PLOS ONE

RESEARCH ARTICLE

An artificial miRNA system reveals that relative contribution of translational inhibition to miRNA-mediated regulation depends on environmental and developmental factors in *Arabidopsis thaliana*

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Citation: von Born P, Bernardo-Faura M, Rubio-Somoza I (2018) An artificial miRNA system reveals that relative contribution of translational inhibition to miRNA-mediated regulation depends on environmental and developmental factors in *Arabidopsis thaliana*. PLoS ONE 13(2): e0192984. https://doi.org/10.1371/journal.pone.0192984

Editor: Sebastien Pfeffer, Institut de Biologie Moleculaire et Cellulaire, FRANCE

Received: October 16, 2017

Accepted: February 1, 2018

Published: February 16, 2018

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: Work at the Max Planck Institute in the Department of Molecular Biology was supported by the Max Planck Society and DFG SFB1101. I.R.-S. is supported by the Spanish Ministry of Economy and Competitiveness (BFU2014-58361-JIN, RYC-2015-19154) and through the "Severo Ochoa Programme for Centres of Excellence in

Abstract

Development and fitness of any organism rely on properly controlled gene expression. This is especially true for plants, as their development is determined by both internal and external cues. MicroRNAs (miRNAs) are embedded in the genetic cascades that integrate and translate those cues into developmental programs. miRNAs negatively regulate their target genes mainly post-transcriptionally through two co-existing mechanisms; mRNA cleavage and translational inhibition. Despite our increasing knowledge about the genetic and biochemical processes involved in those concurrent mechanisms, little is known about their relative contributions to the overall miRNA-mediated regulation. Here we show that co-existence of cleavage and translational inhibition is dependent on growth temperature and developmental stage. We found that efficiency of an artificial miRNA-mediated (amiRNA) gene silencing declines with age during vegetative development in a temperature-dependent manner. That decline is mainly due to a reduction on the contribution from translational inhibition. Both, temperature and developmental stage were also found to affect mature amiRNA accumulation and the expression patterns of the core players involved in miRNA biogenesis and action. Therefore, that suggests that each miRNA family specifically regulates their respective targets, while temperature and growth might influence the performance of miRNA-dependent regulation in a more general way.

Introduction

Control of gene expression is paramount for any organism in order to exist and transit through different developmental stages as well as to interrelate with their surroundings during

R&D" 2016-2019 (SEV-2015-0533) and the CERCA programme from the Generalitat de Catalunya.

Competing interests: The authors have declared that no competing interests exist.

their life cycle. All layers controlling gene expression are tightly regulated, from chromatin state to protein post-translational modifications, including mRNA stability. Small RNAs (sRNAs) have emerged in the last decades as central elements embedded in those regulatory layers. sRNAs come in several flavors depending on the source of RNA used for their biogenesis [1]. MicroRNAs (miRNAs) are a special class of sRNAs that mainly regulate the expression of their targets post-transcriptionally. miRNA-dependent regulation has evolved independently in at least six eukaryotic lineages, including land plants [2]. Most of the current knowledge about plant miRNA biogenesis, action and function comes from studies in the model organism *Arabidopsis thaliana*. Primary miRNA transcripts (pri-miRNA) arise from the RNA polymerase II-dependent expression of independent transcriptional units. Their expression pattern is under the control of specific regulatory sequences as is the case for protein coding genes [3]. Pri-miRNAs are processed by the microprocessor complex in mature miRNA duplexes ranging from 19 to 24 nt at the dicing bodies within the nuclei in a two-step enzymatic reaction [4]. Proteins from the DICER family, mainly DICER-LIKE1 (DCL1; [5]) are the core components of the microprocessor complex and are assisted by accessory proteins such as HYPONASTIC LEAVES1 (HYL1; [6]) or DOUBLE-RNA BINDING PROTEIN 2 (DRB2; [7]), SERRATE (SE; [8]) and C-TERMINAL DOMAIN PHOSPHATASE-LIKE 1 (CPL1; [9]). The resulting mature miRNA duplexes are subsequently protected from degradation through HUA-ENHANCER 1 (HEN1)-mediated methylation [5]. Next, HASTY (HST; [10]) is thought to participate in the transport of the stabilized miRNA duplexes to the cytoplasm where they are loaded into the RNA-Induced Silencing Complex (RISC). Proteins from the ARGONAUTE (AGO) family are the main executive components of the RISC complex. The Arabidopsis genome has 10 *AGO* genes of which AGO1 [11] and AGO10 are considered the main players in post-transcriptional miRNA-mediated gene silencing [12]. Once loaded into the RISC, one of the two duplex strands is degraded while the remaining one serves to scan the cytoplasm seeking for highly complementary mRNAs. miRNAs control the expression of their targets both by mRNA-target cleavage and translational inhibition [12]. Beyond their co-existence, knowledge about the overall contribution of both mechanisms in plants is scarce and suggests that it might be cell-type specific [13]. Noteworthy, the implications of both regulatory mechanisms on miRNA-mediated regulation are critical for its dynamics. While mRNA cleavage and degradation is a non-reversible process, it is thought that translational inhibition might be reverted allowing for a rapid expression of its repressed targets [14].

Plant miRNAs are involved in the regulation of a series of developmental and stress-related genetic programs [15, 16]. Nevertheless, little is known about whether general miRNA biogenesis and action, or the efficiency of their regulation vary during the course of development and/or as consequence of environmental changes. Initial attempts of dealing with such a gap relied on assaying changes of endogenous miRNAs [17, 18]. A major drawback from those studies is that mature miRNAs are usually produced from polygenic families and their accumulation is driven by distinct chromatin modifications, promoter activity and pri-miR structure [19–21]. In addition, most of those studies assessed whole plants sampled at the same time regardless the growth temperature. Arabidopsis developmental timing is temperature-dependent, therefore the significance of their findings might be limited [22].

In order to circumvent such limitations and clearly discern how those parameters might influence miRNA performance, we used an artificial and highly sensitive miRNA reporter system and discrete and equivalent developmental points covering the entire plant life cycle [9]. Our results show that accumulation of mature artificial miRNA (amiR-LUC) and the resulting regulation (mechanism and efficiency) of its target depend on growth temperature and developmental stage. We also show that both factors affect the expression of several key players involved both in miRNA biogenesis and action.

The mechanisms of miRNA-mediated attenuation of gene expression have been harnessed to silence specific genes with amiRs [23, 24]. Therefore, our findings are not only relevant for understanding miRNA regulation, but also instructive for the use of amiR-based gene silencing technology.

Results

Addressing developmental and environmental impact on miRNA-mediated regulation

RNA silencing has been described as an antiviral defense mechanism in both plants and invertebrates [25]. Such defense mechanism is temperature sensitive with higher temperatures leading to increased production of virus-derived sRNAs [26]. In order to study whether miRNA-mediated silencing is also under the influence of environment and/or development, we used an artificial miRNA reporter system that proved to be highly sensitive to perturbations in miRNA biogenesis and action [9]. This reporter system relies on the expression of the Firefly luciferase gene (*LUC*) under the constitutive Cauliflower Mosaic Virus 35S promoter. Simultaneously, the expression of an artificial miRNA (*amiR-LUC*) driven by the very same promoter, specifically silences *LUC* expression. As control, we used a similar reporter system in which synonymous point mutations were introduced within the miRNA-complementary sequence in the *LUC* gene. Those point mutations rendered the LUC mRNA resistant (*rLUC*) to amiR-LUC regulation [27]. Using this artificial approach has clear advantages compared to relying on endogenous miRNAs. Among those advantages is that the production of both miRNA and target are controlled by the same promoter and can be related at all growth conditions and developmental stages to the proper control allowing a fine dissection of all steps of the regulation.

Arabidopsis plants carrying either the *LUC* or *rLUC* reporter systems were grown along at 16˚C and 23˚C. 16˚C is closer to the temperatures Arabidopsis typically experiences in its normal habitats, while 23˚C, despite being commonly used for Arabidopsis growth in controlled chambers, can be considered a stress temperature. Since the speed of Arabidopsis growth is temperature-dependent [22], we established discrete and equivalent time points to collect representative samples spanning the main developmental stages at both temperatures (Fig 1A). Seedlings with the two first true leaves and leaves number 4 and 7 are representative of the transitions from juvenile to adult stages during vegetative development (Fig 1B, [28]). We also assessed inflorescences containing all closed buds (stages 1 to 12 [29]) and pools of the three uppermost siliques after abscission of the senescent floral organs. Levels of the developmental timer miR156 were used to validate the equivalence of the samples collected at the two different growth conditions [30–32]. As expected, miR156 accumulation declined as development progressed confirming that both sets of samples were developmentally equivalent (Fig 1C) [17, 19].

Mature miRNA accumulation has developmental and temperature-dependent components

To study accumulation of mature amiR-LUC, we assayed amiR levels by stem-loop qRT-PCR (Fig 2A) and small RNA blots (Fig 2B).

Independent of growth temperature, amiR-LUC accumulated to higher levels in seedlings than at later stages during vegetative development, *i.e.* leaves 4 and 7 (Fig 2B). Moreover, amiR-LUC levels were higher in siliques and inflorescences at 23˚C when compared to vegetative organs (Fig 2A, Fig 2B).

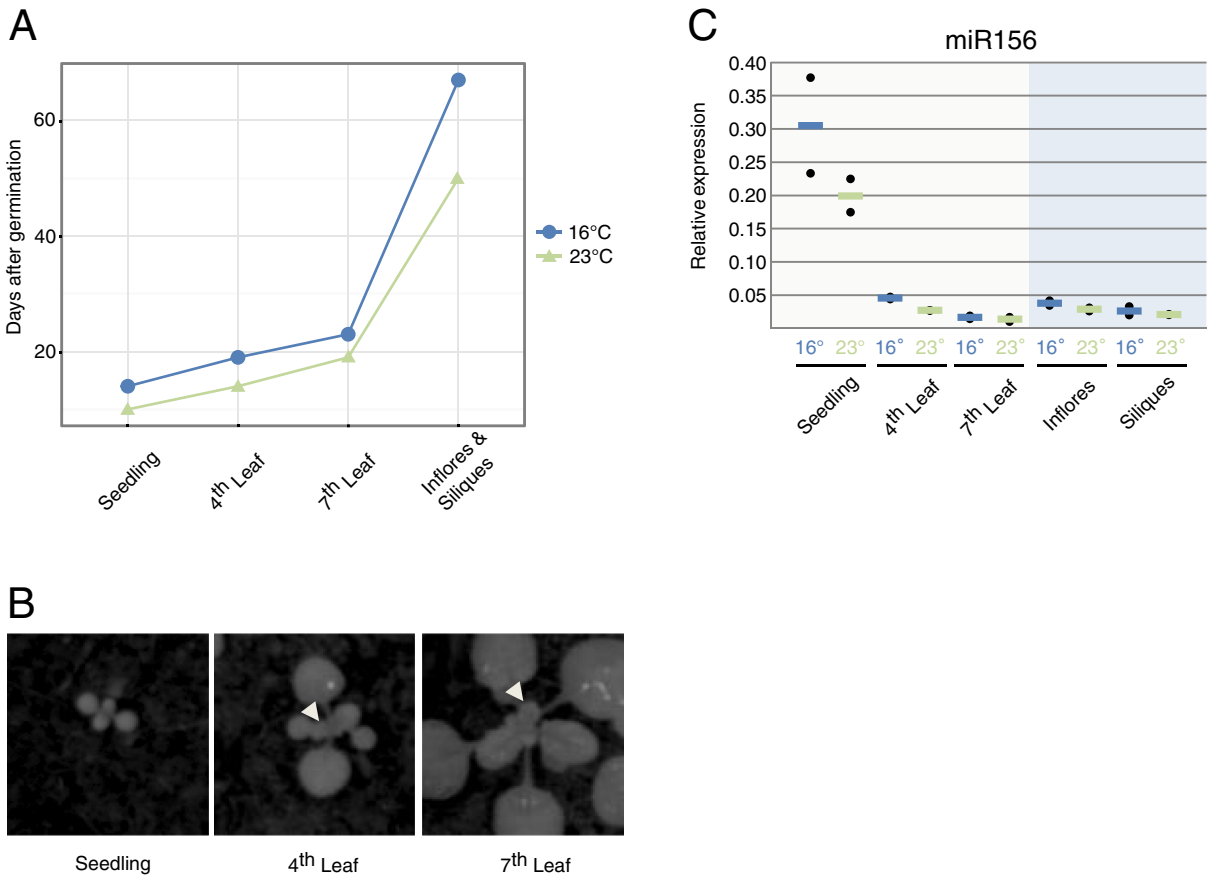

Fig 1. Addressing developmental and environmental influence on miRNA-mediated regulation via a luciferase reporter system. (A) Discrete time points for tissue collection over Arabidopsis life cycle at 16˚C (blue) and 23˚C (green). (B) Representative pictures of the different leaf stages collected spanning vegetative development. Arrows point to the collected leaves. (C) Mature miR156 qRT-PCR to ensure that samples from both datasets (16˚C and 23˚C) were at equivalent developmental points. Black dots represent one biological replicate each, calculated from two technical replicates. Lines, (blue = 16˚C, green = 23˚C) represent the average between two biological replicates. "Inflores" stands for inflorescences.

https://doi.org/10.1371/journal.pone.0192984.g001

Higher temperature was found to increase amiR-LUC levels in late vegetative development (leaf 7) and especially in inflorescence (Fig 2A, Fig 2B).

Discrepancies found between the amiR-LUC levels determined either by qRT-PCR or small RNA blot might be explained by the intrinsic properties of both techniques (Fig 2A, Fig 2B). While stem-loop qRT-PCR monitors only the 21nt long species matching the designed amiR-LUC, small RNA blots can detect isoforms of different length and/or isoforms shifted by a few nucleotides [9].

A simple reason for miRNA accumulation being temperature and stage-dependent could be differential expression of factors involved in miRNA production. We therefore assayed whether the expression of core factors involved in miRNA biogenesis was regulated by development and/or growth temperature. We focused on the core executor DCL1 and in its assistants HYL1, DRB2, SERRATE and CPL1 (Fig 3).

DCL1 mRNA expression levels were similar across all samples, with the exception of a marked increase in inflorescences from plants grown at 23˚C compared to their counterparts grown at lower temperature (Fig 3A).

Regarding to DCL1 helpers, we found that high temperatures (23˚C) led to decreased *HYL1* levels at late vegetative development (leaf 7) compared to 16˚C (Fig 3B). *DRB2* was expressed

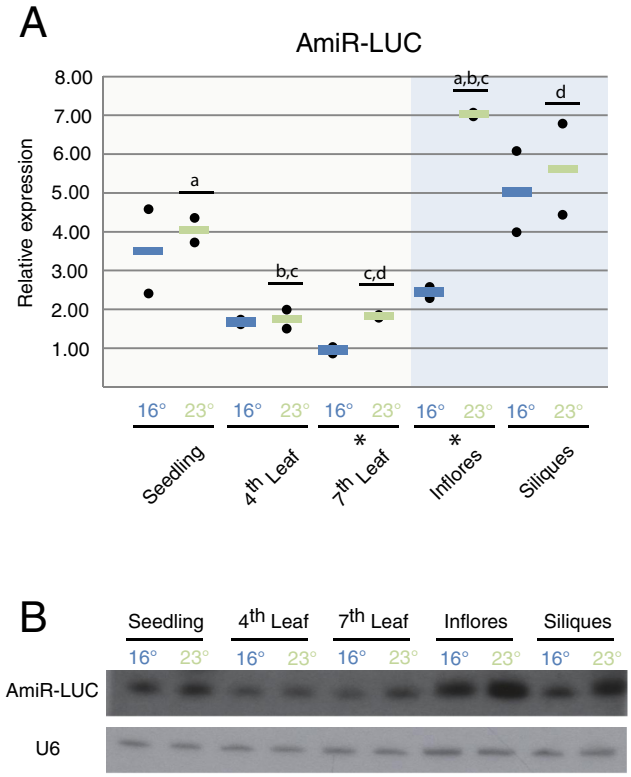

Fig 2. AmiR-LUC accumulation is developmentally and temperature-dependent. *(A) Mature amiR-LUC accumulation assayed by qRT-PCR. Black dots represent one biological replicate each calculated from two technical replicates. Lines, (blue = 16˚C, green = 23˚C) represent the average between two biological replicates. "Inflores" stands for inflorescences. (B) Representative sRNA blot for amiR-LUC accumulation.* * shows tissues in which temperature has a significant effect in a pairwise comparison ($p<0.05$). Letters and lines show significant differences between tissues in ANOVA-test after Tukey correction (adjusted $p<0.05$).

https://doi.org/10.1371/journal.pone.0192984.g002

at higher levels in vegetative tissues from plants grown at 16˚C when compared to inflorescences, while the opposite was true when grown at 23˚C (Fig 3C). Likewise, *SE* was more highly expressed in vegetative than in reproductive organs in plants grown at 16˚C (Fig 3D).

Plants grown at 16˚C presented the same trend of lower levels of *CPL1* expression in vegetative tissues that was also found for *DRB2* (Fig 3E). Collectively, our results show dynamic and heterogeneous expression profiles of different members of the core miRNA biogenesis machinery. We observed little correlation between these patterns and the accumulation of mature amiR-LUC across the different samples with the only exception of inflorescences from plants grown at 23˚C. When compared to plants grown at 16˚C, higher levels of amiR-LUC were paralleled by higher levels of *DCL1 and DRB2*. It is noteworthy that for most of the miRNA biogenesis co-factors, we observed a general tendency to higher expression levels in vegetative organs from plants grown at 16˚C compared to what was found in reproductive organs.

Efficiency and mode of action of miRNA-mediated regulation is temperature dependent

Once we had established that development and temperature affect the accumulation of mature miRNAs, we sought to explore whether miRNA-mediated gene silencing was also developmentally and environmentally regulated.

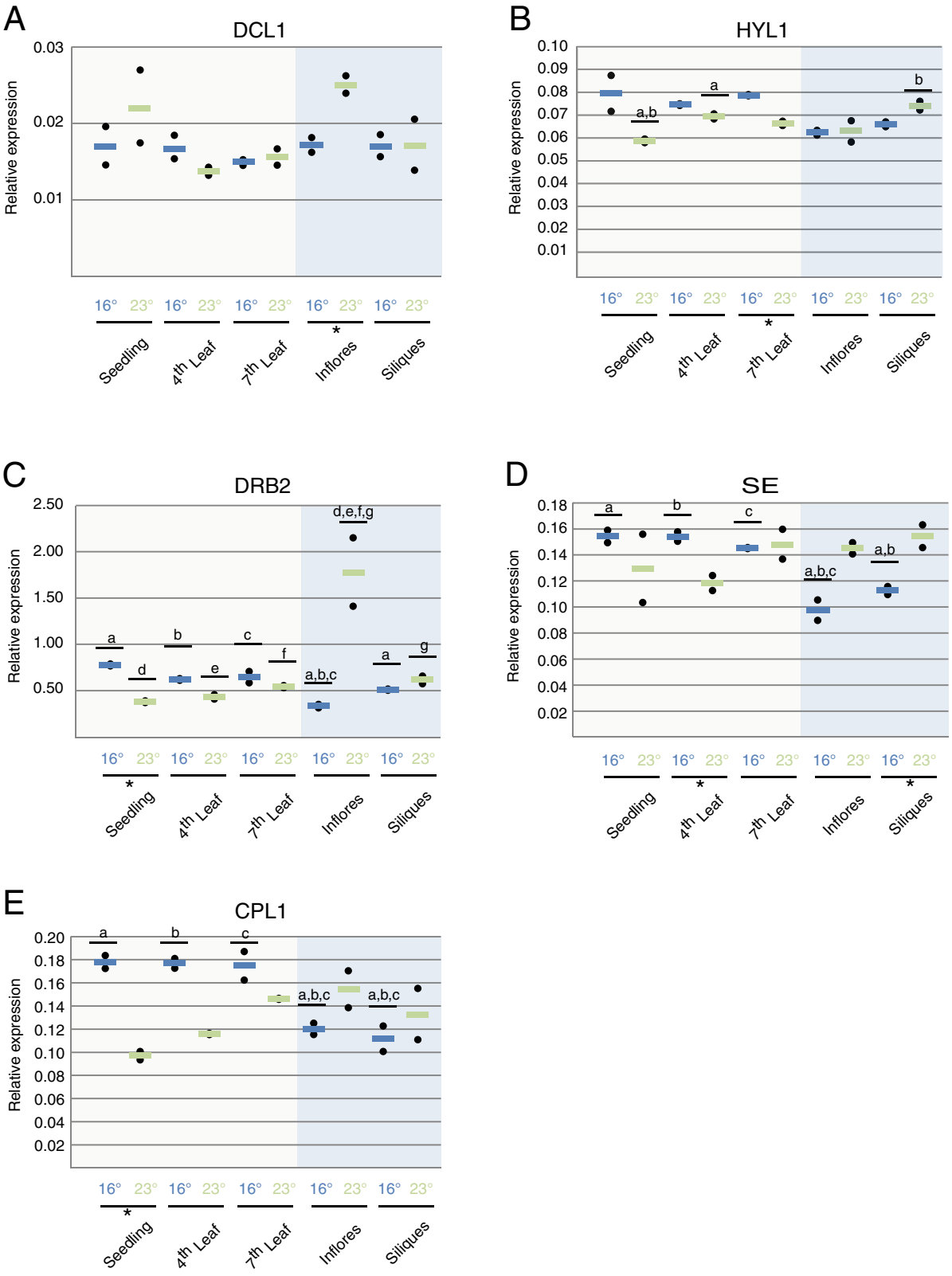

Fig 3. Effect of development and temperature on the expression of miRNA biogenesis factors. *(A) DCL1. (B) HYL1. (C) DRB2. (D) SE. (E) CPL1. Black dots represent one biological replicate each calculated from two technical replicates. Lines, (blue = 16˚C, green = 23˚C) represent the average between two biological replicates. "Inflores" stands for inflorescences.* * shows tissues in which temperature has a significant effect in a pairwise comparison ($p<0.05$). Letters and lines show significant differences between tissues in ANOVA-test after Tukey correction (adjusted $p<0.05$).

https://doi.org/10.1371/journal.pone.0192984.g003

We firstly assayed the contribution of target cleavage regulation in response to different growth temperatures and across development. *LUC* mRNA levels were assayed in the same samples used for qRT-PCR and with primers flanking the miRNA-targeted sequence. *LUC* levels were reduced by 60 to 85% when compared to *rLUC* depending on tissue and growth conditions (Fig 4A). We found that higher levels of mature amiR-LUC (Fig 2) lead to lower levels of *LUC* transcripts in inflorescences from plants grown at 23˚C (Fig 4A).

To assess the contribution of translational inhibition we inferred the levels of LUC protein by measuring LUC activity in protein extracts from samples collected at the same time as the ones used for expression assays (Fig 4B). We observed that during vegetative development LUC levels increased, although to a different extent, both at 16˚C (leaf 4 vs. leaf 7) and 23˚C (seedling vs. Leaf 7) (Fig 4B). We also found that the ultimate effect of miRNA-dependent regulation over the production of functional targeted-protein was temperature dependent. LUC protein levels were clearly higher at 16˚C than the ones found in samples from plants grown at 23˚C in leaf 7 and siliques (Fig 4B).

Next, we studied whether the differential contribution of both regulatory mechanisms was developmentally and/or environmentally determined. We reasoned that translational inhibition mechanisms would lead to a further reduction of LUC protein levels when compared to mRNA levels. Therefore, we created a Co-existence index, representing the ratio between LUC protein levels and LUC mRNA levels. Values higher than 1 indicated a low contribution from translational inhibition to miRNA-dependent regulation, while the opposite was true for values below 1. As seen in Fig 4C, the translational inhibition mechanism was gradually less effective during vegetative development at 16˚C (leaf 4 vs. leaf 7). We observed the same tendency in leaf 7 from plants grown at 23˚C when compared with earlier stages of development (seedlings). In inflorescences and siliques, translational inhibition was more potent at 23˚C when compared to 16˚C.

The two main effectors within miRNA-loaded RISC complexes are AGO1 and AGO10. Both proteins have redundant but also specific roles in miRNA-mediated gene silencing [33]. Thus, it has been suggested that AGO10 has a more prominent role on translational inhibition [33] despite evidence that it is also able to cleave its mRNA targets [34]. To ascertain whether developmental and environmentally-dependent changes on the coexistence index correlated with variations on their expression, we analyzed both *AGO1* and *AGO10* profiles by qRT-PCR (Fig 4D; Fig 4E). We did not find significant differences of expression across the different samples neither for *AGO1* nor *AGO10*. Therefore, we did not observe any correlation between *AGO1* and *AGO10* expression patterns that could explain the differences in the co-existence index.

Altogether, these results show that developmental as well as environmental components influence both miRNA regulation and the balance between cleavage and translational inhibition mechanisms of gene silencing.

Discussion

Our findings show that plant miRNA performance (accumulation, efficiency and co-existence of target cleavage and translational inhibition) is influenced by both development and environment. Our results support that the expression of several central players in miRNA performance also depends on development and temperature in which plants are grown.

The view of the different pathways involved in sRNA production and action was initially rather simplistic and static [35]. It was generally assumed that molecular players devoted to generate each type of sRNA were ubiquitously expressed and, therefore, the main layer of control on sRNA-mediated regulation was orchestrated by the expression patterns of the RNA

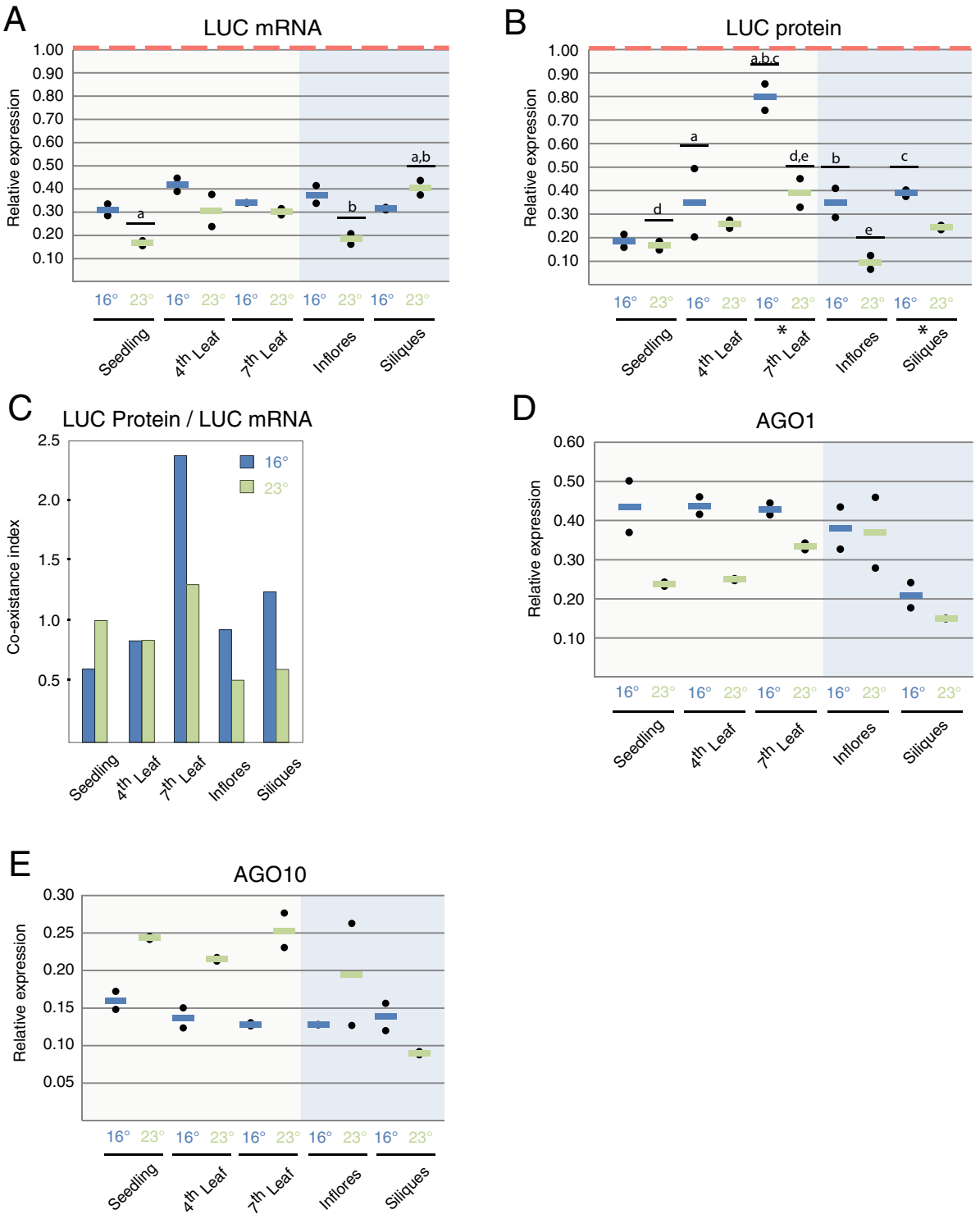

Fig 4. miRNA mode of action is developmentally and temperature-dependent. *(A) LUC mRNA expression levels assayed by qRT-PCR normalized to LUC mRNA in rLUC control plants (red dotted line). Lines, (blue = 16˚C, green = 23˚C) represent the average between two biological replicates. (B) LUC protein levels. Black dots represent one biological replicate each calculated from two technical replicates. Lines, (blue = 16˚C, green = 23˚C) represent the average between two biological replicates normalized to LUC protein levels in rLUC control plants (red dotted line). (C) Coexistence index is the ratio of average protein levels by average mRNA levels from each sample and condition. (D) AGO1 expression levels assayed by qRT-PCR. Black dots represent one biological replicate each calculated from two technical replicates. Lines, (blue = 16˚C, green = 23˚C) represent the average between two biological replicates. (E) AGO10 expression levels assayed by qRT-PCR. Black dots represent one biological replicate each calculated from two technical replicates.*

Lines, (blue = 16˚C, green = 23˚C) represent the average between two biological replicates. (A-E) "Inflores" stands for inflorescences. * shows tissues in which temperature has a significant effect in a pairwise comparison ($p<0.05$). Letters and lines show significant differences between tissues in ANOVA-test after Tukey correction (adjusted $p<0.05$).

https://doi.org/10.1371/journal.pone.0192984.g004

from which they originated. We are currently starting to appreciate that this might be a more dynamic process [36]. Our results support a more dynamic scenario in which the expression of molecular players and mechanisms involved in miRNA-mediated gene silencing are developmentally and environmentally-sensitive.

Although siRNA biogenesis in plants has been reported to be temperature sensitive, with siRNA levels correlating with growth temperature, mature miRNA accumulation has been thought to be largely temperature insensitive [26, 37, 38]. In contrast to studies where whole plants were assayed, our study dissects the temperature effect using discrete samples that encompass the different developmental stages during vegetative and reproductive development. Our analysis shows that amiR-LUC accumulation is temperature-responsive in leaves produced at late stages of development (leaf 7 and inflorescences). That positive temperature effect on amiR-LUC levels is more dramatic in reproductive tissues with a greater accumulation in inflorescences grown at 23˚C (Fig 2A; Fig 2B). Such increased accumulation is likely to be a consequence of the higher expression of the central miRNA biogenesis factor *DCL1* (Fig 3A) and its assistant *DRB2* (Fig 3C).

miRNA-mediated gene silencing relies on two mechanisms that are thought to co-exist, target cleavage and translational inhibition [12]. Nevertheless, beyond their existence little is known about their individual contribution to target gene silencing in plants. In mammals, miRNA-mediated regulation occurs mainly through target degradation [39, 40]. Initial work shows that in plants the contribution of both mechanisms might be cell-type dependent [13]. It is also unknown whether environmental conditions can influence plant miRNA efficiency and their mode of action.

Our results reveal that the efficiency of miRNA regulation decays with age in Arabidopsis (Fig 4C; leaf 4 versus leaf 7) in plants grown at low temperatures. That decline on efficiency is mainly due to the reduction on the contribution from translational inhibition to the overall miRNA-dependent regulation. In contrast, the contribution from this mechanism increases with temperature during reproductive development (Fig 4C). Nevertheless, we could not correlate that temperature and developmental effect in the amiR efficiency or mode of action with the expression patterns of the two main silencing effectors, *AGO1* and *AGO10*.

Recently, the DCL1 partner proteins HYL1 and DRB2 have been suggested to determine whether a miRNA triggers cleavage or translational repression of its targeted mRNAs [41]. While HYL1-mediated miRNA production contributes to degradation of the targeted mRNA, DRB2-dependent miRNA biogenesis triggers translational inhibition. Despite the observed changes in the coexistence between both regulatory mechanisms over development, we could only correlate higher levels of *DRB2* expression to a more pronounced contribution through translational inhibition in inflorescences grown at 23˚C when compared to lower temperature (Fig 2C, Fig 4C).

The general lack of correlation found between the expression patterns of the different genes involved in miRNA biogenesis and action and the amiR levels and mode of action suggests that additional players and/or post-translational modifications of the already known ones might determine the mechanism through which miRNAs regulate the expression of their targets [9, 42]. Indeed, post-translational modifications, such as the phosphor/de-phospho balance, have been shown in plant and animal systems as core events in the regulation of miRNA production and action [9, 43]. Additionally, temperature-dependent subcellular location has

also shown to modulate AGO7 function in Arabidopsis [44]. Therefore, future studies should focus on those modifications to fully understand the molecular basis behind plant miRNA performance depending on the cell-type, developmental stage and growth conditions.

Plants compromised in essential components of the miRNA machinery, such as DCL1 and AGO1 [45, 46], are usually sterile when grown at 23˚C. Nevertheless, a partial restoration of fertility is found when those plants are grown at lower temperatures. According to our results, miRNA regulation efficiency in inflorescences is lower at 16˚C when compared to plants grown at higher temperatures. Consequently, miRNA gene silencing might play a minor role in the general regulation of gene expression at low temperatures in inflorescence thereby explaining fertility restoration in these growth conditions.

Finally, our results are informative for the use of artificial miRNAs to downregulate endogenous genes at late stages of development or as part of crop protection strategies.

Material and methods

Plant material

Plants were grown on soil in long days (16h light/8h dark) under a mixture of cool and warm white fluorescent light at 16˚C and 23˚C and 65% humidity. *LUC* miRNA-activity reporter [9] and *rLUC* control in which synonymous point mutations were introduced to render the firefly luciferase miRNA-insensitive [27] have been previously described. Each reporter was combined in a single T-DNA carrying both the 35S::(r)LUC and the 35S::amiRLUC. Homozygous plants with a single T-DNA insertion were selected and used before [9, 27].

RNA analyses

Total RNA was isolated as described in [47] using tissue pooled from 15 randomized individuals per sample and biological replicate.

Reverse transcription was performed with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) using 200ng of total RNA previously treated with DNase I (Thermo Scientific) following the protocol described in [48].

PCRs were carried out in presence of SYBR Green (Invitrogen) and monitored with the CFX384 Real-Time PCR Detection-System (Bio-Rad) in two technical and two biological replicates. Biological replicates were treated as independent samples. Relative expression changes were calculated using $2^{-\Delta Ct}$ in all assays except in Fig 3A where the $2^{-\Delta\Delta Ct}$ method was applied to normalize *LUC* mRNA levels to the ones of *rLUC*. Expression levels were normalized to *β-TUBULIN2* (At5g62690). Mature miRNA quantifications were performed by stem-loop RT-PCR as described [48].

For small RNA blots, 3 μg of total RNA were used and two biological replicates performed. All primers used are listed in S1 Table.

Protein assays

Proteins were isolated from the corresponding tissues from 15 randomized individuals per sample and biological replicate. After tissue homogenization, the resulting powder was resuspended in protein extraction buffer (PBS, Triton X-100 0.1%, Complete EDTA-free (Roche)). After centrifugation, 50 μl of protein were mixed with the same volume of Beetle-Juice (PJK) Firefly substrate. Luciferase activity from two biological replicates was measured in technical triplicates on a Centro LB 960 (Berthold Technologies) device. Protein concentration of two biological replicates was assessed using the Bradford protein assay kit (BioRad) in technical triplicates. From this, Luciferase activity per μg of protein was calculated and the average of

both biological replicates was used for further analysis. Values were normalized to the ones from rLUC.

Statistical analysis

Significance of the effect of temperature and development as observed on protein expression was assessed statistically for the data shown on Figs 2A, 3A–3E, 4A, 4B, 4D and 4E. Departure from a normal distribution and homogeneity of variance could not be tested due to the number of biological replicates being 2. However, normality could be assumed for the population from which the data was sampled due to the clear pattern observed on the above-mentioned figures. Next, we sought to assess whether development had a global effect on the joint measurements across stages for the two given temperatures. To that end, a one-way analysis of variance was performed for each temperature. Significance of the difference between the two observations for each developmental stage was assessed via two-sample independent t-test at 16˚C and 23˚C.

Analysis of variance was performed to investigate whether development had a global effect on the joint measurements across stages for each temperatures. For those cases in which the anova omnibus test revealed a significant effect of development, a post-hoc Tukey test was performed to identify the single stages responsible for that effect being significant while adjusting for multiple hypothesis testing.

Supporting information

S1 Table. Oligonucleotide primer sequences used in this study.
(DOCX)

Acknowledgments

We greatly acknowledge the support from Detlef Weigel since a substantial part of this work was conducted at the MPI for Developmental Biology. We thank Silvio Collani and Catia Igreja for support with establishing the luciferase quantification assay, and Patricia Lang, Sascha Laubinger, Peter Brodersen, Pablo Manavella and members of the MoRE Lab for critical reading of the manuscript.

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Funding acquisition: Ignacio Rubio-Somoza.

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Methodology: Ignacio Rubio-Somoza.

Supervision: Ignacio Rubio-Somoza.

Writing – original draft: Patrick von Born, Ignacio Rubio-Somoza.

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