Functional Analysis and RNA Sequencing Indicate the Regulatory Role of Argonaute1 in Tomato Compound Leaf Development

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Abstract

Regardless of whether a leaf is simple or compound, the mechanism underlying its development will give rise to a full comprehension of plant morphogenesis. The role of Argonaute1 (AGO1) in the development of simple leaves has been established, but its role in the development of compound leaves remains to be characterized. In this paper, a virus-induced gene silencing (VIGS) strategy was used to dramatically down-regulate the expression of AGO1 ortholog in tomatoes, a model plant for research into compound leaves. AGO1-silenced tomato compound leaves exhibited morphological defects of leaf adaxial-abaxial and trichome development. Analysis of global gene expression profiles indicated that the silencing of AGO1 in tomato compound leaf caused significant changes in the expression of several critical genes, including Auxin Response Factor 4 (ARF4) and Non-expressor of PR5 (NPR5), which were involved in adaxial-abaxial formation and trichome development. Analysis of global gene expression profiles indicated that the silencing of AGO1 in tomato compound leaf caused significant changes in the expression of several critical genes, including Auxin Response Factor 4 (ARF4) and Non-expressor of PR5 (NPR5), which were involved in adaxial-abaxial formation and trichome development. Collectively, these results shed light on the complicated mechanism by which AGO1 regulates compound leaf development.

Introduction

Leaf development is divided into three continuous main stages: initiation, primary morphogenesis, and secondary morphogenesis. First, the leaf arises at the flanks of the shoot apical meristem (SAM) (initiation stage). Then, the blade is initiated and forms (primary morphogenesis stage). Finally, the leaf area grows substantially, forming a leaf (secondary morphogenesis stage) [1]. Although leaves show great diversity in their shape and size, they are normally divided into two major morphogenetic groups: simple and compound. Simple leaves are each a single unit consisting of a petiole and a blade (lamina). Arabidopsis, tobacco, maize, and rice have simple leaves [2]. During the past decades, simple leaf development has been well characterized at the molecular level, and many genes and pathways have been found to be involved [3]. The morphogenesis stage is longer in compound leaves than in simple leaves, allowing for the initiation of leaflets [1]. The blades of compound leaves are divided into several units called...
leaflets, which are attached to the central leaf rachis and each resembles a simple leaf. The formation of a compound leaf requires the balance of indeterminate and determinate growth at the leaf margin to form a leaflet. In this way, compound leaf development may serve as an attractive model for the study of mechanisms underlying complex morphologies, which specified the location and formation of lateral organs. In the past, the fundamental mechanism of regulation on compound leaf development has been demonstrated at the molecular and physiological levels [1, 4, 5].

AGO1 was first identified as a locus control of leaf development in Arabidopsis, and ago1-1 null mutant exhibited severe developmental abnormalities. Because these unusual mutants look like a small squid, argonaut, they were named argonaute [6]. Genetic research suggested that AGO1 may play a critical role in simple leaf adaxial-abaxial, proximal–distal, and venation patterning [7–9] and in the development of stomatal complexes [9, 10]. AGO1 is a core component of RNA-induced silencing complexes (RISCs) [11, 12]. It is responsible for the gene silencing mediated by microRNA (miRNA) and small interfering RNA (siRNA) [13]. In this way, severe simple leaf phenotype of the ago1 mutant might be due to the disruption of miRNA or siRNA-mediated gene regulation. Mutation of AGO1 in Arabidopsis has been shown to induce striking accumulation of targets of miRNA and siRNA, such as miR165/166 targets (Homeodomain-leucine Zipper III (HD-ZIP III)) [14], miR824 targets (Agamous-like 16 (AGL16)) [10], and trans-acting siRNA (ta-siRNA) targets (ARF3 and ARF4) [15]. HD-ZIP III genes and ARFs play essential roles in simple leaf development [16]. This suggests that AGO1 is critical to regulation of simple leaf development and that it does so mainly via control of levels of targets of miRNAs and siRNAs. The tomato is one of the reference systems for the study of compound leaf development [4]. There are two AGO1 orthologs in tomatoes, SlAGO1-1 and SlAGO1-2 [17]. However, the lack of a tomato ago1 mutant impedes functional understanding of SlAGO1 in compound leaf development. Recently, the function of SlAGO1 was indirectly investigated by Polerovirus silencing suppressor (P0)-mediated SlAGO1 destabilization [17]. Knockdown SlAGO1 at protein level by P0 overexpression resulted in abnormal compound leaves that had filament-like shapes [17]. However, the molecular mechanism that SlAGO1 regulates compound leaf development remains unknown. Here, SlAGO1 expression was directly suppressed in tomato compound leaves by VIGS, which resulted in narrow, wiry leaves. RNA sequencing (RNA-seq) profile analysis indicated that large numbers of genes were differentially expressed in SlAGO1-silenced leaves and some of them were involved in compound leaf morphogenesis. In addition, SlAGO1 might play an important role in trichome formation by regulating hormone-related genes. Collectively, these results shed new light on the complicated mechanism by which SlAGO1 regulates the development of compound leaves.

Materials and Methods

2.1 Plant material and growth conditions

Tomato plants of cultivar Ailsa Craig (AC) were planted in commercial tomato-cultivated soil. All plants were grown in a greenhouse at 22°C with 75% relative humidity under a 16 h light/8 h dark cycle.

2.2 Cloning procedures and vector construction

For the VIGS, the tobacco rattle virus (TRV)-based vectors pTRV1 and pTRV2 were adopted. A pTRV2-SlAGO1 recombinant plasmid was constructed by inserting a 498 bp EcoRI/EcoRI DNA fragment (SlAGO1 fragment) into pTRV2 vector. The SlAGO1 fragment was PCR amplified from tomato cDNA using primers SlAGO1-VIGS For and SlAGO1-VIGS Rev. Oligonucleotide primers used are listed in S3 Table.
2.3 VIGS treatments

Agrobacterium tumefaciens strain GV3101 containing pTRV1, pTRV2 or pTRV2-SlAGO1 vectors were cultured at 28°C in LB medium (pH 5.6) containing 10 mM morpholineethanesulfonic acid and 20 μM acetosyringone with kanamycin, gentamycin, and rifampicin antibiotics. After shaking for 16 h, cultures were harvested and re-suspended in infiltration buffer (10 mM MgCl₂, 200 μM acetosyringone) to a final optical density 600 (O.D. 600) of 1.8. Re-suspensions of pTRV1 and pTRV2 or pTRV2-SlAGO1 were mixed at a ratio of 1:1 and allowed to sit at room temperature for 3 h. Agrobacterium was injected into the cotyledons of seedlings with a 1 mL syringe without a needle. Tomato seedlings injected with pTRV1 and pTRV2 served as controls. Each inoculation was carried out three times and each time six different plants were injected. When VIGS produced a visible phenotype, tomato leaf samples were collected and stored at -80°C for further analysis.

2.4 RNA extraction and PCR

Total RNA was isolated from TRV control and TRV-SlAGO1 leaf samples using DeTRNa reagent (EarthOx, U.S.). The RNA concentration and purity were measured using a NAS-99 spectrophotometer (ATCGene, U.S.). The RNA integrity was checked using agarose gel electrophoresis. Genomic DNA was removed from extracted total RNA by DNase treatment. Then 2 μg total RNA was used for the first-strand cDNA synthesis using a TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (Trans, China) with oligo(dT) primer, TRV RT primer and stem-loop RT primers. PCR was performed using EasyTaq PCR SuperMix (Trans, China) with PCR system T-100 (Bio-rad, U.S.). RT-PCR conditions were as follows: 94°C for 10 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s.

Quantitative PCR (qPCR) was performed using SYBR Green PCR Master Mix with a BIO-RAD PCR System CFX96 (Bio-rad, U.S.). SlAGO1 primers that anneal outside the region targeted for VIGS were used to ensure that only the endogenous SlAGO1 would be detected. qPCR conditions were as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Changes in the fluorescence of SYBR Green were monitored automatically in each cycle, and the threshold cycle (Ct) over the background was calculated for each reaction. The relative expression levels were measured using 2^{-ΔΔCt} analysis [18]. All PCR data presented are representative of three independent experiments. Actin and U6 were used as the internal controls for mRNA and miRNA, respectively. The oligonucleotide primers used in this study are listed in S3 Table.

2.5 RNA-seq and data processing

Total RNA was extracted from the leaves of TRV-SlAGO1 plants and TRV control plants (two biological replicates each). The total RNA samples were treated with DNase I to degrade any possible DNA contaminants. The RNA concentration and purity were measured using a NAS-99 spectrophotometer. RNA integrity was confirmed using agarose gel electrophoresis. Then the mRNA was enriched by using oligo (dT) magnetic beads. RNA libraries with inserts approximately 250–500 bp in size were prepared (BGI, China) for 50 bp single-end sequencing on the Illumina HiSeq 2000, at a depth of ≈50 million reads per library (for statistics on read counts and percentage of mapped reads, see Table 1). All RNA-seq data for this article have been deposited at the National Center for Biotechnology Information Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra/) under accession number SRP043486.

RNA-seq reads were checked for quality and trimmed to removing barcode and adaptor sequences. To rule out rRNAs and tRNAs, all reads were aligned to plant rRNA and tRNA sequences using the Short Oligonucleotide Analysis Package (SOAP2, http://soap.genomics.
org.cn/soapaligner.html). Plant rRNAs and tRNAs information were extracted from the NCBI Non-Redundant (NR) Dataset (http://www.ncbi.nlm.nih.gov/). The cleaned reads were then aligned to the tomato reference genome (SGN release version SL2.50, ftp://ftp.sgn.cornell.edu/tomato_genome) using TopHat [19]. The raw counts of aligned reads for each tomato gene model and from each library were derived using an in-house Perl script. Differentially expressed genes (DEGs) between TRV-SlAGO1 and TRV leaf samples were identified using the DESeq package [20]. Genes exhibiting a |fold-change| ≥2 and adjusted P-value <0.05 were selected for DEGs.

2.6 Gene Ontology (GO) enrichment analysis

Because GO terms were not well annotated for tomato genes, the corresponding Arabidopsis best homologues were collected and the WEGO (http://wego.genomics.org.cn/) was used for GO enrichment. GO enrichment analysis provided all GO terms that significantly enriched in DEGs relative to the genome background and filtered the DEGs corresponding to biological processes, cellular components, and molecular functions.

2.7 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis

Pathway enrichment analysis identifies enriched metabolic pathways and signal transduction pathways based on comparison of DEGs to the whole genome background. KEGG is the major public pathway-related database. DEGs were entered into KEGG for analysis (http://www.genome.jp/kegg/).

Results

3.1 Repression of SlAGO1 led to the abnormality of compound leaves

The severe growth arrest in ago1 null mutants prevents functional characterization of AGO1 at later stages of leaf development. VIGS is one of the most powerful tools for the analysis of key genes whose mutations cause embryonic and seedling-lethality [21]. The TRV-based VIGS vector is widely used for efficient silencing of genes in tomato plants [22–24]. Considering that there are two AGO1 homologues in tomatoes, a DNA fragment of almost 500 bp that was found to be common to both SlAGO1-1 and SlAGO1-2 was cloned and inserted into pTRV2 vector, named pTRV2-SlAGO1. In order to silence SlAGO1, Agrobacterium cultures containing pTRV2-SlAGO1 and pTRV1 were injected into the cotyledons of one-week-old tomato seedlings (S1 Fig). Cultures of pTRV2 and pTRV1 served as controls.

After four weeks, all the seedlings injected with TRV-SlAGO1 exhibited a range of phenotypic abnormalities that did not shown in TRV-injected control plants. Typically, most leaflets of upper un-injected compound leaves were small, narrow, asymmetrical, and curled.

| Library       | Raw reads   | Total Mapped Reads | Perfect Mapped Reads | Unique Mapped Reads |
|---------------|-------------|--------------------|----------------------|---------------------|
| TRV-SlAGO1-1  | 58,577,976(100%) | 43,421,744(74.13%) | 33,974,546(58.00%)  | 41,463,159(70.78%)  |
| TRV-SlAGO1-2  | 58,409,202(100%) | 42,939,331(73.51%) | 36,609,869(62.68%)  | 41,017,409(70.22%)  |
| TRV-1         | 50,565,659(100%) | 37,656,833(74.47%) | 29,560,551(58.46%)  | 36,080,591(71.35%)  |
| TRV-2         | 61,363,163(100%) | 45,747,071(74.55%) | 39,167,949(63.83%)  | 43,850,729(71.46%)  |

1, 2, biological replicates.

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downward. Some of them lacked petiolules (Fig 1A–1H). Most of them lost their adaxial–abaxial polarity and had almost smooth margins (Fig 1H). Some TRV-SlAGO1 leaflets were nearly radial and resembled shoestrings, and their midribs were outgrown or detached (Fig 1H and 1I). Moreover, dark green flecks appeared on the abaxial side of some tomato leaflets which
were injected with TRV-SlAGO1 (Fig 1K–1M). In addition, midribs of TRV control leaflets were concave on the adaxial surface whereas those of TRV-SlAGO1 were concave on the abaxial surface (Fig 1N and 1Q). Observed under a scanning electron microscope (SEM), cells of the “shoestring” leaf were columnar in shape, resembling those of a midrib and were distinct from the irregular polygon of TRV control leaf cells (Fig 1O and 1R). SEM analysis also showed that dark green fleck had denser trichomes and glossier surface, which more closely resembled the adaxial side (Fig 1P and 1S, Fig 2).

Since most upper un-injected leaves showed severe phenotype, suggesting that the recombinant TRV-SlAGO1 virus moved from cotyledons to upper leaves and induced gene silencing. TRV2 specific primers were used for both TRV control and TRV-SlAGO1 plants. The different sizes of amplified fragments clearly indicated that both TRV and recombinant TRV-SlAGO1 can efficiently replicate and spread systemically in tomato plants and that the abnormal leaves were caused by TRV-SlAGO1 and not TRV itself (Fig 3A). qPCR analysis indicated that levels of SlAGO1-1 and SlAGO1-2 were suppressed by 95% and 84%, lower in TRV-SlAGO1 than in the TRV control, respectively (Fig 3B). Recently, impairing the biogenesis of ta-siRNAs and misregulating ARF3 and ARF4 in tomato ago7 mutant was found to cause a gradient of leaf-shape aberrations, such as shoestring leaves lacking leaf blade expansion [25], resembling the TRV-SlAGO1 phenotypes. However, the SlAGO7 transcript levels in TRV control and TRV-SlAGO1 injected plants were comparable (Fig 3C). Collectively, these results indicate that the specific silencing of SlAGO1 by VIGS was responsible for the abnormal phenotype of tomato compound leaves.

### 3.2 Global overview of RNA-seq profiles of SlAGO1-silenced compound leaf

To gain insights into the molecular impact of SlAGO1 silencing on compound leaf development, gene expression patterns of SlAGO1-silenced leaves and TRV control leaves were compared using RNA-seq. Two RNA libraries, representing two biological replicates, were prepared and sequenced, generating more than 50 million raw reads, respectively (Table 1). In TRV control and TRV-SlAGO1 plants, a total of 22,773 and 23,116 tomato genes, respectively, were found to be expressed. DESeq analysis showed 1,467 genes to be significantly differentially expressed between TRV-SlAGO1 and the TRV control. Compared with TRV control, 1,051 genes were up-regulated in TRV-SlAGO1, and 416 genes showed dramatic down-regulation (Fig 4A, S1 Table). To determine whether RNA-seq data were reliable, 17 of the DEGs were randomly selected and their expression levels were checked using qPCR. The fold change in the DEGs expression levels of qPCR and RNA-seq was very similar (R² = 0.88, P < 0.001) (Fig 4B).

GO analysis revealed that DEGs covered a wide range of biological processes, cellular components, and molecular function, which could be classified into 21, 10, and 8 categories. Among biological processes, the largest group was metabolic process (19.1%), followed by cellular processes (19%). The cellular component genes were placed mainly in the cell category (29%) and cell part category (29%). In terms of the number of genes, the two largest groups within the molecular function category were catalytic (45%) and binding (36%), making up four-fifths of all the DEGs (Fig 4C). Pathway enrichment analysis was accomplished using the Kyoto Encyclopedia of Genes and Genomes (KEGG), aiming at collecting genes involved in the same biological pathway. These DEGs were involved in as many as 109 pathways, of which the top 15 were shown in Fig 5, including metabolic pathways, biosynthesis of secondary metabolites, and plant hormone signal transduction.
3.3 SiAGO1 influenced expression pattern of tomato genes involved in polarity development

Because TRV-SiAGO1 leaves showed severe adaxial-abaxial abnormalities (Fig 1) and AGO1 was responsible for small RNA-induced gene silencing, it is here hypothesized that the expression levels of a series of miRNA and siRNA target genes, which were involved in the determination of polarity development, were affected by the silencing of SiAGO1. The expression levels of these genes were measured by RT-PCR and qPCR analyses. The results showed that the expression levels of several genes were significantly changed in TRV-SiAGO1 plants compared to TRV control plants (Fig 3).
leaf polarity, might be affected by SIAGO1 knockdown. ARF4 is the target of ta-siRNA3, which participates in construction of the abaxial sides of leaves [26]. ARF4 expression was higher in SIAGO1-silenced plants than in TRV control plants (Fig 6A).

Because the polarity disorder phenotype of our TRV-SIAGO1 plants was reminiscent of rachis-like structure of Arabidopsis BLADE-ON-PETIOLE (bop) mutant in which BOP was found to participate in polarity development [27], the transcription level of NPR5, which is the ortholog of BOP in tomatoes, was checked. As expected, NPR5 level decreased by 90% relative to the TRV control (Fig 6B).

The genes in the HD-ZIP III family are targets of miR166/165, including REVOLUTA (REV), CORONA (CNA), PHAVOLUTA (PHV), PHABULOSA (PHB) and HOMEBOX
### Fig 5. Pathway enrichment analysis of differentially displayed genes from TRV-SlAGO1 plants.

| Pathway                                      | Gene_number | Qvalue |
|----------------------------------------------|-------------|--------|
| Stilbenoid, diaryheptanoid and gingerol biosynthesis | 50          | 0.04   |
| Starch and sucrose metabolism                | 100         | 0.03   |
| Plant-pathogen interaction                   | 150         | 0.02   |
| Plant hormone signal transduction            | 200         | 0.01   |
| Pheny/propanoid biosynthesis                 | 250         |        |
| Phenylalanine metabolism                     |             |        |
| Metabolic pathways                           |             |        |
| Glycerophospholipid metabolism               |             |        |
| Flavonoid biosynthesis                       |             |        |
| Ether lipid metabolism                       |             |        |
| Diterpenoid biosynthesis                     |             |        |
| Cutin, suberine and wax biosynthesis         |             |        |
| Carotenoid biosynthesis                      |             |        |
| Biosynthesis of secondary metabolites        |             |        |
| Amino sugar and nucleotide sugar metabolism  |             |        |

RichFactor: 0.00 0.02 0.04 0.06 0.08 0.10 0.12 0.14 0.16

**Fig 6. qPCR analysis of genes related to leaf development.** (A) *ARF4* showed higher expression levels in SlAGO1-silenced leaves than in controls. (B) *NPR5* were down-regulated in TRV-SlAGO1 control leaves. (C) Target genes of miR166 displayed no significant changes when injected with TRV-SlAGO1. The error bar indicates the standard deviation of three biological replicates. Asterisks indicate significant difference as determined using the student’s t-test (*, P < 0.01).

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GENE 8 (HB8), which govern the formation of the adaxial side of simple leaves. In Arabidopsis ago1 mutants, PHB, PHV, and REV expression levels are increased throughout the leaf [8, 28]. Surprisingly, no significant differences in the accumulation of PHV and REV were observed between SlAGO1-silenced tomato plants or in controls plants (Fig 6C), suggesting that the regulation mechanism of HD-ZIP III family in compound leaf development remained to be characterized.

The accumulation of mature miRNA was also analyzed (Fig 7). Their relative expressions showed various changes. miR159, miR164, miR168, and miR396 declined significantly and miR390 rose visibly. miR156 and the abovementioned miR166 exhibited no visible changes. One possible reason for this might be that AGO1 was involved in miRNA-mediated RNA degradation and did not participate in miRNA biogenesis directly. In this way, miRNAs displayed diverse patterns of expression in SlAGO1-silenced leaves.

3.4 Genes involved in trichome development are affected in SlAGO1-silenced lines

There were denser and longer trichomes on the surfaces of in SlAGO1-silenced leaves than on those of TRV control leaves (Fig 8A–8H). Evidence has suggested that auxin and gibberellin (GA) are involved in trichome development [29, 30]. RNA-seq and KEGG pathway analysis have suggested that many DEGs are clustered in auxin and GA metabolism when SlAGO1 is silenced (Fig 8I and 8J). For these reasons, it is here hypothesized that the anomalous trichomes on SlAGO1-silenced leaves are the result of abnormal hormone response. The expression level of IAA15, whose down-regulation would cause the glabrous phenotype, was assessed [30]. It reached 2.3 times control levels in SlAGO1-silenced plants (Fig 8K). This result indicated that SlAGO1 might affect its expression level, which was involved in trichome development. We also analyzed the expression level of GAI, which was involved in GA pathway [29]. The relative...
expression level of GAI was 4.6 times higher in TRV-SlAGO1 plants (Fig 8K), suggesting that silencing of SlAGO1 affected hormone regulation.

We made a table to compare the orthologous genes from Arabidopsis, rice and maize with those of tomato which were identified in this paper (S2 Table). Although, roles of all these orthologous genes from other species have not been clearly established yet, the comparison will help to identify orthologous genes of other species for controlling the processes of leaf development and hormone regulation.
Discussion

4.1 S/AGO1 can be silenced efficiently by VIGS

VIGS is widely used to identify gene function because it is rapid, reliable, transformation-free, and easy to perform [31, 32]. It can also repress endogenous genes through inoculation of a recombinant virus carrying a specific fragment of the host gene [33]. When the virus infects plants and spreads systemically throughout them, the endogenous gene transcripts, which are homologous to the insert fragment in the virus vector, are degraded by post-transcriptional gene silencing (PTGS) [34]. Mi et al. supposed that VIGS was compromised in the ago1 mutant, with greatly reduced photo bleaching when injected with TRV-PDS [33]. However, the level of endogenous SIAGO1 gene decreased strikingly in response to VIGS, resulting in unusually shaped leaves (Figs 1 and 2). It is here reasoned that, although AGO1 plays pivotal roles in VIGS, other AGOs might also supplement it. For instance, AGO1 and AGO2 have redundant roles in miR408-mediated plantacyanin regulation [35]. In a recent study, Shao et al. used high-throughput sequencing to search small RNAs that enriched in the four AGO proteins, AGO2, AGO5, AGO7, and AGO10. They discovered that AGO2 and AGO5 might be implicated in the miRNA mediated pathway [36]. In tobacco, AGO1 and AGO4 were found to cooperate with VIGS to achieve extensive systemic silencing [37]. All of the evidence given above shows that other AGO proteins might act as substitutions of AGO1 in VIGS. The exact roles of other AGOs in VIGS remain to be determined and will require intensive study. However, it is clear that AGO1 can be silenced by VIGS, at least in tomato plants. This sheds new light on the rapid and functional analysis of AGO family by VIGS.

4.2 Common factors played distinguish roles between simple and compound leaf polarity

Morphogenesis of simple and compound leaves is regulated by distinct genetic mechanisms with shared common key factors [4, 38]. Class I KNOTTED1-LIKE HOMEBOX (KNOX1) transcription factor is one of the most important factors for SAM development in Arabidopsis and maize, whose expression is restricted to the SAM [39]. However, in tomato plants, the KNOXI genes Tomato KNOTTED1 (TKN1) and TKN2 expression is restored in developing primordial leaves [40]. This mainly affects tomato leaf maturation [41].

In Arabidopsis, genes of the HD-ZIP III family are critical to adaxial-abaxial formation, which are targets of miR166/165 [14]. Overexpression of CNA resulted in radialized and trumpet-shaped leaves in Arabidopsis [42]. Both gain-of-function mutants phb and phv led to leaf polarity defects and the formation of meristems in ectopic positions [43], and similar mutations in the REV gene caused polarity disorders in leaves [44]. In this study, SIAGO1-silenced leaves showed disorganized adaxial-abaxial sides, or even loss of polarity, failing to form a blade and developing with radial symmetry (Figs 1H and 8F). However, surprisingly, the transcription expression of HD-ZIP III family genes were not increased in the case of SIAGO1 knockdown but rather unaffected (Fig 6C). There have been few reports that focused on the functions of HD-ZIP III family in tomato leaf construction so far. Hu et al. overexpressed REV in tomato and the plants produced leaves whose edges curled upward [45], but there was no effect on polarity development. P0-mediated SIAGO1 degradation did not result in any change in the level of REV expression [17]. This was consistent with current results showing that REV had no visible differences in TRV-SIAGO1 leaves relative to TRV control leaves (Fig 6C). It is possible that SIAGO1 might regulate HD-ZIP III family genes by translation inhibition [46].
4.3 Diverse regulation of SlAGO1 on target genes of small RNA

AGO1 is involved in the regulation of gene expression via RISCs [47]. In general, target genes of small RNAs, such as ARF4, the target of ta-siRNA, are up-regulated in SlAGO1-silenced plants, in which SlAGO1 acts as a negative regulator (Fig 5). The accumulation of small RNAs may contribute to disorganized polarity in TRV-SlAGO1 leaves. There are also many miRNA target genes that manifesting no significant change in SlAGO1-silenced plants (S2 Fig). These results were consistent with those reported by Martienssen et al., who found that only 1 to 6% of genes displayed significant expression changes in Arabidopsis ago1 mutant in microarrays, in which several known miRNA targets increased remarkably, whereas others showed little or no change [48]. Growth Regulating Factor 1 (GRF1), which was regulated by miR396 and may reach higher levels in SlAGO1-silenced plants showed a marked decrease (S1 Fig) [33]. The phenotype of TRV-SlAGO1 leaflets was similar with those of GRFs loss-of-function mutants, which were smaller than control ones [49]. For this reason, it is here speculated that GRF1 might be controlled by other factors that played more substantial roles than SlAGO1. In addition, AGO1 might partially mediate translational repression of some of the targets of small RNAs [50].

Supporting Information

S1 Fig. Schema of VIGS treatment in tomato cotyledon. Here, 498 bp of SlAGO1 fragment was placed in the vector of pTRV2. The plasmids of pTRV1, pTRV2-SlAGO1 were transferred into Agrobacterium strain GV3101 separately, mixed at a 1:1 ratio, and injected into the cotyledon of tomato. LB: left border of T-DNA; CP: TRV coat protein; Rz: self-cleaving ribozyme; RdRP: RNA-dependent RNA polymerase; MP: movement protein; 16K: 16 kD cysteine-rich protein; RB: right border of T-DNA. The location of TRV2 specific primers is also shown. (TIF)

S2 Fig. qPCR analysis of miRNA-target transcripts. Some miRNA target genes showed no significant differences from inTRV-SlAGO1 plants. The error bar indicates the standard deviation of three biological replicates. (TIF)

S3 Fig. qPCR analysis of GRF1. The error bar indicates the standard deviation of three biological replicates. Asterisks indicate significant difference as determined by the student’s t-test (*, P < 0.01). (TIF)

S1 Table. Differentially expressed genes of RNA-seq. (XLSX)

S2 Table. Orthologous genes from tomato, Arabidopsis, rice and maize. (XLSX)

S3 Table. Oligonucleotide primers used in the study. (XLSX)

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Author Contributions
Conceived and designed the experiments: TW RL HZ DF. Performed the experiments: TW RL LW. Analyzed the data: TW HZ. Contributed reagents/materials/analysis tools: BZ YL DF. Wrote the paper: TW HZ.

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