ARGONAUTE1 and ARGONAUTE4 Regulate Gene Expression and Hypoxia Tolerance[1][OPEN]

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In plants, hypoxia can be induced by submergence, and the lack of oxygen impairs mitochondrial respiration, thus affecting the plant's energy status. Hypoxia has major effects on gene expression; these changes induce key responses that help meet the needs of the stressed plant. However, little is known about the possible role of RNA signaling in the regulation of gene expression under limited oxygen availability. Here, we report the contribution of ARGONAUTE1 (AGO1) to hypoxia-induced gene regulation in Arabidopsis (*Arabidopsis thaliana*). Submergence induced changes in levels of the microRNAs miR2936 and miR398, but this had no obvious effects on their putative target mRNA levels. However, we found that *ago1-27* plants are intolerant to submergence and transcriptome analysis identified genes whose regulation requires functional AGO1. Analysis of mutants affected in various branches of RNA signaling highlighted the convergence of AGO1 signaling with the AGO4-dependent RNA-directed DNA methylation (RdDM) pathway. AGO4-dependent RdDM represses the expression of *HOMOLOG OF RPW8 4* (*HR4*) and alters its response to submergence. Remarkably, methylation of the second exon of *HR4* is not only reduced in *ago4-1* but also in plants overexpressing a constitutively stable version of the oxygen sensor RELATED TO APETALA2 12 (RAP2.12), indicating convergence of oxygen signaling with epigenetic regulation of gene expression. Therefore, our results identify a role for AGO1 and AGO4 RNA-silencing pathways in low-oxygen signaling in Arabidopsis.

The response of plants to hypoxia, often caused by flooding and submergence, includes a dramatic reorganization of gene expression to match the changed energetic status of the plant, suffering from the lack of oxygen as the final electron acceptor in mitochondrial respiration (Bailey-Serres and Voesenek, 2008; Loreti et al., 2016). Hypoxia sensing occurs through the oxygen-dependent destabilization of proteins belonging to group VII Ethylene Responsive Factors (ERF-VII; Gibbs et al., 2011; Licausi et al., 2011a), by the action of Plant Cys Oxidases (Weits et al., 2014; White et al., 2017) that oxidize the Cys residue at the N terminus of the ERF-VII proteins, targeting them for proteasomal degradation (Gibbs et al., 2015; Giuntoli and Perata, 2018). Under hypoxia, stable ERF-VII proteins bind to an evolutionarily conserved cis-motif to regulate hypoxia-responsive gene expression (Gasch et al., 2016). In Arabidopsis (*Arabidopsis thaliana*), hypoxia elevates the mRNA steady-state levels of several hundreds of transcripts, including a group of 49 anaerobic core genes that are ubiquitously expressed in all organs and cell types in response to low oxygen (Mustroph et al., 2009). The genes belonging to the anaerobic core are also associated with ribosomes (Mustroph et al., 2009), whereas selective repression of translation occurs for half of the cellular mRNAs under hypoxia, a phenomenon that is rapidly reversed upon reoxygenation (Branco-Price et al., 2008). Besides transcriptional regulation, the mRNA level or translation of mRNAs into proteins can be regulated at the posttranscriptional level, including posttranscriptional gene silencing (PTGS) and other RNA silencing pathways. In PTGS, small RNAs such as microRNAs (miRNAs) are the major regulators of mRNA degradation or inhibition of translation. miRNAs play important roles in defining

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plant development, plant nutrition, and responses to biotic and abiotic stresses (Koroban et al., 2016). The effects of drought, high temperature, cold, and heavy metals on the expression of miRNAs have been described (Shriram et al., 2016), and *miR398* was identified as a miRNA repressed by several abiotic stresses (Zhu et al., 2011).

Interestingly, miRNAs have been shown to play a role in the response to hypoxia in animals (Crosby et al., 2009), and available evidence suggests that hypoxia modulates the expression of various miRNAs in Arabidopsis and corn roots (Moldovan et al., 2010 Licausi et al., 2011b; Zhai et al., 2013). However, the level of the relative mature miRNA sequences barely changes under oxygen deprivation, suggesting that miRNAs do not play a major role in gene regulation under hypoxia. This conclusion was also supported by the lack of differences in the expression of putative target transcripts of the modulated miRNAs (Licausi et al., 2011b). It was also proposed that, rather than direct sensing of hypoxic conditions, the modulation of miRNA expression results from inhibition of mitochondrial respiration, since treatment with antimycin A, an inhibitor of complex III of the cytochrome pathway, up-regulated the same miRNAs that were found to be responsive to hypoxia (Moldovan et al., 2010).

miRNA-mediated PTGS requires the ARGONAUTE1 (AGO1) protein, a component of the RNA-induced silencing complex that binds miRNAs. RNA-induced silencing complex uses the mature miRNA as a guide for cleavage of homologous mRNAs to repress gene expression. Besides being required for miRNA-mediated PTGS, AGO1 is also required for other RNA silencing pathways. AGO1 associates with 21-22 nucleotide small interfering RNAs (siRNAs) to trigger the biogenesis of RNA-DEPENDENT RNA POLYMERASE 6 (RDR6)-dependent secondary siRNA that may also contribute to the establishment of RNA-directed DNA methylation (RdDM; Chan et al., 2005). Furthermore, it was recently demonstrated that AGO1 binds to the chromatin of transcribed genes, promoting their transcription (Liu et al., 2018). Arabidopsis *ago1* mutants display severe developmental abnormalities and are sterile. However, hypomorphic *ago1* mutants that are completely defective for PTGS, including fertile ones, were isolated (*ago1-25*; *ago1-27*; Morel et al., 2002). The use of mutants defective in RNA signaling is potentially useful for determining the relevance of this pathway for gene regulation under specific environmental conditions. Here, we analyzed the response to hypoxia in *ago1-27* to investigate the role of miRNAs and other RNA silencing pathways in the response of Arabidopsis plants to hypoxia. Together, our results indicated that tolerance to submergence in Arabidopsis requires a functional AGO1 protein that, together with AGO4-dependent noncanonical RNA-directed DNA methylation (RdDM) pathway, influences gene expression under hypoxia.

RESULTS

Submergence Severely Affects Survival in ago1-27 Plants

The *ago1-27* mutant displays rosettes with dark green and serrated leaves (Fig. 1A; Morel et al., 2002). We verified that the expression of some *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) genes, modulated by the *miR156* family, was up-regulated in *ago1-27* (Fig. 1B), as expected in a mutant defective in PTGS. We also checked if PTGS operated in submerged plants by using Arabidopsis plants overexpressing *miR399* and displaying strong repression of its target, *PHOSPHATE 2* (*PHO2*; Bari et al., 2006). *miR399*-dependent repression of *PHO2* was unaffected by 12-h submergence, indicating that PTGS operates under low oxygen conditions (Fig. 1C). In order to verify the contribution of AGO1 to submergence survival, we performed a tolerance experiment and the results showed that *ago1-27* plants were more sensitive to submergence than wild-type plants: most of the *ago1-27* plants died following submergence for 24 h, as shown in Figure 1D, indicating that *ago1-27* plants fail to recover from submergence treatment.

Submergence Induces miR2936 and Represses miR398

The intolerance to submergence of *ago1-27* plants suggests that PTGS triggered by miRNAs could be required for proper responses to low oxygen. This may occur through the action of specific miRNAs; we thus profiled miRNAs in aerobic and submerged wild-type plants. The microarray (Affymetrix miRNA 4.0 array) features 183 probes able to detect both premiRNAs and mature Arabidopsis miRNAs. The results showed that miRNA expression levels vary largely, with *miR159* being the most expressed both in aerobic and submerged plants (Fig. 2A). A scatterplot visualization of the expression level comparing the aerobic (Air) signal value with that of submerged plants indicated that submergence exerted only minor effects on the expression of most miRNAs (Fig. 2A), but miRNAs modulated by submergence were nonetheless identified [fold change (FC) ≥ 2 or ≤ 2; Fig. 2B]. Given that the microarray expression levels represent the sum of expression of the premiRNA and that of the mature miRNA sequence, we performed a quantitative PCR (qPCR) analysis to determine the expression levels of the mature miRNAs identified in Figure 2B. The results confirmed the up-regulation of *miR2936* and repression of *miR398* (Fig. 2C), whereas the other miRNAs were not significantly affected by submergence at the mature miRNA level (Supplemental Fig. S1).

RAP2.12 and Mitochondrial Functionality Regulate miRNA Abundance

To gain additional clues on the signaling mechanism modulating the expression of *miR2936* and *miR398*, we

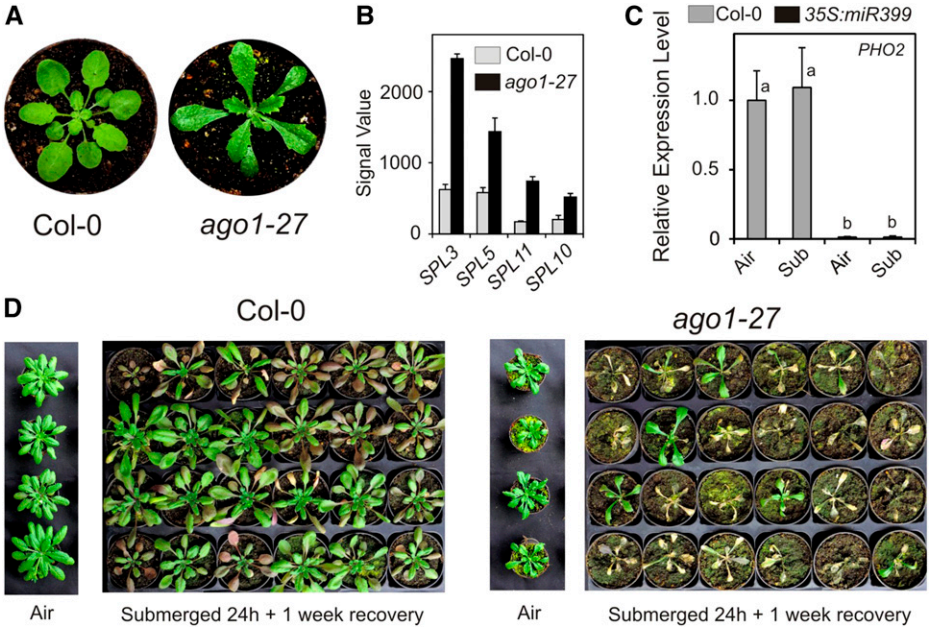

Figure 1. The *ago1-27* mutant is highly intolerant to submergence (Sub). A, Phenotype of Col-0 and *ago1-27* plants. B, Expression of *SPL* genes in aerobic plants. Data are mean ± SD ($n = 3$ biological replicates, each composed by two pooled plants). The expression of *SPL* genes was significantly (P value ≤ 0.05) higher in *ago1-27* plants (MicroArray Suite 5.0, Change Algorithm, Linear models algorithm applying the Benjamini-Hochberg correction to P values). C, Repression of *PHO2* in *35S:miR399* plants is unaffected by submergence. Data are mean ± SD ($n = 5$ plants). Values that significantly differ from each other are indicated by different letters (according to two-way ANOVA test, Bonferroni post hoc test, $P \leq 0.05$) D, Effect of submergence on survival of Col-0 and *ago1-27* plants. Plants were submerged for 24 h in the dark and transferred to normal 12-h light/12-h dark conditions for 1 week.

analyzed the expression level of these miRNAs in plants expressing a constitutively stable version of the oxygen sensor RAP2.12 (*35S:Δ-RAP2.12*) as well as in the quintuple mutant for all five ERF-VII proteins (*erfvii*). The results showed that *miR2936* was similarly regulated by submergence in wild-type, *35S:Δ-RAP2.12*, and *erfvii* plants (Fig. 2D). This indicated that the induction of *miR2936* was independent of the canonical oxygen sensing machinery, based on ERF-VII proteins (Fig. 2D). *miR398* repression by submergence was instead affected by ERFVII proteins, because its level was already very low in the *35S:Δ-RAP2.12* plants (Fig. 2D). However, expression of *miR398* was not altered in *erfvii* plants, indicating that, besides RAP2.12 stabilization, an additional mechanism operates under submergence to suppress *miR398* expression.

We next explored whether this mechanism relied on inhibition of mitochondrial respiration, as proposed by Moldovan et al. (2010). We indeed found that antimycin A, an inhibitor of mitochondrial complex III, upregulated *miR2936* and repressed *miR398* (Fig. 2E). Similarly, inhibition of mitochondrial complex II by carboxin induced *miR2936* and repressed *miR398* (Fig. 2E). In contrast, rotenone, a mitochondrial complex I inhibitor, was not effective in regulating *miR2936* and *miR398*, but azide (NaN_3), an inhibitor of complex IV, repressed *miR398* (Fig. 2E). Plants treated with the mitochondrial inhibitors responded by inducing an oxidative stress marker (*GSTU24*), confirming uptake and efficacy of the inhibitors (Fig. 2F). Overall, repression of *miR398* by inhibitors of mitochondrial respiration complexes explains the lack of a response in *erfvii* for *miR398*, because this miRNA can be also repressed independently by ERF-VII proteins through a mechanism dependent on inhibition of mitochondrial activity. These results indicate that partially overlapping hypoxia signaling pathways operate to modulate submergence-responsive miRNAs.

The Putative Targets of miR2936 and miR398 Are Not Regulated by Submergence

The expression of *miR2936* targets *AT1G76720* and *SU(VAR)3-9 HOMOLOG 5* (*SUVH5*; Grant-Downton et al., 2009) in the wild type was not in agreement with an increase in *miR2936* levels under submergence (Fig. 3A). Furthermore, expression of *AT1G76720* and *SUVH5* was unchanged in *ago1-27*, suggesting that, under the experimental conditions, these two genes are not influenced by PTGS (Fig. 3A). Submergence repressed *miR398* (Fig. 2, B and C), which targets the cytosolic *COPPER/ZINC SUPEROXIDE DISMUTASE1* (*CSD1*), the chloroplastic *CSD2*, and the *COPPER CHAPERONE FOR SUPEROXIDE DISMUTASE* (Sunkar et al., 2006) that delivers the copper cofactor to the

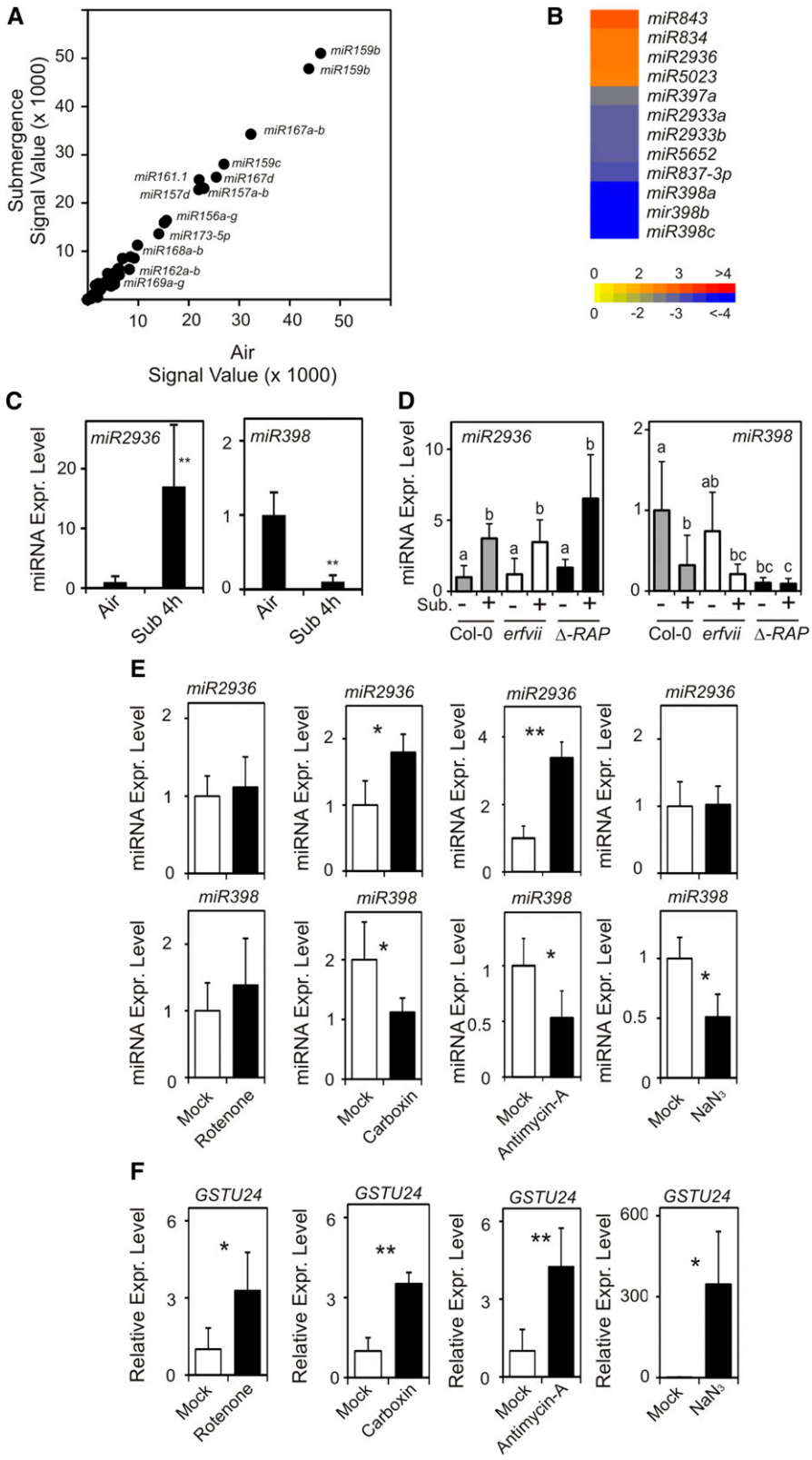

Figure 2. Effects of submergence (Sub) on the expression (Expr.) of miRNAs. A, Scatterplot of the signal value (microarray data) of miRNAs under either aerobic or anaerobic conditions. B, Heatmap of the fold change (microarray data) of the miRNAs that were found to be differentially regulated by submergence. C, miRNA expression level by RT-qPCR ($n = 5$ plants; t test, $^{**}P < 0.01$). D, Effect of submergence in wild type (Col-0), *erfvii*, and *35S:Δ-RAP2-12* (*Δ-RAP*) on the expression of *miR2936* and *miR398*. Plants were submerged for 4 h in the dark. Data are mean ± SD ($n = 5$ plants). Values that significantly differ from each other are indicated by different letters (according to two-way ANOVA test, Bonferroni post hoc test, $P < 0.05$). E, Effect of inhibitors (100 μM) on the expression of miRNAs in air. F, Effect of inhibitors (100 μM) on the expression of *GSTU24* in air. Data in (E) and (F) are mean ± SD of four biological replicates, composed by two pooled plants each (t test, $^{**}P < 0.01$; $^{*}P < 0.05$).

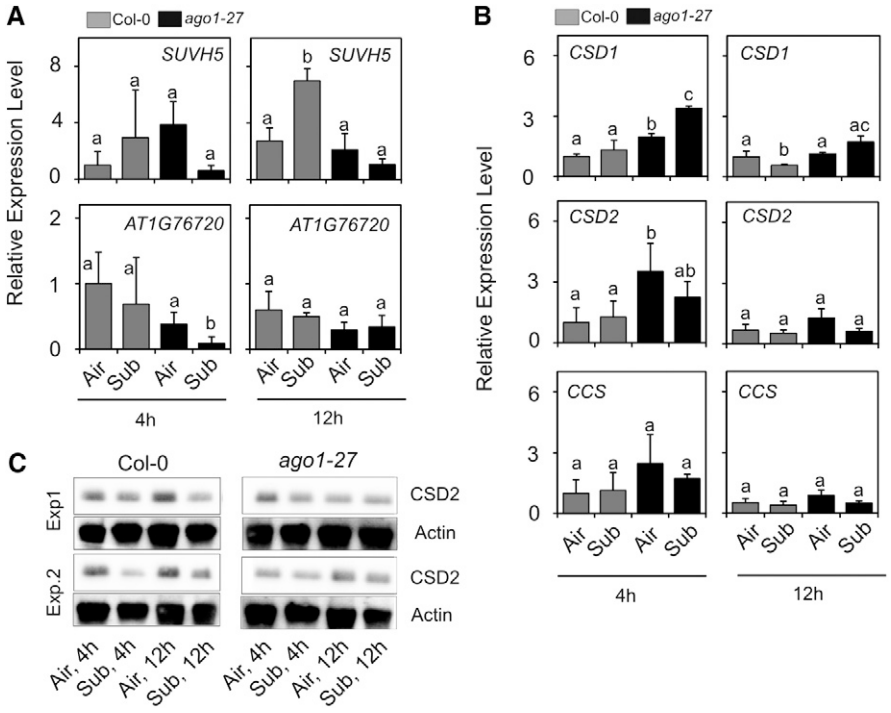

Figure 3. Expression of the putative target genes of miRNAs differentially expressed under submergence (Sub). A, Analysis of the expression of the putative target genes of *miR2936* in either wild-type (Col-0) and *ago1-27* plants. The mRNA level was analyzed by RT-qPCR. The relative expression level is calculated with the Col-0 sample at 4-h Air set to one. Data are mean ± SD ($n = 5$ plants). Values that significantly differ from each other are indicated by different letters (according to two-way ANOVA test, Bonferroni post hoc test, $P < 0.05$). B, Same as in (C) for the targets of miR398. C, Immunoblot of CSD2 protein level. Actin was used as loading control. The results from two independent experiments (Exp) are shown.

three Cu/Zn superoxide dismutases (SODs; *CSD1*, *2*, and *3*). These genes, however, were largely unaffected by submergence (Fig. 3B). Expression of *CSD1* and *CSD2* was slightly higher in *ago1-27*, but this difference was lost after 12 h (Fig. 3B). We speculated that instead of acting on mRNA stability, miRNAs could repress translation. However, immunoblot analysis of the CSD2 protein confirmed that its level was unchanged in the wild type and *ago1-27*, with only a slight reduction in CSD2 level upon submergence (Fig. 3C). Therefore, in line with previous results, changes in the expression of target genes do not correlate with those of the hypoxia-modulated miRNAs (Moldovan et al., 2010; Licausi et al., 2011b).

AGO1 Is Not Required for Expression of Core Anaerobic Genes

Given the minor impact of hypoxia on miRNAs and their target genes, we speculated that under submergence AGO1 could also be involved in RNA signaling pathways independent of PTGS. Besides PTGS mediated by miRNAs, AGO1 can directly affect RNA transcription (Chan et al., 2005) and contributes to the establishment of RNA-directed DNA methylation (RdDM). All these processes may impact the ability of Arabidopsis to adapt to submergence (Fig. 1D). We therefore explored the transcriptome of wild type (Col-0) and *ago1-27* plants, and the results (Fig. 4A) showed that 27 genes were more expressed in *ago1-27* and 14 genes are less expressed in *ago1-27*. This indicates that the transcriptome of the *ago1-27* mutant is quite similar to that of the wild type and that small RNA signaling, under normal aerobic control conditions, does not dramatically affect gene expression.

We then sampled plants after 12-h submergence and identified genes that are differentially regulated in the two genotypes by setting a threshold for the air versus submergence FC in gene expression at Log_2 FC ≥ 2 or ≤ -2 (Fig. 4B). The Venn diagram in Figure 4C shows that 435 and 103 genes were up- and down-regulated, respectively, in both wild-type and *ago1-27* plants, but also that 167 genes were specifically up-regulated in *ago1-27* plants and 98 were up-regulated only in wild-type plants (Fig. 4C). The expression of the 49 core anaerobic genes (Mustroph et al., 2010) in wild-type and in *ago1-27* plants is shown in Figure 4D. All 49 genes behaved very similarly in both genotypes and, indeed, we found that only one gene, namely FAR-RED-ELONGATED HYPOCOTYL1-LIKE displayed a FC ≥ 2 (P value ≤ 0.05) when comparing the signal values of submerged wild type with that of *ago1-27* plants (Fig. 4E). All other genes were expressed at the same level in the two genotypes, as shown for a selection of genes in Figure 4E.

AGO1 Influences Starch Content and the Sugar Starvation Status of Plants under Hypoxia

We noticed that several of the genes differentially regulated in *ago1-27* by submergence are typically induced in plants experiencing sugar starvation. Given the importance of sugar availability for Arabidopsis tolerance to submergence (Loreti et al., 2018), we compared the transcriptome of *ago1-27* with that of *pgm*, a starchless mutant that is intolerant of submergence and

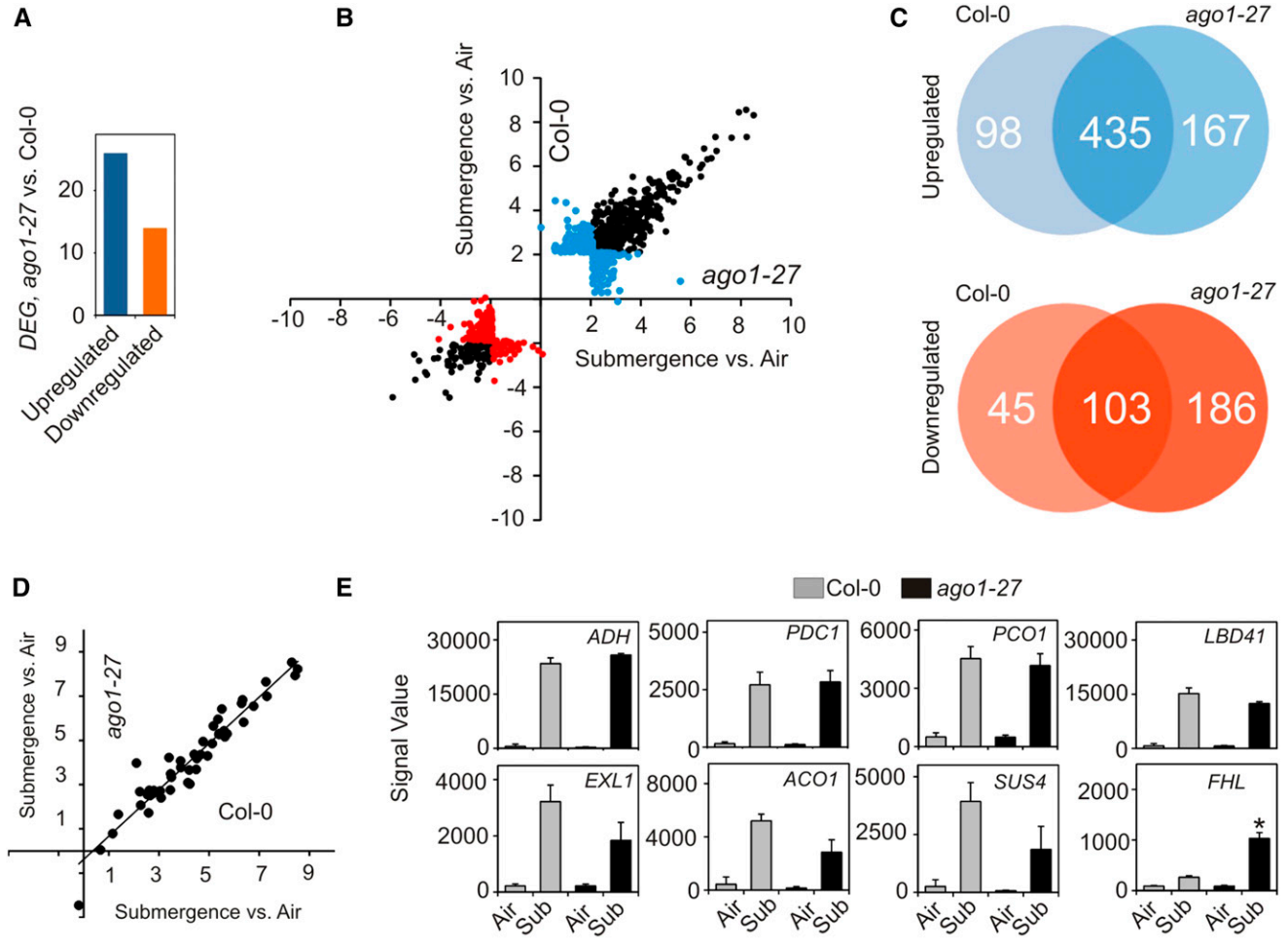

Figure 4. Analysis of the transcriptome of wild-type and *ago1-27* plants under submergence (Sub) for 12 h. A, Number of differentially expressed genes (DEG) in aerobically grown Col-0 and *ago1-27* plants. DEG are defined as genes that display a Log FC (Log_2 FC) ≥ 2, *P* value ≤ 0.05 (up-regulated) or ≤ -2, *P* value ≤ 0.05 when comparing the signal value of Col-0 with that of *ago1-27* ($n = 3$). B, Scatterplot of Log_2 FC of gene expression, submergence vs. air, in Col-0 and *ago1-27* plants. Blue dots represent genes that are exclusively induced with a Log_2 FC ≥ 2, *P* value ≤ 0.05 in Col-0 or *ago1-27* plants. Red dots represent genes that are repressed with a Log_2 FC ≤ -2, *P* value ≤ 0.05 exclusively in Col-0 or *ago1-27* plants. Black dots represent genes that are induced or repressed in both Col-0 and *ago1-27* plants. C, Venn-diagram showing the number of genes differentially regulated in Col-0 and *ago1-27* plants. D, Scatterplot of Log_2 FC of the 49 anaerobic core gene expression submergence vs. air in Col-0 and *ago1-27* plants. E, Expression level (Signal Value from the microarray data) of a selection of anaerobic genes. The expression was significantly (*P* value ≤ 0.05) higher under submergence in wild-type (Col-0) and *ago1-27* plants. Statistical difference (*P* value ≤ 0.05) between the signal value under submergence between the two genotypes is indicated by (*). Significance calculated by the MAS 5.0 Change Algorithm, Linear models algorithm applying the Benjamini-Hochberg correction to *P* values. Data are mean ± SD ($n = 3$ biological replicates, each composed by two pooled plants).

induces starvation–related genes as a consequence of starch deficiency. We found that approximately 25-30% of the genes up-regulated by submergence and darkness in *ago1-27* were also strongly up-regulated in *pgm* (Fig. 5A). A cluster of 17 genes is up-regulated in submergence in both *ago1-27* and *pgm*, eight of which had an expression pattern in *ago1-27* closely matching that of *pgm* (Fig. 5B). Interestingly, *FAR-RED-ELONGATED HYPOCOTYL1-LIKE*, the only gene belonging to the 49 core anaerobic genes (Mustroph et al., 2010) displaying altered expression in *ago1-27* (Fig. 4E), belongs to the group of genes that are likely altered in their expression because of a sugar starvation condition in *ago1-27* plants (Fig. 5B). We investigated whether the different behavior of *Col-0* and *ago1-27* under submergence conditions could be due to a difference in starch content or alternatively to a different rate of starch degradation. The results showed that the level of starch in *ago1-27* is less than half of that observed in the wild-type rosettes (Fig. 5, C and D). However, starch degradation in *ago1-27* proceeds normally under both aerobic and submerged conditions (Fig. 5E). These data suggest that

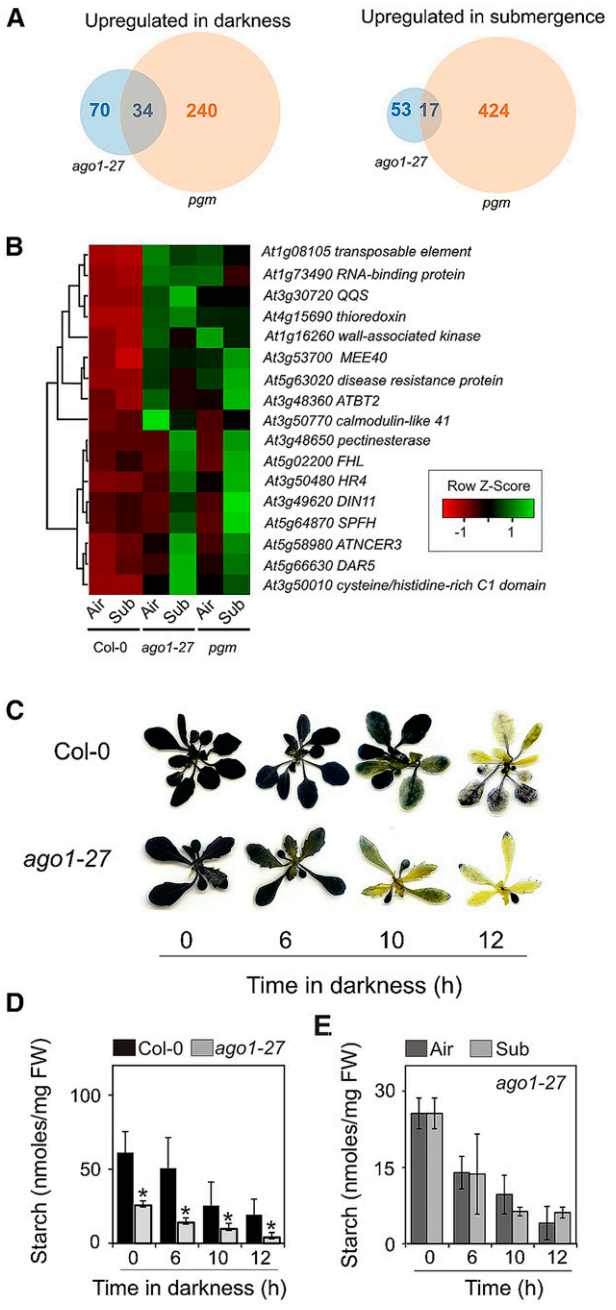

Figure 5. *ago1-27* plants suffer from sugar starvation under submergence (Sub). A, Venn-diagram showing the number of genes differently regulated in *pgm* and *ago1-27* plants when compared with the wild type. B, Heatmap showing the pattern of expression of the 17 genes that are up-regulated in *pgm* and *ago1-27* plants when compared with the wild type. C, Iodine staining of starch in wild-type and *ago1-27* plants during the night. Rosette images were digitally abstracted and made into a composite for comparison. D, Starch content in aerobic wild-type and *ago1-27* plants. T0 is at the end of the day. E, Starch degradation is aerobic and submerged *ago1-27* plants. T0 is at the end of the day. See Loreti et al. (2018) for data on starch degradation in the wild type. Statistical difference [(D) and (E); *t* test, *P* value ≤ 0.05) between the signal value under submergence between the two genotypes is indicated by (*). D and E, data are mean ± SD of four biological replicates. FW = fresh weight.

AGO1 may affect submergence tolerance through its impact on starch levels (Loreti et al., 2018).

AGO1 Is Required for the Correct Expression of Several Genes under Hypoxia

Additional analysis of the microarray dataset allowed us to identify genes whose expression was higher (Log_2 FC ≥ 2, *P* value ≤ 0.05) in submerged *ago1-27* plants compared with the submerged wild-type plants (Fig. 6A). This cluster of genes includes *HOMOLOG OF RPW8 4* (*HR4*), previously identified as up-regulated in plants overexpressing a stable form of the oxygen sensor RAP2.12 (Giuntoli et al., 2017) and coregulated in *ago1-27* and *pgm* plants (Fig. 5B). Conversely, we also identified genes, not classified as core anaerobic genes, which are induced by submergence in the wild-type but not in *ago1-27* plants (Fig. 6B), which included *SELENIUM-BINDING PROTEIN3* (*SBP3*). In mammalian systems *SBP1* is a target of hypoxia-inducible factor-1α (HIF-1α; Scortegagna et al., 2009), but there is currently no evidence of an involvement of SBP proteins in plant responses to hypoxia. We selected *HR4* and *SBP3* as markers of these two distinct responses in *ago1-27*. The impact of *ago1-27* on the expression of *HR4* and *SPB3* were validated by reverse transcription (RT)-qPCR in an independent experiment and also confirmed using a distinct *AGO1* mutant allele, namely *ago1-25* (Fig. 6C).

We next investigated how the RNA interference (RNAi) pathway regulating *HR4* and *SPB3* expression was connected to oxygen sensing. Plants expressing a constitutively stable version of RAP2.12 (*35S:Δ-RAP2.12*) expressed higher levels of both *ALCOHOL DEHYDROGENASE1* (*ADH1*) and *HR4* already in air (Fig. 7A). *SBP3* was instead unaffected by RAP2.12 (Fig. 7A). Analysis of the expression of *ADH1*, *SBP3*, and *HR4* in the quintuple mutant defective in all five ERF-VII (*erfvii*) showed that although *ADH1* and *HR4* induction were absent in *erfvii*, *SBP3* was normally induced in the mutant (Fig. 7B). These results allowed us to place *HR4* on the same oxygen signaling pathway as *ADH1*, whereas *SBP3* is regulated by submergence independently of ERF-VII proteins (Fig. 7).

AGO1-Dependent Repression of HR4 Is Mediated by AGO4

In order to dissect the signaling pathway leading to misregulation of *HR4* and *SPBP3* in *ago1-27*, we analyzed several mutants defective in PTGS or transcriptional gene silencing (TGS) through RdDM (Fig. 8; see Cuerda-Gil and Slotkin, 2016 for a review on PTGS and RdDM). The mutants analyzed included *rna-dependent rna polymerase 2* (*rdr2*) and *dicer-like 3* (*dcl3*), which are required for the generation of 24-nucleotide (nt) siRNA required for the canonical RdDM pathway. RDR6, DICER-LIKE 1 (DCL1) and DCL4, and AGO1 contribute

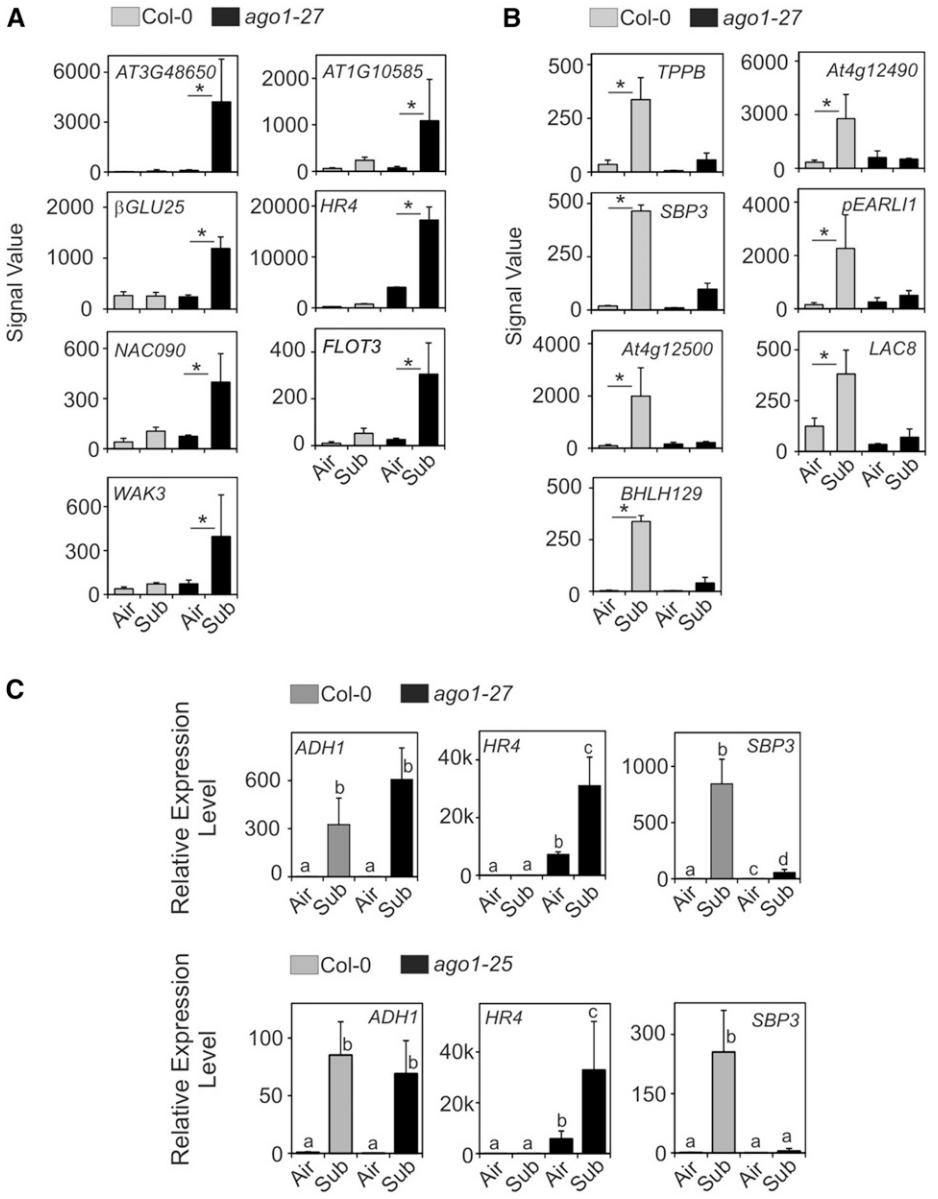

Figure 6. Effects of submergence (Sub) on gene expression in Col-0 and *ago1-27* plants. A, Expression level (Signal Value from the microarray data) of genes displaying higher expression (*P value $\leq$ 0.05) under submergence in *ago1-27* but not in wild-type (Col-0) plants (MAS 5.0 Change Algorithm, Linear models algorithm applying the Benjamini-Hochberg correction to P values). Data are mean $\pm$ SD (n = 3). Abbreviations of gene names are as follows: β-GLU25 = β GLUCOSIDASE 25; NAC090 = NAC DOMAIN CONTAINING PROTEIN 90; WAK3 = WALL ASSOCIATED KINASE 3; FLOT3 = FLOTILLIN 3. B, Expression of genes displaying higher expression (*P value $\leq$ 0.05) under submergence in Col-0 but not in *ago1-27* plants (MAS 5.0 Change Algorithm, Linear models algorithm applying the Benjamini-Hochberg correction to P values). Data are mean $\pm$ SD (n = 3). Abbreviations of gene names are as follows: TPPB = TREHALOSE-6-PHOSPHATE PHOSPHATASE B; SBP3 = SELENIUM BINDING PROTEIN3; pERAL1 = PHOSPHOLIPASE LIKE PROTEIN; LAC8 = LACCASE8; BHLH29 = BASIC HELIX LOOP PROTEIN 29. C, RT-qPCR validation of selected genes in wild-type plants and in the *ago1-27* and *ago1-25* mutants. Data are mean $\pm$ SD of four biological replicates, each composed by two pooled plants. Values that significantly differ from each other are indicated by different letters in figures (according to two-way ANOVA test, Bonferroni post hoc test, $P < 0.05$).

to the establishment of the noncanonical RDR6-DCL3 RdDM. AGO4 binds DCL3-generated 24-nt siRNAs and directs the activity of DOMAINS REARRANGED METHYLTRANSFERASE 1-2 (DRM1 and DRM2).

The expression of *ADH* was unaffected in the mutants tested, although its induction by submergence was reduced in *ago4-1*. Induction by submergence of *SBP3* was repressed in *ago1-27* and in *hen1-1*, which is defective in a methyltransferase required for the methylation of miRNAs and siRNAs, (Supplemental Fig. S2), but not in the other mutants used (Fig. 8), indicating that *SPB3* expression is influenced by PTGS but not RdDM. *HR4* is not expressed in *hen1-1* (Supplemental Fig. S2), which is expected because this mutant in the ecotype Landsberg *erecta* of Arabidopsis (L*er*) background lacks the *HR4* locus (Xiao et al., 2004).

HR4 expression and modulation by submergence were clearly influenced in mutants affected in RdDM (Fig. 8). The canonical pathway for RdDM involves the production of double-stranded RNA by RDR2 followed by the action of DCL3, producing 24-nt siRNAs that are loaded to AGO4 driving DRM1/DRM2 DNA methylation. Interestingly, *HR4* was highly expressed and also submergence-inducible in *dcl3-1*, *ago4-1*, and *drm1/drm2*, but its expression was unaffected in *rdr2-1* (Fig. 8A), indicating that *HR4* is regulated by a noncanonical RdDM pathway (Cuerda-Gil and Slotkin, 2016). Indeed, the RDR6-DCL3 noncanonical RdDM pathway controls *HR4* expression, as highlighted by the strong response observed in *HR4* expression in *rdr6-15* (Fig. 8B). All the other components required for the RDR6-DCL3 pathway were needed to keep *HR4* repressed, including AGO1 (Fig. 6A), DCL2, and DCL4

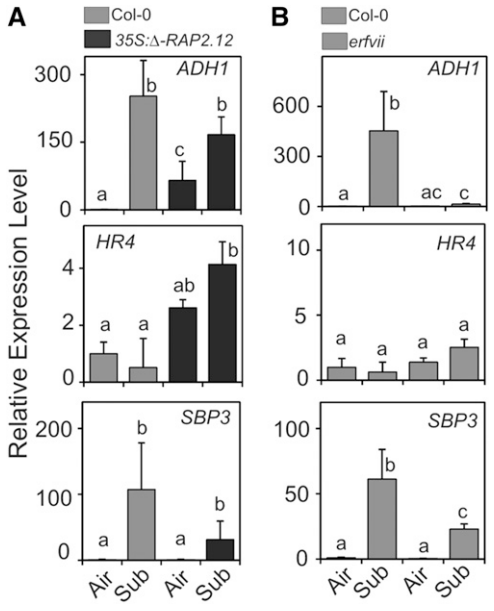

Figure 7. Expression level of selected genes under submergence (Sub) in Arabidopsis lines affected in oxygen sensing. The mRNA level was analyzed by RT-qPCR in an experiment performed submerging the plants for 4 h in the dark. A, Comparison of the response between the wild type (Col-0) and the *35S:Δ-RAP2-12* line. B, Comparison of the response between the wild type (Col-0) and the *erfvii* mutant. The relative expression level is calculated with the wild-type (Col-0) sample at 4-h Air set to one. Data are mean ± SD of four biological replicates. Values that significantly differ from each other are indicated by different letters (according to two-way ANOVA test, Bonferroni post hoc test, $P < 0.05$).

(Fig. 8B), which are required for the RNAi loop in the RDR6-DCL3 RdDM (Cuerda-Gil and Slotkin, 2016).

RNA Signaling Pathways and their Contribution to Submergence Tolerance

The observation that distinct RNA signaling pathways operate under submergence raised an important question about their contributions to submergence tolerance. The AGO1-HUA-ENHANCER1 (HEN1) pathway is required to ensure the correct induction by submergence of the gene cluster to which *SBP3* belongs (Fig. 6B). In the absence of AGO1 or HEN1 these genes are no longer induced under submergence, thus rendering the anaerobic response of Arabidopsis plants incomplete. This may predict increased intolerance to submergence in *hen1-1*, as described for the *ago1-27* mutant (Fig. 1D). We indeed found that *hen1-1* is extremely intolerant to submergence (Supplemental Fig. S3). On the other hand, *ago4-1* plants were highly tolerant to submergence (Fig. 9A), suggesting that DNA methylation driven by AGO4 is detrimental for submergence tolerance.

We then checked if methylation of *HR4* was influenced in *ago4-1*, given that the second exon of *HR4* is strongly methylated in the wild type (Dowen et al., 2012). We used methylation sensitive restriction enzyme qPCR to estimate the methylation status of *HR4* in wild-type, *ago4-1*, and *35S:Δ-RAP2.12* plants. The results showed that the second exon of *HR4* is strongly methylated in the wild type but much less in *ago4-1* and, remarkably, also in *35S:Δ-RAP2.12* plants (Fig. 9, B and C). The demethylation of *HR4* observed in *ago4-1* and *35S:Δ-RAP2.12* plants corresponds with the higher expression of this gene in these lines when compared with the wild type (Figs. 7A and 8A). The possibility that oxygen sensing mediated by RAP2.12 can affect DNA methylation opens new perspectives in hypoxia signaling.

DISCUSSION

Although knowledge about transcriptional regulation of gene expression under hypoxia increased considerably during the last decade, our understanding of the possible role of RNA silencing pathways under hypoxia remained limited. Hypoxic Arabidopsis roots display altered expression of various miRNAs, but a clear relationship between the expression of miRNAs and expression of their respective target genes is missing (Moldovan et al., 2010; Licausi et al., 2011b). None of the miRNAs that Moldovan et al. (2010) and Licausi et al. (2011b) identified as modulated by hypoxia in Arabidopsis roots were found to be modulated by submergence in Arabidopsis leaves, and only *miR391* was found as hypoxia-modulated in roots by both by Moldovan et al. (2010) and Licausi et al. (2011b), indicating that miRNA expression might vary depending on the experimental conditions. Interestingly, we found that several inhibitors of mitochondrial functionality up-regulate *miR2936* and repress *miR398*, suggesting that submergence modulates miRNA expression through a signaling pathway linked to mitochondrial functionality, in agreement with data obtained by Moldovan et al. (2010) using antimycin-A.

Repression of *miR398* by submergence in leaves (Fig. 2C) is of interest given that this miRNA was identified as being involved in the response of plants to several abiotic stresses (Zhu et al., 2011). *miR398* targets SODs (Sunkar et al., 2006), and its repression during submergence could induce the expression of SODs to mitigate the adverse effects of superoxide radicals produced during submergence. However, we did not observe changes in the expression of the *miR398* targets after submergence, neither at the mRNA nor at protein level (Fig. 3, B and C).

Given that previous studies on miRNAs related to hypoxia did not identify a clear relationship between the expression levels of miRNAs and their relative targets (this work; Moldovan et al., 2010; Licausi et al., 2011b), it would be tempting to speculate that miRNA contribution to plant responses to hypoxia is limited or that miRNAs and target genes are expressed in separate, distinct cell types. Alternatively, given that

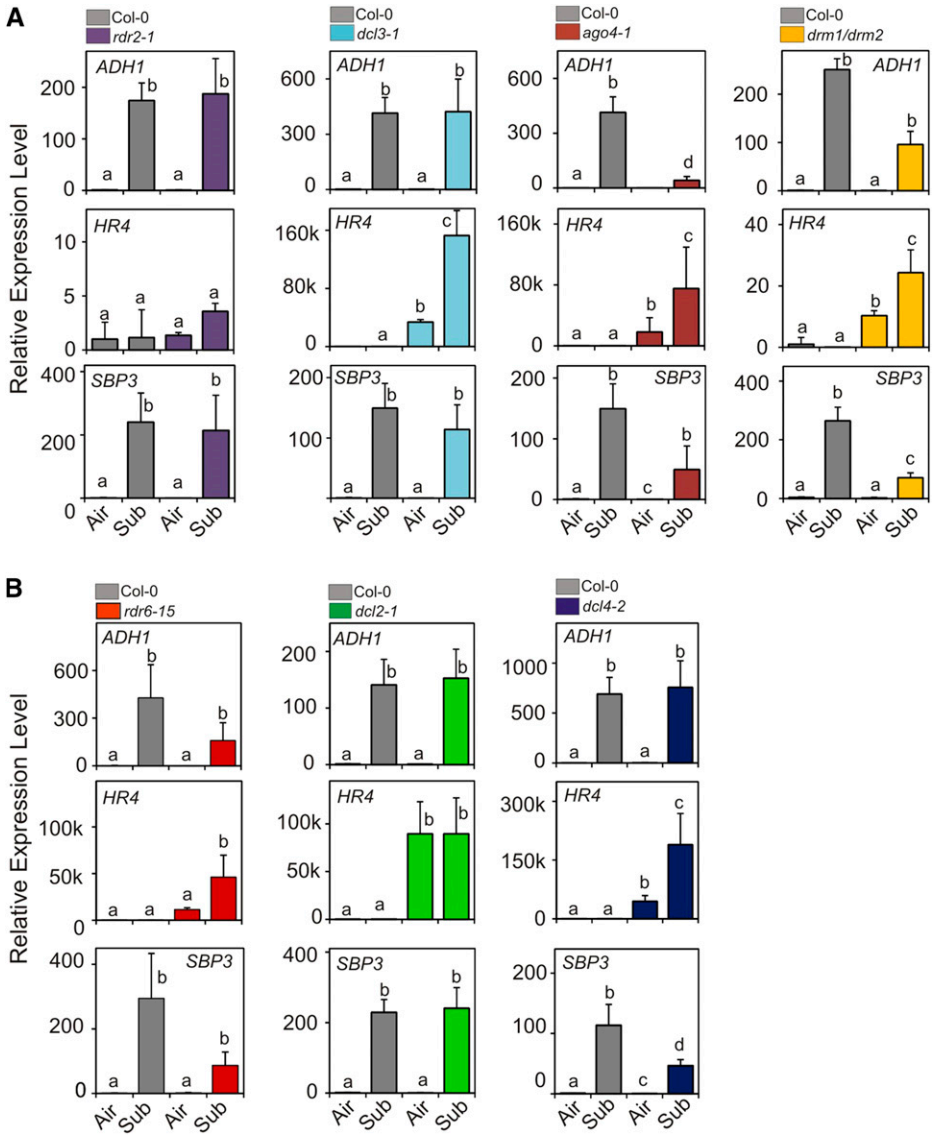

Figure 8. Expression level of selected genes under submergence (Sub) in RNA silencing mutants. The mRNA level was analyzed by RT-qPCR in an experiment performed submerging the plants for 4 h in the dark. A, Comparison of the response between the wild type (Col-0) and mutants affected in the canonical pathway for RdDM. B, Comparison of the response between the wild type (Col-0) and mutants affected in the noncanonical pathway for RdDM. The relative expression level is calculated with the wild type (Col-0) sample at 4 h Air set to one. Data are mean ± SD of four biological replicates. Values that significantly differ from each other are indicated by different letters (according to two-way ANOVA test, Bonferroni post hoc test, $P < 0.05$).

41,965 genome-wide miRNA target sites and 10,442 miRNA target genes were identified in the Arabidopsis genome (Bülow et al., 2012), a number largely exceeding the miRNA target genes described and validated to date, it is likely that the target genes modulated under hypoxia by *miR398* and *miR2936* are yet to be identified.

Intolerance to submergence in *ago1-27* (Fig. 1D) is likely caused by the inability of this mutant to induce a set of anaerobic genes (Fig. 6B), or by its reduced starch content (Fig. 5C), given the similarity of the *ago1-27* transcriptome with that of the highly submergence-intolerant *pgm* plants (Loreti et al., 2018). Interplay between RNA signaling and carbohydrate metabolism was reported to occur in relation to a complex mechanism in which different DCL proteins compete for their substrate (Wu et al., 2017). *dcl4* mutants show increased starch and sugar content, possibly because competition among DCLs affects the expression of *SUPPRESSOR OF MAX-LIKE 4* (*SMXL4*) and *SMXL5* (Wu et al., 2017). Besides *SMXL4* and *SMXL5*, four other protein-coding genes were found to be direct substrates of DCL2 because they predominantly generate 22-nt small RNAs in *dcl4* (Wu et al., 2017). Among these, the authors identified *BETA-AMYLASE3* (*BAM3*; Wu et al., 2017), coding for one of the major β-amylases that degrade transitory starch in Arabidopsis (Streb and Zeeman, 2012). Interestingly, *BAM3* was substantially upregulated in *ago1-27*, together with the noncatalytic, regulatory *BAM4* (Supplemental Fig. S4). Higher expression of β-amylases can reduce the total starch content in leaves and could explain the lower starch content observed in *ago1-27* (Fig. 6B).

Under submergence, core anaerobic genes (Mustroph et al., 2010) were unaffected in *ago1-27* (Fig. 4, D and E), but other submergence-related genes were identified in *ago1-27* with altered expression when compared with the wild type (Fig. 6, A and B). The group of genes that is normally induced by hypoxia and submergence

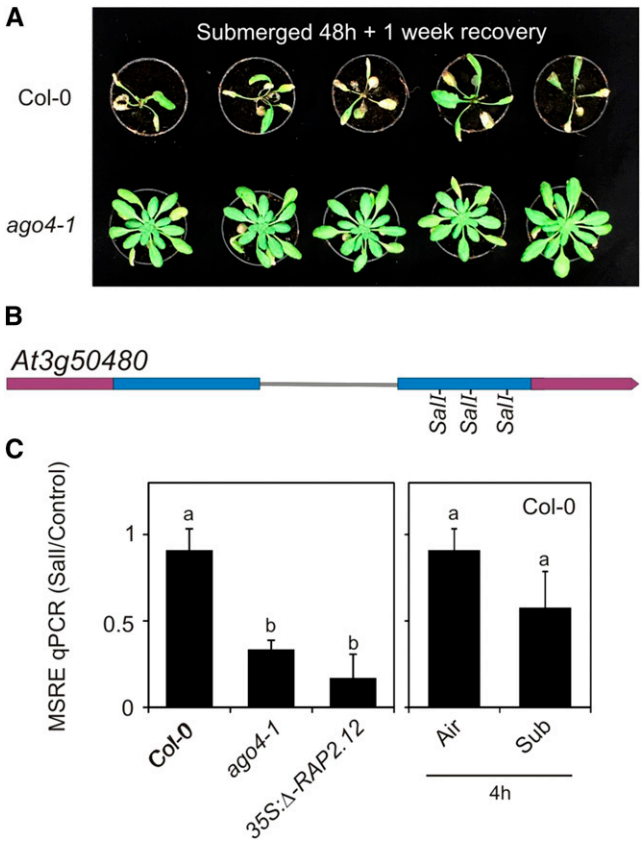

Figure 9. AGO4 and RAP2.12 influence the methylation status of *HR4*. A, Effect of submergence (Sub) on survival of *Col-0* and *ago1-27* plants. Plants were submerged for 48 h in the dark and transferred to normal 12-h light/12-h dark conditions for 1 week. B, Schematic representation of the *HR4* gene. The two exons are represented in blue color. The methylations sensitive *Sal*I sites are shown on the second exon. C, Methylation sensitive restriction enzyme (MSRE) qPCR of the second *HR4* exon. Changes in DNA methylation level of *HR4* are shown as ratio between the *Sal*I-treated and untreated DNA. Data are mean ± SD of four biological replicates (two pooled plants for each biological replicate). Values that significantly differ from each other are indicated by different letters in figures (according to two-way ANOVA test, Bonferroni post hoc test, $P < 0.05$).

but fails to do so in *ago1-27* (Fig. 6B) may require a step involving PTGS/TGS for their hypoxic induction. *SBP3* also fails to be induced by submergence in *hen1-1* (Supplemental Fig. S2), but it is normally induced in the wild type and in *ago4-1* (Fig. 8A), indicating that PTGS, rather than TGS, regulates *SBP3*. *SBP3* up-regulation in submerged plants depends on a pathway requiring both AGO1 and HEN1 independently of the ERF-VII–dependent oxygen sensing machinery (Fig. 8). It is tempting to speculate that *SBP3* is indirectly modulated under hypoxia by indirect, AGO1-dependent de-repression of its transcription, because *SBP3* is less induced in *ago1-27*.

HR4 encodes a biotic-stress related protein that is up-regulated by relatively long submergence treatments in the wild type (Supplemental Fig. S5). Interestingly, *HR4* is up-regulated in in *35S:Δ-RAP2.12* seedlings and plants (Fig. 7A; Giuntoli et al., 2017). Loss of DNA methylation enhances resistance to bacteria, and *HR4* was shown to be de-methylated in response to *Pseudomonas syringae* (Dowen et al., 2012). We indeed found that the second exon of *HR4* is highly methylated in the wild type but not in *ago4-1* and, interestingly, in *35S:Δ-RAP2.12* plants (Fig. 9). Regulation of *HR4* by submergence is emphasized in mutants affected in RdDM (Fig. 8), indicating that when de-methylated, the gene is hypoxia responsive. De-methylation of *HR4* in *35S:Δ-RAP2.12* plants suggests the existence of a mechanism linking ERF-VII stabilization to pathogen tolerance. Evidence for a role of the oxygen-dependent N-end rule pathway in the plant responses to pathogens, as well evidence for a direct role of RAP2.3 in resistance to *Botrytis*, indeed suggests that the de-methylation of *HR4* by RAP2.12 could be of importance in the context of the still largely unexplored role of ERF-VII stabilization in the infection zone (Zhao et al., 2012; de Marchi et al., 2016; Vicente et al., 2019). *HR4* was up-regulated in *rdr6-15* but not in *rdr2-1* (Fig. 8), thus indicating that it is normally repressed by a non-canonical pathway for RdDM requiring the biogenesis of RDR6-dependent secondary siRNAs. In this context AGO1, together with DCL2 and DCL4, would be required for the RNAi loop that generates the RNA template for RDR6 action, followed by 24-nt siRNA production through the action of DCL3 (Cuerda-Gil and Slotkin, 2016). The 24-nt siRNAs bind to AGO4 to direct the action of DRM2 on the target genes to be methylated.

Here, we explored the contribution of AGO1, a major determinant of posttranscriptional gene regulation on gene expression under hypoxia. Convergence of mitochondrial signaling with the canonical oxygen sensing machinery contributes to hypoxia modulation of miRNA expression. The miRNA-dependent PTGS pathway can operate under submergence, but we could not identify the actual targets of the modulated miRNAs, indicating that further work is required in this field. Besides the role of AGO1 in the miRNA pathway, other AGO1-dependent small RNA signaling pathways affect the expression of a group of genes under hypoxia. Interestingly, we found that TGS operates, via AGO4, to silence genes that would be otherwise regulated under hypoxia, opening the door for further studies on the impact of hypoxia on DNA methylation in plants. In animals, hypoxia induces DNA and histone Lys hypermethylation (Gallipoli and Huntly, 2019). Two recent articles showed that two Lys-specific demethylases (KDMs) are oxygen sensitive, and de-methylate histones in an oxygen-dependent manner (Batie et al., 2019; Chakraborty et al., 2019). Similar to the highly conserved hypoxia-inducible factor (HIF) pathway (Kaelin and Ratcliffe, 2008), KDMs require oxygen for their activity and were therefore identified as additional oxygen sensors in animals. Recently, an oxygen-regulated component of the histone methylating polycomb repressive complex 2 was identified in plants,

which is controlled through the N-end rule pathway like RAP2.12 (Gibbs et al., 2018). Polycomb repressive complex 2 may thus be acting as a transducer of oxygen availability into methylation changes in an analogous way to the animal KDMs.

Interestingly, the HIF and KDMs pathways may have coevolved to coordinate cellular response to low oxygen tensions. The immediate hypermethylation of H3K4 at the promoters of HIF target genes indeed indicates convergence of the two pathways (Gallipoli and Huntly, 2019). Whether in plants transcriptional regulation under hypoxia also involves ERF-VII-dependent transcriptional activation together with DNA and histone methylation or de-methylation is a fascinating hypothesis requiring further studies.

MATERIALS AND METHODS

Plant Material

Genotypes of Arabidopsis (*Arabidopsis thaliana*) used included Col-0 ecotype (wild type), L*er* (background of *hen1-1*), and the hypomorphic *ARGONAUTE1* mutants *ago1-27* and *ago1-25* (Morel et al., 2002). *Ago1-27* and *ago1-25* are hypomorphic mutants that are as impaired in PTGS as the previously identified strong *ago1* mutants without showing the marked developmental defects of *ago1* (Morel et al., 2002). It was proposed that AGO1 could have a dual role in PTGS and development, with the latter independent of PTGS (Morel et al., 2002). Other mutant lines used are reported in Supplemental Table S1. We used a line constitutively expressing a version of the RAP2.12 protein that is also stable in air (*35S:Δ-RAP2.12*; Licausi et al., 2011a). The quintuple *erfvii* mutant (Marín-de la Rosa et al., 2014) is a null mutant for *RAP2.3*, *RAP2.12*, *HYPOXIA RESPONSIVE ERF1*, *HYPOXIA RESPONSIVE ERF2*, and a knock-down for *RAP2.2* (Giuntoli et al., 2017). The starchless mutant *pgm* (Caspar et al., 1985) was also used. Plants were grown in pots for 3 to 4 weeks at 23°C with a 12/12-h photoperiod at 120 μmol photons m^{-2} s^{-1} before being used for experiments. Plants were submerged in tanks with a water level of 10 cm above leaf level. The submergence treatment was carried out in the dark, starting at 7 PM (11 h after the commencement of the light cycle). Oxygen concentration was measured in the tanks used for submerging the plants using a FireStingO2 high precision, personal computer–controlled fiber-optic oxygen meter (Pyro Science;). The oxygen probe used was OXROB10. Water temperature was contemporarily measured using the Dipping-Probe Temperature Sensor TDIP15. The oxygen levels measured were as previously reported (Loreti et al., 2018). Inhibition of the mitochondrial electron transport chain was achieved by spraying 100 μM inhibitors dissolved in ethanol (Sigma-Aldrich) on adult plants 2 h before the anoxic treatment (Fig. 2E). Treatments with the inhibitors were performed under anoxia to avoid dilution of the inhibitors in the water used to submerge the plants. Anoxia treatments were carried out using an enclosed anaerobic workstation (Anaerobic System model 1025; Forma Scientific) as previously described (Loreti et al., 2018).

Total RNA Extraction, qPCR, and RNA Gel Blots

Total RNA was extracted as previously described (Perata et al., 1997) with a minor modification (omission of aurintricarboxylic acid) to make the protocol compatible with the subsequent PCR procedures. Electrophoresis using a 1% (w/v) agarose gel was performed for all RNA samples to check for RNA integrity, followed by spectrophotometric quantification. Reverse-transcription was performed using the Maxima First Strand complementary DNA (cDNA) Synthesis Kit for RT-qPCR, with dsDNase (Thermo Fisher Scientific). Expression analysis was performed by RT-qPCR using an ABI Prism 7300 sequence detection system (Applied Biosystems). RT-qPCR was performed using 30 ng cDNA and iQ SYBR Green Supermix (BioRad Laboratories), according to the manufacturer's instructions. Expression of *UBIQUITIN10* (*At4g05320*) and *ACTIN2* (*At3g18780*) were used as endogenous controls for Col-0 and L*er* genotypes, respectively. Relative expression levels were calculated using GeNorm (http://medgen.ugent.be/~jvdesomp/genorm). For a list of the primers used and designed by Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/), see Supplemental Table S2. Mature miRNA RT-qPCR was performed using the stem-loop technique (Chen et al., 2005). After contaminating DNA was removed with RQ1 RNase-free Dnase (Promega), the cDNA was processed using Superscript III (Invitrogen). For RT-qPCR, we analyzed 50 ng of cDNA. Primers were designed using the miRNA Primer Design Tool (Czimmerer et al., 2013). See Supplemental Table S3 for the list of primers.

RNA Isolation, cRNA Synthesis, and Hybridization to Affymetrix GeneChips

RNA was extracted as described in the section "Total RNA Extraction, qPCR, and RNA Gel Blots" RNA quality was assessed by agarose gel electrophoresis and spectrophotometry. RNA was processed for use on Affymetrix GeneChip Arabidopsis ATH1 Genome Arrays, as previously described (Loreti et al., 2005). Hybridization, washing, staining, and scanning procedures were performed by Genopolis (University of Milano-Bicocca), as described in the Affymetrix technical manual. Microarray analysis was performed using an R/Bioconductor (Gentleman et al., 2004) and Robin (Lohse et al., 2010). Normalization was performed using MAS5.0. The miRNA profiling was performed using the GeneChip miRNA 4.0 Array. In this case, small RNAs were extracted using the mirVana Kit (Thermo Fisher Scientific), and RNA quality was assessed by Agilent BioAnalyzer. Hybridization, washing, staining, and scanning procedures were performed by University of Milano-Bicocca, as described in the Affymetrix technical manual. Publicly available microarray datasets were analyzed using Genevestigator (Hruz et al., 2008).

Extraction of Proteins, SDS-PAGE, and Immunoblots

Plant material was extracted by grinding in liquid nitrogen precooled samples with a pestle to a fine powder. The extraction buffer (50 mm Tris-HCl, pH 7, and 1% (w/v) SDS with Sigma P9599 protease inhibitor cocktail) was added, vortexed vigorously, and then centrifuged for 30 min at 14,000 rpm to obtain a supernatant. Protein content in the supernatant was quantified with Bio-Rad DC reagent (Lowry method). Samples were dissolved in 5× Laemmli buffer, treated at 95°C for 10 min, and loaded (30 mg) to Invitrogen NuPAGE gels (10% Bis-Tris Midi Gels). After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane using the Bio-Rad Trans-Blot turbo transfer pack. An anti-CSD2 chloroplastic Cu/Zn superoxide dismutase antibody (Agrisera AS06 170) and secondary goat antirabbit IgG horseradish peroxidase conjugated antibody (Agrisera AS09 602) were used. The antiactin11 antibody (Agrisera AS10 702) was used together with secondary rabbit antimouse IgG horseradish peroxidase conjugated antibody (Agrisera AS09 627). Detection was performed using the LiteAblot Turbo Chemiluminescence substrate (Euroclone).

Methylation Analysis

Genomic DNA from 40 mg of Arabidopsis leaves was extracted using Wizard Genomic DNA Purification Kit (Promega). Electrophoresis using a 1% (w/v) agarose gel was performed for all DNA samples to check for DNA integrity, followed by spectrophotometric quantification. DNA methylation analysis of GpC sites for second exon of HR4 (*At3g50480*) was performed using the methylation-sensitive enzyme *Sal*I (Thermo Fisher Scientific). The enzymatic digestion was performed on 1 μg of purified genomic DNA using 10 U of *Sal*I in a 20 μl final volume with buffer O (33 mM Tris-acetate [pH 7.9], 10 mM Mg-acetate, 66 mM K-acetate, 0.1 mg/ml bovine serum albumin). The reaction was incubated at 37°C for 4 h followed by an incubation step at 65°C for 30 min for *Sal*I thermal inactivation. After digestion, the treated and nontreated DNA were amplified by qPCR using an ABI Prism 7300 sequence detection system (Applied Biosystems). qPCR was performed using 30 ng DNA and iQTM SYBR Green Supermix (BioRad Laboratories), according to the manufacturer's instructions. *UBIQUITIN10* (*At4g05320*) was used as an endogenous control. Changes in DNA methylation level of HR4 are shown as ratio between the *Sal*I-treated and nontreated DNA. The primers used to amplify the second exon of HR4 are reported in Supplemental Table S2.

Statistical Analyses

Values that significantly differ from each other are indicated by different letters in figures (according to two-way ANOVA test, Bonferroni post hoc test, $P < 0.05$) unless differently stated in the figures.

Accession numbers

Microarray datasets were deposited in a public repository with open access (ATH1 Genechip, accession no. GSE116996; miRNA4.0 Array, accession no. GSE116998; http://www.ncbi.nlm.nih.gov/projects/geo).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Effect of 4-h submergence on the expression of miRNAs.

Supplemental Figure S2. Expression level of selected genes under submergence in Arabidopsis lines affected in RNA silencing.

Supplemental Figure S3. Effect of submergence on survival of Col-0, L*er*, *hen1-1* plants.

Supplemental Figure S4. Expression of *BAM3* and *BAM4* in wild type and *ago1-27* plants.

Supplemental Figure S5. Expression of *HR4* obtained using Genevestigator to interrogate the Affymetrix Genechip datasets.

Supplemental Table S1. Arabidopsis lines utilized.

Supplemental Table S2. Primers used for gene expression analysis using RT-qPCR.

Supplemental Table S3. Stem-Loop primers used for miRNA expression analysis using RT-qPCR.

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