li et al., 2013; Michaeli et al., 2019).

However, compared to the even distribution of AGO1 in the root cells without exogenous BR (Figure 5A, BR−), GFP-AGO1 formed condensates at the ER under BR treatments (Figure 5A, BR+), and the correlation coefficient was significantly decreased to 0.29 ± 0.09 (Figure 5B). Of note, the total fluorescence intensity of GFP-AGO1 relative to ER-mCherry was not changed by BR treatments (Figure 5C). BKI1-YFP, a positive control for the BR treatments (Wang and Chory, 2006), was significantly dissociated from the plasma membrane under BR treatments (Figure S2B), showing that BR treatments were effective. Collectively, these results indicate that BR treatments decreased the degree of localization of AGO1 at the ER.

BR-deficient mutants exhibited increased localization of AGO1 at the ER

Since BR treatments resulted in decreased localization of AGO1 at the ER, we then investigated how the localization of AGO1 at the ER was regulated in the BR-deficient mutants. Western blot analysis showed that the total levels of AGO1 were significantly increased in the rot3-5, rot3-2, and bri1-5 mutants (the left four lanes in Figure 6A), indicating that the dysfunction of the BR pathway has obvious effects on the

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**Figure 5.** Brassinosteroid (BR) treatments caused reduced localization of ARGONAUTE1 (AGO1) at the endoplasmic reticulum (ER)

(A) Localization of GFP-AGO1 and ER-mCherry in the root cells with or without BR treatments. Four- to five-day-old seedlings of the transgenic plants expressing AGO1p::GFP-AGO1 and 35S::ER-mCherry were treated with (BR+) or without (BR−) 1 μM epiBL for 30 min, and the fluorescence of the root tip cells was observed by microscopy. Scale bars, 10 μm. (B) Pearson’s correlation coefficient analysis showing the co-localization of GFP-AGO1 and ER-mCherry in the root cells with or without BR treatment. Small triangles indicate the value for one image, and the mean value was calculated from 12 individual images for each case. *** represents *P* value < 0.001 between groups evaluated with Student’s t-test. (C) Mean value for gray intensity of total GFP-AGO1 relative to ER-mCherry in the root cells with or without BR treatments. Approximately 100 cells from 10 plants were analyzed.
accumulation of AGO1. A microsome fractionation assay further showed that the association of AGO1 with the microsomes was significantly higher in the BR-deficient mutants than in Col-0 (the right four lanes in Figure 6A). As the cytosolic materials and the free polysomes are usually enriched in the soluble fraction while all the endomembrane compartments mainly containing the ER debris and the endomembrane-bound polysomes are mainly collected in the pellet fraction (microsomes), the increased levels of AGO1 in the microsomes of the BR-deficient mutants indicated that the association of AGO1 with the ER might be stimulated when the BR pathway is shut down.

Figure 6. The localization of ARGONAUTE1 (AGO1) at the endoplasmic reticulum (ER) is increased in the brassinosteroid (BR)-deficient mutants. (A) Levels of AGO1 in subcellular compartments assessed via microsome fractionation assay in two-week-old Col-0, rot3-5, rot3-2, and bri1-5 seedlings. Phosphoenolpyruvate carboxylase (PEPC) and Hsc70 are a cytosolic and an ER lumenal protein, respectively. (B) Subcellular localization of GFP-AGO1 in Col-0, rot3-2, and bri1-5 root cells. Root cells of four-day-old seedlings were stained with ER-Tracker and the fluorescence for both GFP-AGO1 and ER-Tracker was observed using confocal microscopy. Scale bars, 10 μm. (C) Pearson’s correlation coefficient analysis showing the co-localization of GFP-AGO1 and ER-Tracker in the root cells of Col-0, rot3-2, and bri1-5. Small triangles indicate the value for one image, and the mean value was calculated from 14 individual images for each case. (D) Mean value for gray intensity of total GFP-AGO1 relative to ER-Tracker in the root cells of Col-0, rot3-2, and bri1-5. Approximately 100 cells from 10 individual plants for each genotype were analyzed. ** and *** represent P-value < 0.01 and 0.001 between groups evaluated with Student’s t-test, respectively.
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Unexpectedly, BR treatments had no detectable effect on the levels of AGO1 in the microsomes (Figure S2C), which might be due to the minor effect of exogenous BRs and/or the limited sensitivity of the microsome fractionation assay.

To further visualize the localization of AGO1 at the ER in the BR-deficient mutants, we constructed the transgenic plants expressing AGO1p::GFP-AGO1 in the rot3-5, rot3-2, and bri1-5 mutant backgrounds by genetic crosses. We then assessed the localization of AGO1 at the ER in the root cells by analyzing the fluorescent signals of GFP-AGO1 and the ER-Tracker blue-white DPX (Michaeli et al., 2019), a cell-permeant dye that is highly selective for the ER in living cells. Staining followed by fluorescence microscopy showed that GFP-AGO1 co-localized with ER-Tracker (Figure 6B), and their correlation coefficient was also somewhat high (0.25 ± 0.04 in Col-0, Figure 6C), further indicating that AGO1 localizes at the ER. Importantly, the correlation coefficients of GFP-AGO1 and ER-Tracker were significantly increased in the BR-deficient mutants (0.32 ± 0.05 in rot3-2 and 0.35 ± 0.04 in bri1-5; Figure 6C), suggesting that the decrease of BR function indeed causes increased localization of AGO1 at the ER, resulting in enhanced efficiency of miRNA-mediated translational repression. Of note, the correlation coefficient (~0.25) of AGO1 relative to ER-Tracker (Figure 6C) was lower than that of AGO1 relative to ER-mCherry (~0.42) under the BR – condition (Figure 5B), which might be due to the difference of indicative sensitivity between ER-mCherry and ER-Tracker. In addition to our finding that the degree of localization of AGO1 at the ER was increased in the BR-deficient mutants, the fluorescence intensities of GFP-AGO1 at the ER were also increased in the rot3-2 and bri1-5 mutants (Figure 6B, D), which is consistent with the finding of increased AGO1 in the microsomes of the BR-deficient mutants (Figure 6A). Taken together, we conclude that BRs negatively regulate miRNA-mediated translational repression via interfering with the subcellular localization of AGO1 at the ER.

AGO1 interacts with ROT3 to maintain the function of ROT3

To understand whether miRNA-mediated translational repression is involved in the BR pathway, we hypothesized that AGO1 might interact with the key components of the BR pathway, especially those factors that localize at the ER. Most of the enzymes involved in BR biogenesis are associated with the ER in higher plants (Hartmann and Benveniste, 1987; Asami and Yoshida, 1999), including DWF4 (Kim et al., 2006). We observed that ROT3 co-localized with AGO1 at the ER in N. benthamiana (Figure S3A). Next, by performing reciprocal co-immunoprecipitation (Co-IP) using transgenic Arabidopsis seedlings expressing ROT3p::ROT3-Flag, we further showed that ROT3 interacts with AGO1 (Figure 7A). Notably, the anti-AGO1 antibody specifically recognized AGO1 of Arabidopsis, as no AGO1 was detected in the null allele mutant ago1-36 (Figure S3B). Because ROT3 harbors two GW motifs (Figure 7B), which mediates the interaction with AGOs (El-Shami et al., 2007; Karlowski et al., 2010), we generated variants (Figure 7B) in which the GW motifs were mutated to AA (ROT3m1 with the first GW motif mutated, ROT3m2 with the second GW motif mutated, and ROT3GW with both GW motifs mutated) and tested their capacity to interact with AGO1. Both the biomolecular luciferase complementation (BiLC) assay using transiently expressing N. benthamiana leaves (Figure S3C) and Co-IP experiments using the stable transgenic Arabidopsis plants (Figure 7C) showed that the ROT3-AGO1 interaction was GW motif-dependent, since the GW mutations blocked its interaction with AGO1, while protein levels of ROT3 and ROT3GW were comparable after they were introduced into the plants (Figures 7C, S3D). Moreover, microsome fractionation assay followed by western blotting analysis confirmed that AGO1 and ROT3 co-accumulated in the microsome fraction (Figure S3E). To investigate the biological significance of the ROT3-AGO1 interaction, we transformed a ROT3p::ROT3GW-YFP construct into rot3 mutants and examined whether ROT3GW complemented the mutants. Unexpectedly, while the wild-type ROT3 fully rescued both rot3 mutants (rot3-2 and rot3-5) (Figure 7D, E), ROT3GW-YFP failed to complement the rot3 mutants (Figure 7D, E). Western blot analysis showed that the levels of the transgenic ROT3 and ROT3GW proteins were comparable in their corresponding T1 lines (Figure 7F), excluding the possibility that the failure of the genetic complementation was due to compromised expression of the ROT3GW-YFP transgene. Therefore, AGO1 itself, or in combination with miRNA-mediated translational repression might play a role in BR biosynthesis in a feedback loop manner. Overall, we conclude that BRs negatively regulate miRNA-mediated translational repression, most likely via interfering with the ER–cytosol partitioning of AGO1.

DISCUSSION

MiRNAs play essential roles in biological processes by regulating the expression of their target genes. Their two major modes of action in plants are endonucleolytic cleavage in the cytoplasm and translational repression at the ER (Trolet et al., 2019). Based on the findings that AGO1, the major effector of miRNA action in Arabidopsis (Baumberger and Baulcombe, 2005), localizes not only in the cytoplasm (Bajczyk et al., 2019; Dalmadi et al., 2019; Michaeli et al., 2019; Trolet et al., 2019) but also at the ER (Brodersen et al., 2008, 2012; Li et al., 2013) and the nucleus (Wang et al., 2011; Liu et al., 2018), we reasoned that the subcellular partitioning of AGO1 might be critical to miRNA-directed gene silencing. In this study, we demonstrated that BRs negatively regulate miRNA-mediated translational repression via interfering with the subcellular distribution of AGO1 at the ER (Figure 8). Since miRNAs and BRs are involved in many aspects of plant development, our findings provide insight into how these two important biological processes integrate at the level of cellular compartments.
MiRNAs as well as other small RNAs are associated with the ER (Li et al., 2016a), prompting the question of how AGO1 itself and/or the AGO/small RNA cargo is loaded onto the ER. Our findings of the involvement of BRs in miRNA-mediated translational repression and AGO1 localization provide an important clue to answer this question. We surmise that BRs could mediate inhibition of miRNA-mediated translational repression via several possible scenarios: (i) BR biosynthesis and miRNA-mediated translational repression might compete for the ER at the subcellular compartment level, thus leading to the inhibition of miRNA-mediated translational repression by BRs. This possible scenario is consistent with the ER localization of BR biosynthetic proteins (Choe et al., 1998). (ii) BRs inhibit miRNA-mediated translational repression possibly by regulating endosomal signaling. This hypothesis is supported by the involvement of BR1 in endosomal signaling (Geldner et al., 2007) and the highly dynamic association/trafficking of AGO1 with the endomembranes (Brodersen et al., 2005).

Figure 7. ARGONAUTE1 (AGO1) interacts with ROTUNDIFOLIA3 (ROT3) and promotes the function of ROT3
(A) Reciprocal Co-IP between ROT3 and AGO1. ROT3p::ROT3-Flag plants were used for either AGO1-IP or Flag-IP. No Ab (IgG) was the negative control. (B) Schematic showing ROT3 harboring two GW motifs. (C) Co-IP showing the interaction between AGO1 and ROT3 or ROT3△GW. The rot3-5 mutant expressing ROT3p::ROT3-YFP or ROT3p::ROT3△GW-YFP was used for AGO1-IP. No Ab (IgG) was the negative control. (D) Representative images showing the complementation of rot3-5 and rot3-2 mutants by ROT3 and ROT3△GW. (E) Statistical analysis showing the complementation of rot3-5 and rot3-2 mutants by ROT3 and ROT3△GW. For each transgene, more than 30 independent T1 lines for each transgene were analyzed. (F) Western blots showing levels of ROT3-YFP and ROT3△GW-YFP in the rot3-5 mutant. Total proteins extracted from inflorescences of six randomly selected T1 plants were analyzed. Col-0 was used as the negative control, and Hsc70 was used as the loading control. Numbers below blots indicate relative protein levels as calculated from three biological replicates, and standard deviations are shown. *** represents P-value < 0.001 between groups evaluated with Student's t-test; others without note indicate that the difference was not significant.
et al., 2012; Li et al., 2013; Bologna et al., 2018; Trolet et al., 2019). (iii) Based on the active crosstalk of BR pathway with intracellular signals and environmental stimuli, BRs might regulate the expression of some unknown genes whose functions may be necessary for miRNA-mediated translational repression. Moreover, AGO1 may play a positive role in BR biosynthesis according to its interaction with ROT3. However, whether AGO1 regulates BR biosynthesis and whether its regulation by AGO1 requires miRNA-mediated translation repression need further investigation. Overall, we conclude that BRs negatively regulate miRNA-mediated translational repression, most likely via interfering with the ER-cytosol partitioning of AGO1.

In addition, it remains unexplored whether miRNAs and AGO1 are loaded onto the ER together or separately. AMP1, an integral ER membrane protein, promotes miRNA-mediated translational repression by facilitating the loading of mRNAs of miRNA-target genes onto the endomembrane-bound polysomes instead of the ER (Li et al., 2013), indicating that AMP1 might act at the very late stage of the miRNA-mediated translational repression pathway, such as the recruitment of the AGO1/miRNA/target gene mRNA cargo to the ER. During this process, cytoplasmic HYL1 was observed to be associated with AMP1 at the ER to promote miRNA-mediated translational repression, possibly by facilitating the loading of AGO1 on polysomes (Yang et al., 2021). By contrast, our observation of the change of AGO1 localization at the ER by BRs (Figures 5, 6) suggests that the regulatory role of BRs on miRNA-mediated translational repression might occur at an earlier step, particularly the subcellular compartmentalization of AGO1. Finally, it was unexpected that no antagonistic effects on miRNA-mediated endonucleolytic cleavage were observed in the BR mutants and under BR treatments, indicating that the balance between the two miRNA modes of action is more complicated than previously thought.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana Columbia (Col-0) is the wild type. Seeds of rot3-2 (cs3728), CUC1p::CUC1-GFP (cs65829), and CUC1p::mCUC1-GFP (cs65830) were obtained from the Arabidopsis Biological Resources Center. Seeds of CSD2p::CSD2-HA and CSD2p::mCSD2-HA (Li et al., 2013) TOE1p::TOE1-Myc and TOE1p::mTOE1-Myc (Zhang et al., 2015), det2-1 (Chory et al., 1991), bri1-5 and bri1-301 (Clouse et al., 1996), ago1-36 (Baumberger and Baulcombe, 2005), ago1-27 (Morel et al., 2002), dcl1-14 (Salk_056243) (Zheng et al., 2011), fry1-6 (Salk_020882) (Gy et al., 2007), amp1-30 (Li et al., 2013), 35S::BKI1-YFP (Wang and Chory, 2006), and...
AG01p::GFP-AGO1 (Wang et al., 2011) were reported previously. Seeds of 35S::ER-mCherry were generated in our laboratory. Seeds were sterilized and plated on Murashige and Skoog medium, kept at 4°C for 3 d before being moved to the growth chamber. For the detection of CSD2 by western blots, the ATS medium was used to allow the addition or omission of 10 µM CuSO4 (Li et al., 2013). Plants were grown in a plant growth chamber at 23°C under 16 h light/8 h dark cycles.

Plasmid construction
35S::YFP-AGO1 and 35S::ER-mcherry plasmids were reported previously (Nelson et al., 2007; Li et al., 2013). To construct the ROT3p::ROT3-Flag plasmid, ROT3 was amplified from Col-0 genomic DNA with primers ROT3F1/R1, cloned into the plant expression vector pCambia1306. To clone the ROT3-pEN, the CDS of ROT3 was amplified from Col-0 cDNA with primers ROT3F2/R2, and cloned into the pENTRY1A vector. Then the ROT3-pEN plasmid was transferred into the modified pB7YW2. For the plasmid of ROT3△GW-pEN, point mutagenesis of the ROT3-pEN plasmid was done using primers ROT3F3/R3 and ROT3F4/R4. To construct the ROT3p::ROT3-YFP and the ROT3p::ROT3△GW-YFP plasmids, the 35S promoter of pB7YW2 was replaced by the ROT3 promoter amplified using primers ROT3F5/R5. Then the ROT3△GW-pEN plasmid was transferred into the modified pB7YW2. For ROT3-nLUC series plasmids, the CDS of ROT3, ROT3m1, ROT3m2 and ROT3△GW was amplified from the corresponding pEN plasmids with primers ROT3F6/R6, and cloned into the plant expression vector 35S::nLUC. Primer sequences are listed in Table S1.

Antibodies
Anti-Hsc70 (SPA-018; Stressgen), anti-GFP (#MMS-118R; Covance), anti-Myc (#sc-70463; Santa Cruz), anti-Flag (F7425; Sigma), anti-HA (#H6908; Sigma), anti-PEPC (As09458; Agrisera), anti-AP2 (As122609; Agrisera), and anti-CSD2 (As06170; Agrisera) antibodies were purchased. The anti-AGO1 antibody (the peptide MVRKRTDAP5 as the antigen) was generated according to Qi et al. (2005) by our lab.

miRNA northern blot
Total RNA was extracted using Trizol reagent (Invitrogen) from 2-week-old seedlings or inflorescences. Ten micrograms of total RNAs were separated by denaturing 15% (w/v) PAGE gel and transferred to a nylon membrane; 5′ radioactivity-labeled oligo nucleotide sequences complementary to miRNAs were synthesized as probes. Hybridization was performed using hybridization buffer (Ambion), and signals were detected using the laser scanner Typhoon FLA 9500 (GE Healthcare). rRNA was used as a loading control. The probes are listed in Table S1.

Co-immunoprecipitation
Transgenic plants were ground in liquid nitrogen and homogenized in lysis buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.2% NP-40, 2 mM DTT, 5% glycerol, and proteinase inhibitor) and centrifuged for 15 min at 13,200 rpm. The lysate was incubated with Flag-M2 beads (Sigma), GFP-Trap beads (ChromoTek) or the anti-AGO1-coupled protein A beads at 4°C for 2 h. The immune complexes were then washed with lysis buffer. Proteins retained on the beads were resolved on SDS-PAGE. Anti-AGO1, anti-Flag or anti-GFP antibodies were used to do western blot analysis.

Chemical treatments
The hormone Epibrassinolide (epiBL, Cat. No. 1641; Sigma-Aldrich) was dissolved in dimethylsulfoxide (DMSO) to yield a 1 mM stock solution. For BR treatments in roots of Arabidopsis plants, 1 µM epiBL were added to 1/2x MS liquid medium and incubate roots on a microslide. Fluorescent images were taken before and after BR treatments of the same sample.

A 1-µM ER-Tracker blue-white DPX (E12353; Thermo Fisher) was applied on roots of Arabidopsis for 5 min followed by a quick wash in distilled deionized water. Then, examine the stained roots by confocal microscopy (FV3000; Olympus) using 405 nm laser for excitation and the signal was collected between 570 and 640 nm.

Microsome fractionation assay
Microsome fractionation preparation was performed as previously described (Brodersen et al., 2012). Five hundred microliters of seedlings ground to fine powder was lysed in 1 mL of microsome buffer (50 mM MOPS, pH 7.6, 0.5 M Sorbitol, 10 mM EDTA, 0.5% polyvinyl pyrrolidone, protease inhibitor mixture (Roche), 1 µM MG132), filtered through Miracloth, and centrifuged at 8,000 g for 10 min at 4°C. Supernatants were then centrifuged at 100,000 g for 30 min in a Beckman TLS-100 rotor and separated into supernatants containing soluble material and pellets containing insoluble material. Pellets were washed in 500 µL of microsome buffer, and collected after an additional spin at 100,000 g for 30 min. For comparison of AGO1, phosphoenolpyruvate carboxylase (PEPC), and Hsc70 protein content in total, supernatant and pellet fractions, pellets were resuspended in PBS, and 20% of the resulting volume loaded on gel (compared with 3% of total extracts and supernatants).

Western blot
For extraction of total proteins, seedlings were ground to fine powder in liquid nitrogen, and homogenized in two volumes of lysis buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.2% Nonidet P-40, 2 mM DTT, 10% glycerol, protease inhibitor) and centrifuged for 15 min at 13,200 rpm. The supernatant was mixed with 5x SDS loading buffer and boiled at 95°C for 5 min. After centrifugation at 12,000 rpm for 10 min, the supernatant containing total proteins was then separated on SDS-PAGE gels, and then followed by regular western blots using corresponding antibodies. The band intensity was measured with Tanon-5200 Gel Image System (Version 4.2.5) software.
BRs inhibit miRNA-mediated translational repression

**Gene expression analysis by quantitative RT-PCR**
Total RNAs were extracted using Trizol reagent (Invitrogen) from seedlings, and 2 μg of Dra I-treated total RNA was used for reverse transcription (PrimeScript™ II reverse transcriptase; TaKaRa). cDNAs were combined with SYBR master mix (Bio-Rad). Quantitative RT-PCR (qRT-PCR) was performed in triplicate with a Bio-Rad C1000 Thermocycler. The data were collected and analyzed with Bio-Rad real-time PCR detection systems and software. Primers are listed in Table S1.

**miRNA expression analysis by qRT-PCR**
Quantitative RT-PCR to detect miRNAs in *Arabidopsis* was done as described (Varkonyi-Gasic et al., 2007). Briefly, the stem-loop RT primers designed according to (Chen et al., 2005; Varkonyi-Gasic et al., 2007), and 200 ng total RNAs extracted from seedlings were used to do a pulsed RT reaction. Next, a forward primer specific to a miRNA and a reverse primer specific to the stem-loop RT primers were used to amplify the RT products with iTaq Universal SYBR Green Supermix (172-5124; Bio-Rad). Primers used for miRNA qRT-PCR were listed in Table S1.

**Confocal microscopic and imaging data analysis**
The subcellular localization of AGO1p::GFP-MYO1, 35S::ER-mCherry and 35S::BK11-YFP in plants were observed with a FV3000 (Olympus) confocal microscope. The excitation/emission wavelengths were 488 nm/500–560 nm for GFP/YFP, and 561 nm/590–660 nm for mCherry. For Figures 5B and 6C, the correlation coefficient was analyzed by cellSens Imaging Software (Olympus) according to (Hao et al., 2016). For Figures 5C and 6D, mean value of gray intensities were analyzed using cellSens Imaging Software (Olympus).

**Biomolecular luciferase complementation assay**
The BiLC assay was performed as described (Chen et al., 2008). Plasmids were introduced into *Agrobacterium* strain GV3101, and the OD values were measured using an ultraviolet-visible spectrophotometer, then co-infiltrated with indicated combinations into young tobacco leaves. Plants were grown in the dark for 1 d and then transferred to long-day conditions (16 h light/8 h dark) for 2 d. Luciferin (1 mM) was sprayed evenly onto infiltrated leaves and LUC activity was monitored with LB985 NightShade with indiGo software (Berthold Tech).

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**AUTHOR CONTRIBUTIONS**
T.W. and B.Z. designed the research. T.W. performed most of the experiments. Y.Z. isolated the rot3-5 mutant. Q.T. and S.Z. helped with the plasmid constructions. W.S. supervised manipulations of the BR treatments. T.W. and B.Z. wrote the manuscript. All authors read and approved of this manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article: http://onlinelibrary.wiley.com/doi/10.1111/jipb.13139/suppinfo

Figure S1. Effect of brassinosteroids (BRs) on microRNAs (miRNA) biogenesis and target gene expression

(A) Transcript levels of miRNA-target genes in Col-0 and the BR-deficient mutants assessed by reverse-transcription quantitative PCR (RT-qPCR). RNAs from 2-week-old seedlings were analyzed. UBQ5 was the internal control. (B) Transcript levels of miRNA target genes with or without BR treatment assessed by RT-qPCR. Two-week-old seedlings were treated with (BR+) or non-treated (BR–) 1 μM 2, 4-epibrassinolide (epiBL) for 2 h. UBQ5 was the internal control. Standard deviations are shown from three biological replicates. (C) Northern blot analysis showing miRNA levels in the indicated plants and Col-0 with or without BR treatment. Total RNAs extracted from 2-week-old seedlings were analyzed, except that miR172 was detected from inflorescences. RNA was used as the loading control. Numbers below blots indicate relative protein levels as calculated from three biological replicates, and standard deviations are shown. *, **, and *** represent P-value <0.05, 0.001, and 0.0001 between groups evaluated with Student’s t-test, respectively.

Figure S2. Genetic interaction between ROTUNDIFOLIA3 (ROT3) and ALTERED MERISTEM PROGRAM1 (AMP1), and the effect of brassinosteroid (BR) treatment on BKI1 and ARGONAUTE1 (AGO1)

(A) Levels of endogenous CSD2 protein in Col-0, amp1-30, rot3-5, and rot3-5 amp1-30 plants detected by western blots. Total proteins from 2-week-old seedlings were analyzed. Hsc70 was used as the loading control. ** represents P-value <0.01 between groups evaluated with Student’s t-test. (B) Localization of BKI1-YFP driven by the 3SS promoter in Arabidopsis root cells with or without BR treatment. Scale bar, 10 μm. (C) Distribution of AGO1 in the subcellular compartments assessed by microscopic fractionation assay with or without BR. Two-week-old seedlings of Col-0 were treated with (BR+) or without (BR–) 1 μM epiBL for 2 h, and a fractionation assay followed by western blotting was performed using total extracts, soluble fractions, or pellets. PEPC and Hsc70 are a cytosolic and an endoplasmic reticulum (ER) luminal protein, respectively.

Figure S3. Relationship between ROTUNDIFOLIA3 (ROT3) and ARGONAUTE1 (AGO1)

(A) The combinations of 3SS::ROT3::YFP with 3SS::ER-mCherry, and 3SS::YFP:AGO1 with 3SS::ER-mCherry were transiently co-expressed in N. benthamiana leaves. Scale bar, 10 μm. (B) Western blot analysis showing the specificity of anti-AGO1. Total proteins were extracted from Col-0, ago1-1 (a null allele of ago1), and ago1-27 (a weak allele of ago1). Hsc70 was used as the loading control. (C) Biomolecular luciferase complementation (BiLC) assays showing the interaction between AGO1 and ROT3. Interaction of paired proteins results in purple chemiluminescence (purple in images). More than five leaves were examined for each pair, and a graph is shown. (D) Western blot analysis showing the expression of AGO1 and ROT3 variants used in (C). Hsc70 was the internal control. (E) ROT3 and AGO1 partially co-exist in a crude membrane fraction. PEPC and Hsc70 are a cytosolic and an endoplasmic reticulum (ER) luminal protein, respectively.

Table S1. Primers used in this study