MicroRNAs (miRNAs) are small molecules that can regulate many biological processes such as development, differentiation and stress response in plants (Voinnet 2009). In Arabidopsis, efficient miRNA biogenesis requires the DICER-LIKE1 (DCL1)--HYPONASTIC LEAVES1 (HYL1) complex, which processes primary miRNA precursor (pri-miRNA) with a hairpin structure into mature miRNAs (Kurihara and Watanabe 2004, Kurihara et al. 2006, Dong et al. 2008, Tagami et al. 2009). On the other hand, the Arabidopsis genome encodes 10 AGO genes (AGO1–10). ARGONAUTE (AGO) proteins possess two domains, the PAZ domain which is a binding cassette with small RNAs and the PIWI domain which has endonuclease activity. Of 10 AGO proteins, AGO1 is a major endonuclease which targets mRNAs complementary to the miRNA sequence, because AGO1 preferentially binds small RNAs with a uridine at the 5′ end, which is a feature of most miRNAs (Baumberger and Baulcombe 2005, Ronemus et al. 2006, Mi et al. 2008, Takeda et al. 2008).

Several hyl1 and ago1 mutants have been obtained. Of them, ago1-25 has a point mutation (G758S) at the PIWI domain, a catalytic center of endonuclease activity, and compromises transgene silencing (Morel et al. 2002). However, the mutated AGO protein still possesses endonuclease activity in vitro (Baumberger and Baulcombe 2005). On the other hand, hyl1-2, a putative knockout mutant, has a T-DNA insertion in the second exon (Vazquez et al. 2004). Both plants showed reduced growth and some morphological defects at 15 d after germination (Fig. 1). ago1-25 shows
developmentally mild and fertile phenotypes compared with ago1 null mutants such as ago1-3 (Vaucheret et al. 2004). In addition, only hyl1-2 plants have violently curled leaves (Fig. 1, arrows).

To reveal the miRNA-deficient effect on the transcriptome pattern, we performed whole genome tiling array analyses in 15-day-old seedlings of ago1-25 and hyl1-2 mutants. First, we examined the accumulation levels of known pri-miRNAs. Using the tiling array, we can particularly detect expression of pri-miRNAs, which are non-coding primary transcripts. Many pri-miRNA structures (exon–intron junction and length of transcribed region) registered in The Arabidopsis Information Resource 8 (TAIR8) genome are imperfect; for example, the gene model of pri-miR156c in TAIR8 nearly coincides with the signal pattern in the tiling array analysis, but the real gene structure of pri-miR166a is probably longer than that in TAIR8 (Fig. 2A). Therefore, the average intensities of pri-miRNAs were calculated in the region from 100 bases upstream of the hairpin sequence registered in Sanger miRBase (http://microrna.sanger.ac.uk/sequences/index.shtml) to 100 bases downstream (Fig. 2A, black bars; Supplementary Table S1). Among all known pri-miRNAs, expression of 59 pri-miRNAs was confirmed (P-initial ≤ 10^{-6}) in the wild type or hyl1-2. The expression-confirmed pri-miRNAs were classified into six categories based on their fold changes (Fig. 2B). Accumulation of about 70% of the expression-confirmed pri-miRNAs dramatically increased ≥1.5-fold in hyl1-2 compared with those in the wild type (Fig. 2A, B). Increased accumulation of five pri-miRNAs in hyl1-2 was verified by quantitative reverse transcriptase–PCR (qRT–PCR) (Fig. 2C). In ago1-25, no remarkable changes in the pri-miRNA accumulation were observed on the array data (Fig. 2A, C). This result is consistent with the proposed

![Fig. 1](https://example.com/image1.png) Fifteen-day-old wild-type (WT, Col-0 ecotype), ago1-25 and hyl1-2 plants. Arrows indicate curled rosette leaves in hyl1-2.

![Fig. 2](https://example.com/image2.png) Effects of ago1-25 and hyl1-2 mutations on accumulation of pri-miRNAs. (A) Examples of expression profiles of pri-miRNAs on OmicBrowse (http://omicspace.riken.jp/gps/group/psca6). The deep-blue regions are exons and the light-blue regions are introns in the TAIR8 gene model. The red and green bars indicate the relative signal intensity of probes (red ≥ 400, green < 400). Horizontal black bars indicate the regions used for calculation of average intensities of pri-miRNAs. (B) Classification of expression-confirmed pri-miRNAs (P-initial ≤ 10^{-6}) into six categories based on the respective fold changes. (C) Quantitative RT–PCR analysis of pri-miRNAs. The average values of fold changes in hyl1-2 on tiling array analysis are shown in brackets. ACT2 mRNA was used as an internal control. (D) Northern blot analysis of five miRNAs. An image of 5S rRNA and tRNAs was used as an internal control.
model that HYL1 assists DCL1 in processing of pri-miRNAs and AGO1 functions after miRNA maturation (Voinnet 2009).

We checked the accumulation of five miRNAs (miR156, miR164, miR166, miR168 and miR319) among the wild type, ago1-25 and hyl1-2 (Fig. 2D). In hyl1-2, the miRNA accumulation decreased as reported previously (Han et al. 2004), although accumulation of miR168 was similar to that in the wild type. On the other hand, little difference (miR156, miR168 and miR319) in the miRNA accumulation was observed in ago1-25. This is probably because the mutated AGO1 protein in ago1-25 has a perfect PAZ domain that is a small RNA-binding cassette and might stabilize some miRNAs (Morel et al. 2002).

Next, we analyzed changes in genes except some kinds of functional RNAs such as miRNAs, small nucleolar RNAs (snoRNAs), tRNAs and rRNAs. The tiling array analyses confirmed the expression of 17,419 genes in ago1-25 and 17,602 genes in hyl1-2 (P-initial ≤10^{-4}). The expression-confirmed genes were classified into six categories based on their fold changes (Fig. 3A). The patterns of expression changes were very similar in ago1-25 and hyl1-2. In ago1-25 and hyl1-2, accumulation of 944 and 2,627 transcripts, respectively, was up-regulated ≥1.5-fold [Fig. 3B, false discovery rate (FDR) α = 0.05; Supplementary Tables S2, S3], and accumulation of 259 and 1,170 transcripts, respectively, was down-regulated <0.66-fold (Fig. 3C, FDR α = 0.05; Supplementary Tables S4, S5). Of the up-regulated and down-regulated transcripts, 542 and 188, respectively, overlapped, and were equivalent to 57 and 73% of the up-regulated and down-regulated transcripts, respectively, in ago1-25. More profound changes were observed in hyl1-2 than in ago1-25 (Fig. 3B, C). This result supports the fact that hyl1-2 plant showed a more severe phenotype than the ago1-25 plant (Fig. 1).

Defects in miRNA-mediated gene silencing should be linked with increased accumulation of their target transcripts (Kasschau et al. 2003). We predicted miRNA-targeted transcripts from the up-regulated transcripts shown in Fig. 3B (see Materials and Methods). In ago1-25 and hyl1-2, 91 and 214 transcripts, respectively (total 248), were predicted as target miRNA candidates (score ≤3, Supplementary Tables S2, S3). They included many previously confirmed targets such as DCL1 (targeted by miR162), ARF8 (miR167), CSD1 (miR398) and CMT3 (miR823) (Fahlgren et al. 2007). Of them, 57 transcripts in ago1-25 and hyl1-2 overlapped, and were equivalent to 63% of the up-regulated transcripts in ago1-25. This result indicated that AGO1 is a major endonuclease center that cleaves target miRNAs (Baumberger and Baulcombe 2005, Ronemus et al. 2006).

Here, we noticed that ago1-25 and hyl1-2 might show different accumulation patterns of some predicted targets regardless of the accumulation levels of the corresponding miRNAs. For example, we predicted that miR156 targets six mRNAs for squamosa promoter-binding family proteins and that miR166 targets three mRNAs for class III HD-Zip family proteins as done previously (Fahlgren et al. 2007). Accumulation patterns of miR156 and miR166 were very similar in ago1-25 and hyl1-2. That is, the accumulation in ago1-25 was similar to that in the wild type, and the accumulation in hyl1-2 was apparently down-regulated (Fig. 2D). However, accumulation of miR156 targets in ago1-25 was higher than that in hyl1-2 (Fig. 4A, B). On the other hand, accumulation of miR166 targets in ago1-25 was lower than that in hyl1-2 (Fig. 4A, B). The accumulation pattern of other miRNA-targeted mRNAs such as a target of miR159, MYB33 (At5g06100) mRNA, was similar to that of miR166 targets (Supplementary Tables S2, S3).

Previous reports showed that some miRNAs, including miR166, were immunoprecipitated with AGO2, AGOS and
AGO7 proteins (Mi et al. 2008, Montgomery et al. 2008, Takeda et al. 2008). These results indicate that some miRNAs act not only with AGO1 protein but also with other AGO proteins. However, it is difficult to conclude that the paradox between the accumulation of miRNA and that of its target is only due to differentiation of incorporation into AGO proteins. One deduced reason is that the amount of the mutated AGO1 proteins might increase in ago1-25, because miR168 targets AGO1 mRNA and, as a result, the activity of AGO1 is supposed to be depressed in the wild type (Vaucheret et al. 2004; Supplementary Tables S2, S3). Alternatively, as ago1-25 is a weak allele among some ago1 mutants, more dynamic changes in the transcriptome might be observed in severe ago1 allele mutants than in ago1-25 as described previously (Vaucheret et al. 2004). In this context, the paradox shown in Fig. 4 might be dissolved using severe ago1 mutants. Thus, diverse effects should build up the transcriptome patterns in the mutant plants.

Previous reports showed that some miRNAs function through translation repression of their targeted mRNAs like animal miRNAs (Chen, 2004, Brodersen et al. 2008). It is not possible to capture any effects on translation from our tiling array data, because they can detect RNA accumulation, but not protein accumulation. Individual analysis at the protein level is necessary to examine the large-scale effect of miRNA-mediated translation repression.

In this study, we supplied whole genome transcriptomes in seedlings of ago1-25 and hyl1-2 mutants using tiling arrays. Our data revealed that defects in the miRNA pathway influenced the accumulation levels of hundreds or thousands of transcripts including pri-miRNAs and miRNA-targeted mRNAs (Figs. 2–4) and that there was a difference in the accumulation patterns of some target mRNAs between ago1-25 and hyl1-2 (Fig. 4). The information presented herein should be useful for future miRNA research and help elucidate the relationship between the miRNA pathway and its effect on transcriptomes.

**Materials and Methods**

The mutants ago1-25 and hyl1-2 (SALK_064863) were as described previously (Morel et al. 2002, Vazquez et al. 2004). Plant growth conditions were as described previously (Kurihara et al. 2008, Kurihara et al. 2009).

The GeneChip Arabidopsis tiling array set (1.0F Array and 1.0R Array, Affymetrix, Santa Clara, CA, USA) was used...
(Zhang et al. 2006). We used 8 µg per array of the total RNA extracted from 15-day-old seedlings for probe synthesis. Probe synthesis, array hybridization and computational analyses of RNA expression were as described previously (Matsui et al. 2008). We used three independent biological replicates for each strand array. The ARTADE-based method was used to detect the expressed genes from the expression data (Toyoda and Shinozaki 2005). We identified the transcripts predominantly up-regulated or down-regulated in the mutants by a modified Mann–Whitney U-test (FDR $\alpha = 0.05$) as described previously (Mann and Whitney 1947, Storey and Tibshirani, 2003).

The Arabidopsis genome annotation used in this analysis was based on the TAIR8 genome version (ftp://ftp.arabidopsis.org/home/tair/Genes/TAIR8_genome_release/TAIR8_functional_descriptions) as of May 5, 2008.

The method used for qRT–PCR was as described previously (Kurihara et al. 2009). The respective pri-miRNA-specific primer sets are listed in Supplementary Table S6.

Total RNA was extracted from 15-day-old seedlings using Isogen reagent (Nippon gene, Chiyoda, Tokyo, Japan), resolved with electrophoresis on a denaturing 15% polyacrylamide gel (7 M urea) in 0.5× TBE buffer and electroblotted onto Hybond-N+ membranes (GE Healthcare, Little Chalfont, UK) in 0.5× TBE buffer. Radiolabeled DNA oligonucleotide probes with antisense sequences against miRNA sequences were constructed by end labeling with $[\gamma-32P]ATP$ using T4 polynucleotide kinase (TOYOBO, Osaka, Japan). Hybridization was performed at 40°C using Perfect Hyb plus hybridization buffer (Sigma, St Louis, MO, USA).

The miRNA population registered in the Arabidopsis small RNA project (ASRP, http://asrp.cgrb.oregonstate.edu/db/microRNaFamily.html, Fahlgren et al. 2007) was used in this study. The miRNA sequences used here were downloaded from the miRBase (http://microrna.sanger.ac.uk/). The miRNA-targeted mRNAs were predicted according to the method described previously (Jones-Rhoades and Bartel 2004). In the miRNA–mRNA pairing, G+U pair, mismatch and gap were scored as 0.5, 1.0 and 2.0, respectively. The mRNAs with a score of <3.0 were regarded as miRNA-targeted mRNA candidates.

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**References**


**Supplementary Material**

Supplementary Table S6.


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