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Arabidopsis miR156 Regulates Tolerance to Recurring Environmental Stress through *SPL* Transcription Factors[C][W]

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Plants are sessile organisms that gauge stressful conditions to ensure survival and reproductive success. While plants in nature often encounter chronic or recurring stressful conditions, the strategies to cope with those are poorly understood. Here, we demonstrate the involvement of *ARGONAUTE1* and the microRNA pathway in the adaptation to recurring heat stress (HS memory) at the physiological and molecular level. We show that *miR156* isoforms are highly induced after HS and are functionally important for HS memory. *miR156* promotes sustained expression of HS-responsive genes and is critical only after HS, demonstrating that the effects of modulating *miR156* on HS memory do not reflect preexisting developmental alterations. *miR156* targets *SPL* transcription factor genes that are master regulators of developmental transitions. *SPL* genes are posttranscriptionally downregulated by *miR156* after HS, and this is critical for HS memory. Altogether, the *miR156-SPL* module mediates the response to recurring HS in *Arabidopsis thaliana* and thus may serve to integrate stress responses with development.

INTRODUCTION

To cope with changes in their physical environment, plants have evolved physiological responses allowing them to tolerate stressful conditions such as extreme temperatures, drought, and high salinity (Jenks and Hasegawa, 2005). These abiotic stresses are a major limiting factor for the geographical distribution and productivity of plants; hence, stress tolerance is a central goal in crop breeding. So far, research has focused on the immediate responses to abiotic stress, leading to important insights into the molecular and genetic basis of stress tolerance. However, how plants respond to enduring and recurring stress conditions has received little attention, although this arguably constitutes the more "natural" stress scenario. Plants can be primed by stress, such that their responses to repeated stress exposure or their further development are modified (Pecinka et al., 2010; Ito et al., 2011; Ding et al., 2012; Sani et al., 2013). Priming precedes a memory phase, during which information on a past stress exposure is stored. A related phenomenon in animals is hormesis, where a low-level stress treatment enhances resistance against the same and other stresses and increases growth, fecundity, and longevity (Gems and Partridge, 2008).

Here, we studied priming by high-temperature stress in *Arabidopsis thaliana* as a model case for environmental stress memory. A plant acquires thermotolerance through a moderate (priming) heat stress (HS) and is then able to survive a severe HS that is lethal to an unadapted plant (Mittler et al., 2012). Acquired thermotolerance is maintained during several days, even if the plant is not exposed to elevated temperatures after the priming HS (Yeh et al., 2012). We call this maintenance of acquired thermotolerance short HS memory; it is an active process that can be separated genetically from the acquisition of thermotolerance (Charng et al., 2006, 2007; Meiri and Breiman, 2009). Across all kingdoms, HS induces the expression of various types of heat shock proteins (HSPs) that protect cellular proteins from denaturation (Larkindale et al., 2005; Richter et al., 2010). Generally, *HSP* expression is activated by HEAT SHOCK TRANSCRIPTION FACTORs (HSFs), peaks early after HS, and declines within a day to base levels (Scharf et al., 2012). *HSFA2* is required specifically for HS memory (Charng et al., 2007). Mutants in two additional heat-induced genes (*HEAT-STRESS-ASSOCIATED32* [*HSA32*] and *ROTAMASE FKBP1*) are specifically impaired in HS memory (Charng et al., 2006; Meiri and Breiman, 2009). Thus, while a few players have been identified, no mechanistic model of HS memory has emerged.

MicroRNAs (miRNAs) have been reported to modulate plant stress responses. miRNAs are ~21-nucleotide-long single-stranded RNA molecules that guide effector proteins, most commonly ARGONAUTE1 (AGO1), to complementary mRNAs, causing either mRNA cleavage or translational inhibition (Voinnet, 2009; Axtell, 2013). miRNAs are generated from primary transcripts that are transcribed by RNA polymerase II and fold into a hairpin structure that is cleaved by DICER-LIKE1 (DCL1) to release the active miRNA (Rogers and Chen, 2013). miRNAs play important roles during plant growth, developmental transitions,

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and determination of cell identity (Jones-Rhoades et al., 2006; Voinnet, 2009). miRNAs are also involved in the modulation of plant responses to biotic and abiotic stresses (Sunkar and Zhu, 2004; Fujii et al., 2005; Navarro et al., 2008; Khraiwesh et al., 2012; Kruszka et al., 2012; Sunkar et al., 2012). It has been suggested that plant miRNAs that regulate growth and development may also mediate modified growth and development after stress exposure (Sunkar et al., 2012).

Here, we report a function for the miRNA pathway and more specifically for *miR156* in the adaptation to recurring HS in *Arabidopsis*. We show that *miR156* is induced by HS and that *miR156* levels after HS are critical for HS memory and affect expression of HS memory–related genes through *SPL* genes.

RESULTS

The miRNA Pathway Is Specifically Required for HS Memory

To test whether miRNAs are involved in the maintenance of acquired thermotolerance (i.e., HS memory), we first tested whether *ago1-25* and *ago1-27* seedlings had a defect in this process. The *ago1-25* and *ago1-27* alleles are hypomorphic (i.e., they retain some residual activity) and display only mild developmental alterations in comparison to a full *ago1* knockout (Morel et al., 2002). To assay HS memory (Charng et al., 2007), we induced thermotolerance in 4-d-old seedlings by a priming HS treatment, consisting of 1 h at 37°C, 1.5 h recovery at 23°C, and 0.75 h at 44°C (acclimation [ACC]; Figure 1). Two or three days after the priming HS, a tester HS (44°C for 80 to 115 min) was applied that is lethal to a plant lacking the priming HS (Figure 1C and Methods). A temperature of 44°C is commonly used as severe HS in *Arabidopsis* stress tolerance studies (Yeh et al., 2012). *Arabidopsis* does encounter such temperatures in nature where full insolation causes leaf temperatures to rise much higher than air temperatures (Salisbury and Spomer, 1964). In consequence, *Arabidopsis* is well adapted to withstand exposure at 44°C after HS priming. Following the described tester HS after priming, both *ago1* mutants showed reduced seedling fresh weight and a higher proportion of highly damaged seedlings compared with the Columbia-0 (Col-0) wild type and only slightly better survival than the memory-deficient *hsfa2* mutant, suggesting a defect in HS memory (Figures 1C, 1D, and 2A to 2C).

To test whether the observed phenotype was specific, we assayed acquisition of thermotolerance and basal thermotolerance (Figures 1A and 1B). The acquisition of thermotolerance is reflected in the amount of severe HS that a plant survives directly after a priming HS (Figure 1B). Basal thermotolerance reflects the dose of severe HS that an unprimed plant will survive (Figure 1A). Mutants with specific defects in the maintenance of acquired thermotolerance are expected to display normal acquisition of thermotolerance and normal basal thermotolerance (Figure 1D). *ago1* mutants acquired thermotolerance to a level comparable to the wild type (Supplemental Figure 1A), and basal thermotolerance was similar to the wild type (Supplemental Figure 1B), indicating that *ago1* is specifically required for the maintenance of acquired thermotolerance.

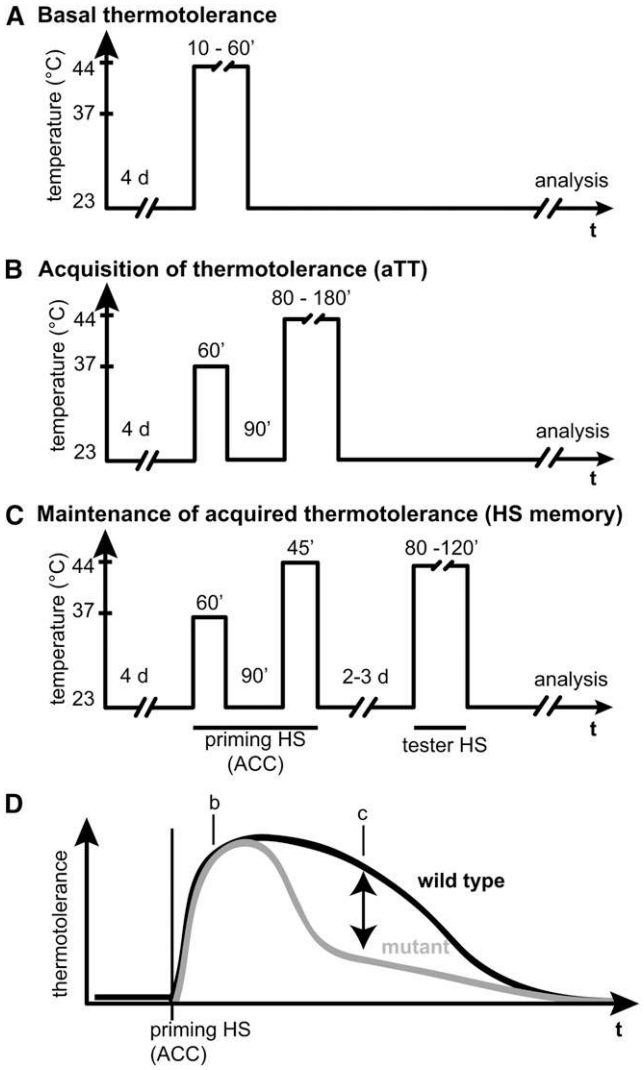

Figure 1. Representation of Different Types of Thermotolerance, Their Assay Conditions, and Schematic Representation of the Thermotolerance Profile of a Mutant with a Specific Defect in the Maintenance of Acquired Thermotolerance (i.e., HS Memory).

(A) Basal thermotolerance. ', minutes; t, time.

(B) Acquisition of thermotolerance.

(C) Maintenance of acquired thermotolerance (HS memory) is assayed by a tester HS of 80 to 120 min 2 or 3 d after a priming HS (ACC), which induced thermotolerance.

(D) Schematic representation of the temporal profile of acquired thermotolerance in the wild type and a mutant with a specific defect in its maintenance (such as *hsfa2*). There is no defect in the acquisition of thermotolerance (b), but a clear difference (see double-pointed arrow) in its maintenance (c).

We next analyzed the hypomorphic *dcl1*-9 mutant that is specifically required for the biogenesis of miRNAs (Jacobsen et al., 1999). As homozygous *dcl1-9* mutants are sterile, we used progeny of a heterozygous plant. Two weeks after the tester HS, individual seedlings were classified according to their phenotype and genotyped. We found more individuals with severe damage

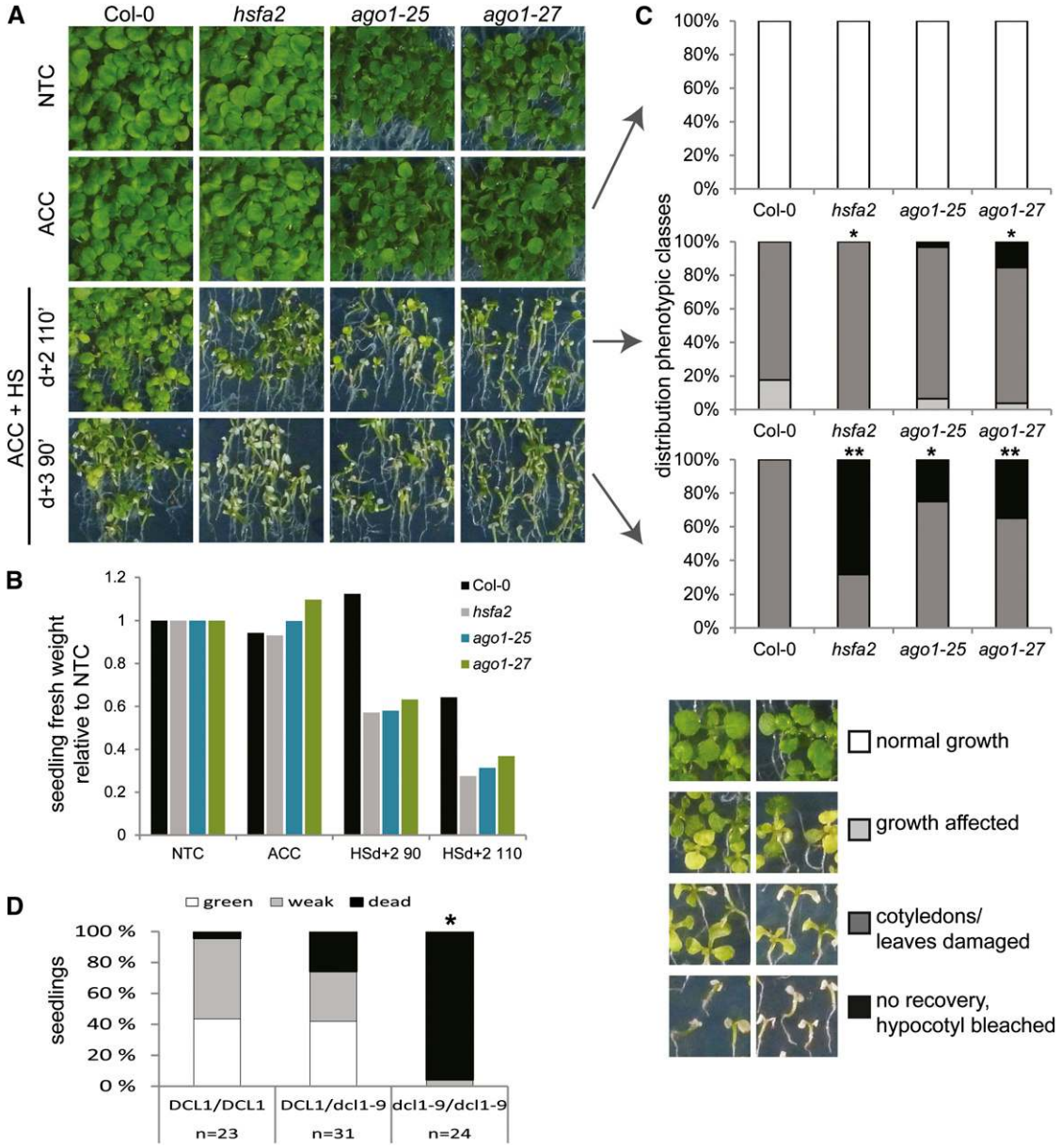

Figure 2. The miRNA Pathway Is Required for the Maintenance of Acquired Thermotolerance.

(A) *ago1-25* and *ago1-27* mutants were assayed for the maintenance of acquired thermotolerance by applying a tester HS two (+2, 110 min) or three (+3, 90 min) days after a priming HS (ACC); *hsfa2* was included as a control. Photographs were taken 14 d after ACC. All plants of one treatment were grown on the same plate. One representative replicate of at least three independent biological replicates is shown. For quantification, see **(B)** and **(C)**. ACC, priming HS.

(B) Quantification of data shown in **(A)**; average fresh weight from 59 ± 14 seedlings was determined 14 d after ACC. Due to very low weight, d+3 samples were not analyzed. Instead, ACC + HS d+2 90 min samples were included, which were omitted in **(A)** due to space constraints.

(C) Quantification of data shown in **(A)**; the percentage of seedlings in different phenotypic classes was determined for different genotypes and heat treatments. Gray arrows indicate the corresponding plates from **(A)**. Panels are (top to bottom): ACC, ACC + HS d+2 110', ACC + HS d+3 90'. Two photographs with representative seedlings of each class are shown to illustrate the damage classes. Asterisk, significantly different distribution compared with Col-0 (Fisher's exact test, *$P < 0.05$; **$P < 0.005$).

(D) *dcl1-9* mutants show reduced maintenance of acquired thermotolerance (Fisher's exact test, $P < 0.001$). Individual seedlings were phenotypically categorized 14 d after a ACC + HS d+2 90' treatment as green, weak (bleached out cotyledons but green hypocotyl and meristem), and dead and genotyped for *dcl1-9* ($n = 78$).

among *dcl1-9* homozygous seedlings than their wild-type siblings, and this altered distribution of phenotypes was statistically highly significant (Figure 2D; $P < 0.001$, Fisher's exact test). Thus, *dcl1-9* is defective in HS memory, suggesting that miRNA biogenesis is required for HS memory.

Global Changes in the Transcriptional Response to HS in *ago1* Mutants

We next investigated the transcriptome of *ago1* during the maintenance of acquired thermotolerance. To this end, we performed ATH1 GeneChip analysis of Col-0 wild type and *ago1-25* RNA extracted from seedlings at 4 and 52 h after a priming HS and from nontreated control (ntc) seedlings harvested at the same time as the 4-h HS seedlings. In the wild type, 401 genes were changed at 4 h at least 4-fold, and 96 genes at 52 h after HS (Figure 3A; Supplemental Data Set 1). Of these 96 genes, 87 (91%) were induced by HS and 41 (43%) were induced also at 4 h. We classified these 41 genes as HS memory related. Of the genes differentially expressed at 4 h, only 42% were induced. Selected memory-related genes (*APX2*, *HSA32*, *HSP17.6A*, and *HSP22.0*; $\log_2$ fold change [ATH1, 52 h] 5.1, 2.4, 6.6, and 5.3, respectively) were further analyzed using quantitative RT-PCR (qRT-PCR) and a more detailed time course (Figure 3B). This revealed that these genes reached their maximal induction 1 d (24 h) after HS and remained activated for 2 d (48 h) and to a lesser extent 3 d (72 h) after HS. This distinguishes the class of HS memory–related genes from classically studied *HSP* genes, such as *HSP70*, which were found in the "4 h only" class and were not analyzed further during this study. Of note, Gene Ontology term analysis for the three classes ("4 h only," "52 h only," and "4 h and 52 h") revealed strong enrichment of HS-related terms (GO0009266, 0009408, and 0010286) for both the "4 h only" and the "4 h and 52 h" classes (Supplemental Figure 1C).

We next identified HS-responsive genes whose expression pattern after HS depended on *AGO1* activity. To this end, we searched for genes whose expression fold changes after HS were at least 2-fold changed in *ago1* compared with Col [(Col HS/Col ntc)/(*ago1* HS/*ago1* ntc) >2 or <2]. Strikingly, 21% of upregulated and 28% of downregulated genes in the "4 h only" class fulfilled this criterion. This fraction increased to 51 and 100%, respectively, in the "4 h and 52 h" class and to 93 and 60%, respectively, in the "52 h only" class (Figure 3C). Compared with this, only 3% of all other genes were accordingly affected in *ago1* mutants. Thus, *ago1* seedlings show a defect in the dynamics of the response to HS at the transcript level, and this effect is most pronounced during later stages. These results were confirmed for selected genes over a more detailed time course (Figure 3B). Thus, *ago1* is required for the later stages of the HS response.

Identification of HS-Responsive miRNAs

The requirement for *AGO1* and *DCL1* in HS memory suggests that miRNAs function in this process. We next identified miRNAs that were specifically induced by HS at early or late stages using a qRT-PCR platform (Pant et al., 2009) for 204 known primary miRNA (pri-miRNA) transcripts (Supplemental Data Set 2). Comparing samples from 4 and 52 h after a priming HS with ntc samples, we identified a number of heat-responsive pri-miRNAs (Figure 4A). Using $\log_2$ fold change >2 as a cutoff, 22 pri-miRNA transcripts were upregulated and 17 downregulated at 4 h, while at 52 h, seven pri-miRNAs were upregulated and four downregulated. Four upregulated pri-miRNAs and one downregulated pri-miRNA were common to both time points. Focusing on upregulated pri-miRNAs, we selected the three pri-miRNAs most strongly upregulated at either time point and the four pri-miRNAs upregulated at both time points and analyzed their induction profile in more detail. *pri-miR156h* and *pri-miR831* transcript levels peaked at 4 h after HS, while *pri-miR391* peaked only after 28 h (Figure 4B; Supplemental Figure 2A). We then tested whether induction of the pri-miRNA corresponded to elevated levels of the mature miRNA (Figure 4C). *miR156a-f* peaked at 4 h but was elevated up to 52 h after HS (relative to the respective ntc; the decline of *miR156a-f* expression between 4 and 52 h ntc samples is developmentally regulated; Wu and Poethig, 2006; Wang et al., 2009); *miR156h* was increased between 0 and 76 h; *miR391* was increased at 52 and 76 h; and *miR831* was increased between 18 and 76 h. For other miRNAs, confirmation of HS induction at either the pri-miRNA level or the mature miRNA level could not be obtained (possibly due to their low abundance close to the detection limit) and they were not analyzed further. Thus, for *miR156h*, *miR391*, and *miR831*, mature miRNA levels generally followed pri-miRNA levels, yet appeared more sustained, possibly because of the higher stability of miRNAs compared with their precursor transcripts in the cell.

The HS induction was most robust for *miR156*. The *miR156* clade is extremely well conserved among flowering plants and is also found in mosses (Arazi et al., 2005; Axtell et al., 2007). It plays important roles in the endogenous timing of leaf initiation and developmental transitions by targeting *SQUAMOSA-PROMOTER BINDING-LIKE* (*SPL*) transcription factors (Cardon et al., 1999; Rhoades et al., 2002; Wang et al., 2008, 2009; Wu et al., 2009; Huijser and Schmid, 2011). The *miR156* clade in *Arabidopsis* is encoded by eight genes producing three different miRNA isoforms: *miR156a-f* are identical, *miR156g* has a single nucleotide substitution at the first position, and *miR156h* has two nucleotide substitutions at positions 11 and 14 relative to *miR156a-f* (Jones-Rhoades and Bartel, 2004). miRNAs of the *ath-miR156h* isoform have also been found in *Arabidopsis lyrata* and *Sellaginella moellendorfii* (Fahlgren et al., 2010; Ma et al., 2010; Kozomara and Griffiths-Jones, 2011). As the *miR156a-f* could be derived from any of six *MIR156(a-f)* genes, we also tested other *MIR156* transcripts and found a HS-induced upregulation for *MIR156c* and *d* (Figure 4B). For these reasons, we focused further analyses on *miR156*.

SPL Transcripts Are Transiently Downregulated by HS through *miR156*

We next searched our transcriptome data for putative *miR156* targets with HS-dependent expression. The transcripts of four putative target genes were downregulated at least 2-fold 4 h after HS (*SPL2*, *SPL9*, *SPL11*, *At5g38610* [predicted pectin methylesterase inhibitor]), and one gene was downregulated at 52 h after HS (*At5g40510*, predicted protein kinase). Of these, *SPL2*, *SPL9*, and *SPL11* were likely targeted by *miR156a-f* and *miR156h*, while *At5g38610* and *At5g40510* were predicted to be specific for *miR156h* (Supplemental Table 1 and Supplemental Figure 3D).

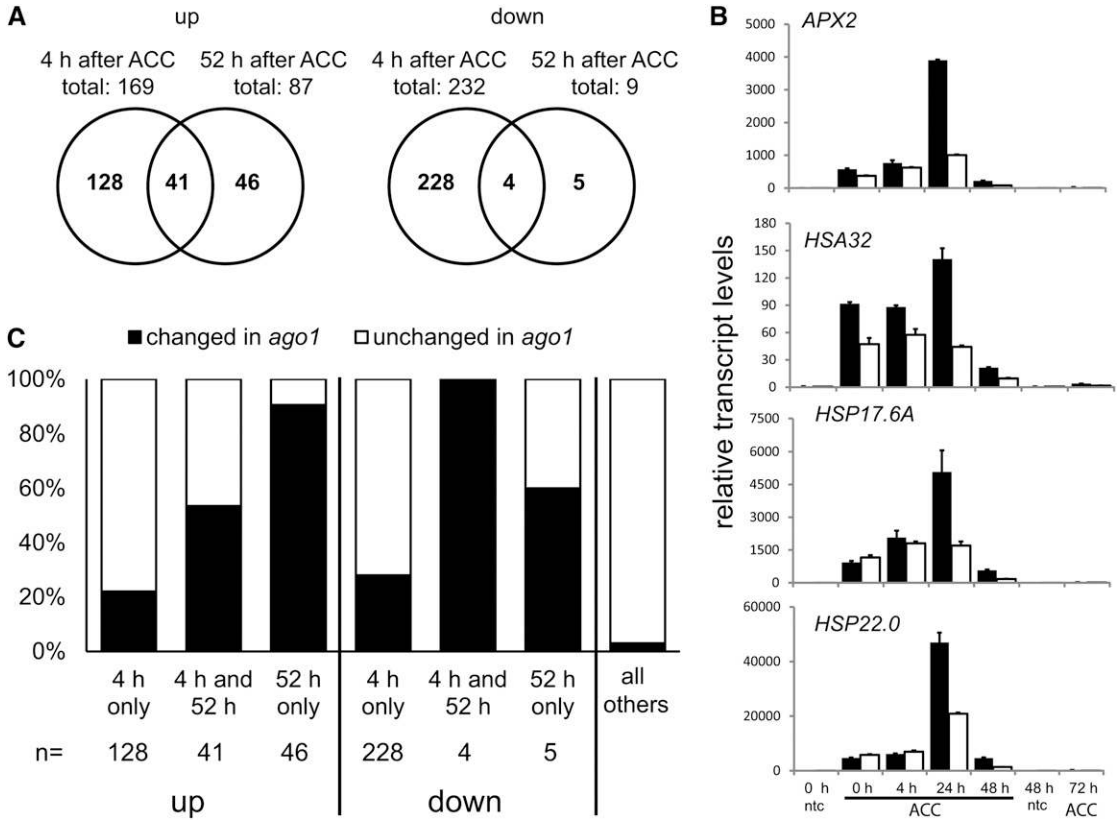

Figure 3. The Effect of *ago1-25* on the Transcriptome Is Most Pronounced during the Late Stages of the HS Response.

(A) Venn diagram representation of ATH1 GeneChip transcriptome profiling of Col-0 samples after a priming HS (ACC). Samples were harvested 4 or 52 h after the end of the ACC; the number of genes that are up- or downregulated at either 4 or 52 h or at both time points relative to a 4 h ntc are shown.
(B) Experimental validation of the indicated HS memory–related genes in Col-0 (black bars) and *ago1-25* (white bars) over a detailed time course after ACC or mock treatment using qRT-PCR. Expression values were normalized to *TUB6* and to Col-0 0 h ntc [(GENE OF INTEREST/*TUB6*)$_x$/(GENE OF INTEREST/*TUB6*)$_{\text{Col0 h ntc}}$]. Error bars are SE of the mean of three replicates. One representative of at least three independent experiments is shown.
(C) Percentage of genes whose induction or repression differed at least 2-fold in *ago1-25* compared with Col-0 in the classes described in **(A)** or among all remaining genes on the microarray (all others), respectively.

Cleavage of *At5g38610* mRNA by *miR156* is supported by degradome data (Addo-Quaye et al., 2008). HS-regulated expression of *SPL2*, *SPL9*, *SPL11*, *At5g38610*, and *At5g40510* was confirmed in a more detailed time course (Figure 5A; Supplemental Figures 2B and 3A). *SPL2*, *SPL9*, *SPL11*, and *At5g38610* expression was strongly reduced immediately (0 h) and 4 h after HS and transcript levels recovered by 24 h, while *At5g40510* transcripts were reduced at 48 h after HS. *At5g38610* encodes a predicted pectin methylesterase inhibitor. Pectin methylesterase activity is required to loosen the cell walls at the sites of organ initiation at the shoot apical meristem (Peaucelle et al., 2008). Inhibiting this activity blocks organ initiation, suggesting that *At5g38610* acts similarly to SPL proteins to limit organ formation.

If downregulation of target transcripts after HS was dependent on the miRNA, this would be expected to be reduced in *ago1* mutants. Indeed, for all three examined *SPL* genes, downregulation was lost in *ago1-25* mutants (Figure 5A, top and bottom panels). For *SPL2* and *SPL11*, this loss was incomplete, which could be due to either residual AGO1 activity in the hypomorphic *ago1-25* mutant or to an additional miRNA-independent mechanism of repression. miRNAs typically regulate their target genes posttranscriptionally through transcript cleavage. Thus, we investigated whether the HS-dependent repression occurred at the transcriptional or posttranscriptional level by quantifying unspliced *SPL* transcripts as a proxy for transcriptional activity (Bäurle et al., 2007; Le Masson et al., 2012). Unspliced transcript levels of *SPL2* and *SPL11* were not repressed by HS in the wild type or *ago1-25* mutants (Figure 5A, middle and bottom panels; for primer positions, see Supplemental Figure 2C). For *SPL9*, a slight reduction of the unspliced transcript was observed, representing a small fraction of the reduction observed for the spliced transcript (Figure 5A, middle and bottom panels). Thus, HS-mediated repression of the tested *SPL* genes occurs largely or exclusively at the posttranscriptional level.

To directly test whether the *miR156* binding site of *SPL* genes is required for the HS-dependent reduction of their transcript levels, we generated transgenic plants expressing a miRNA-resistant *SPL2* or *SPL11* gene under the control of its own promoter (i.e., *ProSPL2:rSPL2* and *ProSPL11:rSPL11*, respectively)

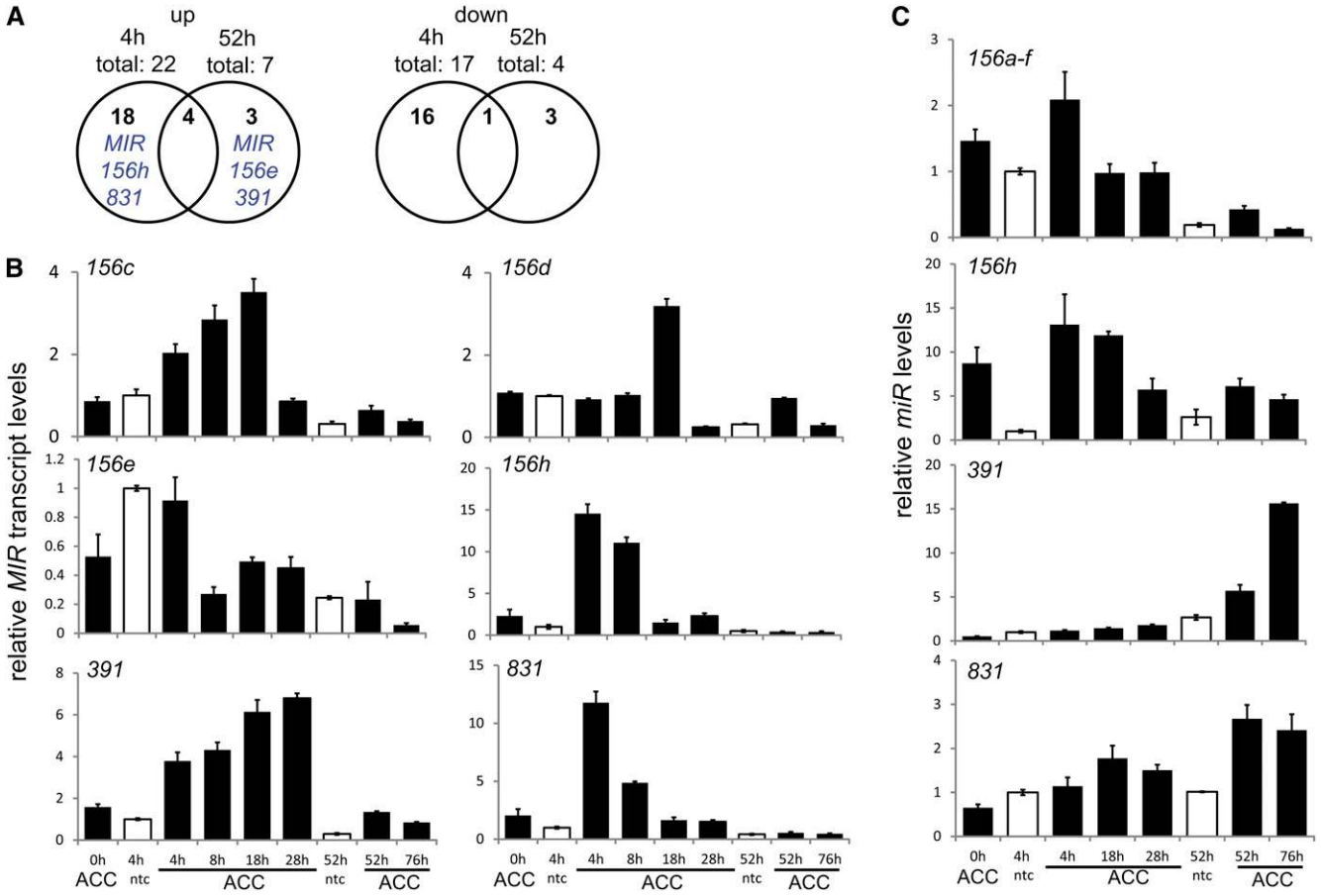

Figure 4. Primary *MIR* Profiling and Transcript Levels of HS-Responsive miRNAs during Maintenance of Acquired Thermotolerance.

(A) Venn diagram representation of primary *MIR* transcript levels after a priming HS (ACC); samples were taken 4 or 52 h after the end of ACC. Up- or downregulated pri-miRNAs at either 4 or 52 h or at both time points are displayed. In blue are the miRNAs further analyzed in **(B)** and **(C)**.

(B) qRT-PCR of the indicated primary *MIR* transcripts.

(C) qRT-PCR of the indicated mature miRNAs.

Expression values were normalized to *TUB6* and the respective 4 h ntc [(*GENE OF INTEREST*/*TUB6*)$_x$/(*GENE OF INTEREST*/*TUB6*)$_{4\ h\ ntc}$]. Black and white bars indicate HS-treated (ACC) and control (ntc) samples, respectively. Error bars are SE of the mean of three replicates. One representative of at least three independent experiments is shown.

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and compared transcript levels from the endogenous *SPL* gene and the transgenic *rSPL* gene by specific qRT-PCR. Comparing the native and miRNA-resistant version of *SPL2* and *SPL11*, respectively, we found that the *miR156*-resistant transcript escaped HS-dependent downregulation (Figure 5B), demonstrating that an intact *miR156* binding site is required for HS-mediated repression of *SPL2* and *SPL11*.

We next analyzed whether GUS activity of a miRNA-resistant *ProSPL9:SPL9-GUS* line (*ProSPL9:rSPL9-GUS*) would be affected by HS (Yang et al., 2012). Indeed, relative GUS activity was progressively increased after HS in *rSPL9-GUS* compared with *SPL9-GUS* seedlings in a quantitative 4-methylumbelliferyl β-D-glucuronidase (MUG) assay (Figure 5C). Accordingly, *suo-2* mutants, which are defective in miRNA-mediated translational repression (Yang et al., 2012), had reduced HS memory (Supplemental Figure 3B), suggesting HS-dependent miRNAs repress their targets in part through translational repression. Taken together, our results indicate that the HS-dependent downregulation of *SPL2*, *9*, and *11* is controlled by *miR156*.

miR156 Is Required for HS Memory and High *miR156h* Levels Enhance the Memory

To investigate whether the induction of *miR156* after HS correlated with a functional role in the HS response during the later stages, we generated a knockdown line of *miR156a-f* (*Pro35S:MIM156a-f*) (Franco-Zorrilla et al., 2007; Todesco et al., 2010). *Pro35S:MIM156a-f* plants were deficient in the maintenance of acquired thermotolerance (Figures 6A and 6B compared with Figures 1C and 1D), while acquisition of thermotolerance and basal thermotolerance were not affected (Supplemental Figures 4A and 4B compared with Figures 1A and 1B). Efficiency of the knockdown was

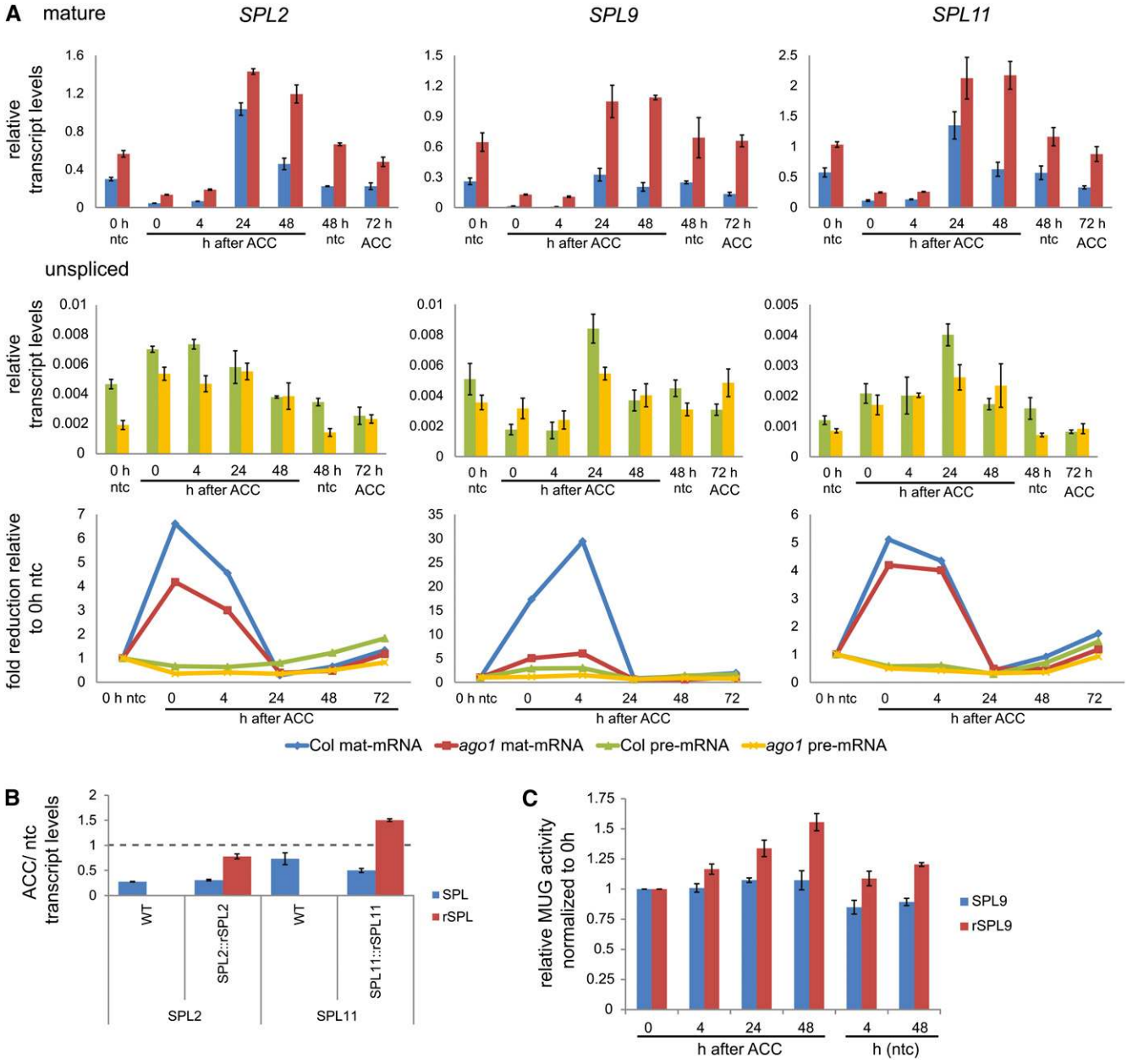

Figure 5. Levels of Mature (Spliced) and Unspliced *SPL* Transcripts during the Maintenance of Acquired Thermotolerance and Their Dependency on Functional *AGO1* and the *miR156* Binding Site.

(A) Mature (top panel) and unspliced (middle panel) *SPL2*, *SPL9*, and *SPL11* transcript levels relative to *TUB6* (*SPLn*/*TUB6*)$_x$ in Col-0 and *ago1-25* at the indicated times after a priming HS. Transcript level for the mature but not the unspliced *SPL2*, *9*, and *11* was increased in *ago1-25*. Bottom panel: fold reduction of *SPL* transcript levels relative to the 0 h control. The repression depends on functional *AGO1* and is evident for the mature mRNAs, but not the unspliced mRNAs, indicating that regulation occurs at the posttranscriptional level. The color code below the third panel applies to all panels in **(A)**. Error bars are SE of the mean of three replicates. The experiment was repeated three times independently, and one representative is shown.
(B) Reduction of *SPL2* and *SPL11* mRNA levels after HS is dependent on an intact *miR156* binding site. *ProSPL2:rSPL2* and *ProSPL11:rSPL11* plants were subjected to a priming HS or no treatment (ntc) and harvested directly after the end of the treatment. Levels of the indicated endogenous *SPL* or *rSPL* transcripts were determined and the ACC/ntc ratio calculated. Error bars are SE of the mean of three replicates. The experiment was repeated three times independently, and one representative is shown.
(C) GUS protein activity after a priming HS in *ProSPL9:SPL9-GUS* and *ProSPL9:rSPL9-GUS* as determined by MUG activity assay. Activity relative to the respective ntc and normalized to the 0-h time point averaged over three independent experiments ± SE indicates that *miR156* represses SPL9-GUS protein levels after HS.

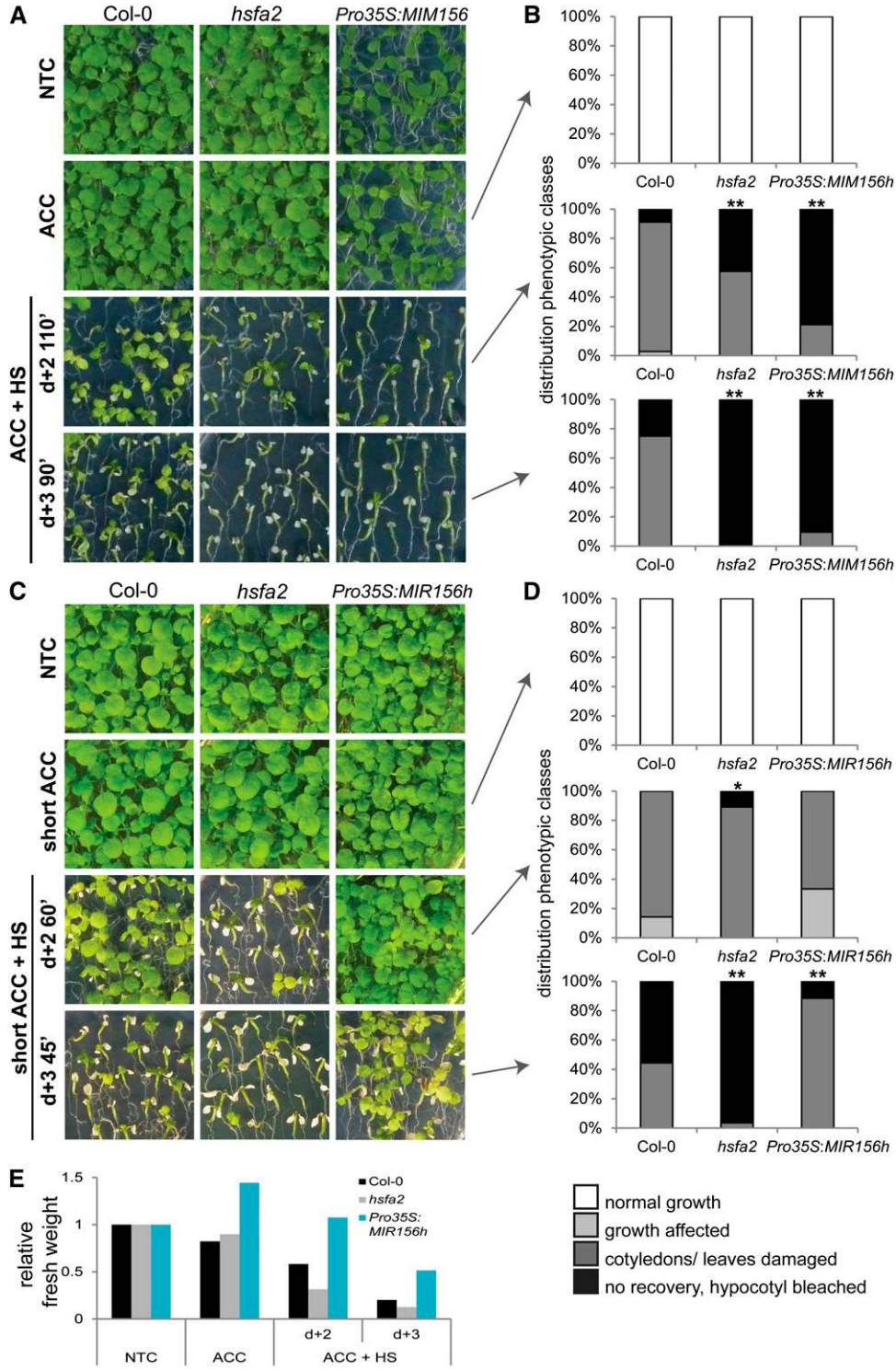

Figure 6. *miR156* Regulates the Maintenance of Acquired Thermotolerance.

(A) Knockdown of *miR156a-f* in *Pro35S:MIM156* impairs HS memory, assayed by applying a tester HS two (+2) or three (+3) days after a priming HS (ACC). Photographs were taken 14 d after ACC.

(B) Quantification of data shown in **(A)**; the percentage of seedlings in different phenotypic classes was determined for different genotypes and heat treatments. Gray arrows indicate the corresponding plates from **(A)**. Panels are (top to bottom): ACC, ACC + HS d+2 110', and ACC + HS d+3 90'. Damage classes are described in **(D)** and Figure 2C.

confirmed by determining *miR156a-f* and *miR156h* levels in *Pro35S:MIM156a-f* after HS (Supplemental Figure 4C). Levels of *miR156a-f* were severalfold reduced, while *miR156h* levels were only slightly lower, suggesting a high specificity of the construct and of the stem-loop qRT-PCR. However, we cannot exclude a weak effect of *Pro35S:MIM156a-f* on *miR156h*. Plants expressing a mimicry construct against *miR156h* were also defective in HS memory (*Pro35S:MIM156h*; Supplemental Figure 4D). Thus, *miR156* isoforms are required for HS memory.

We then asked whether overexpression of *MIR156h* was sufficient to enhance HS memory by generating transgenic plants expressing *MIR156h* under the control of the *35S* promoter (Supplemental Figure 4C). *Pro35S:MIR156h* plants displayed an enhanced maintenance of acquired thermotolerance as evidenced by their increased survival and growth after a tester HS 2 or 3 d after the priming HS (Figures 6C to 6E). Conversely, acquisition of thermotolerance and basal thermotolerance were unaffected (Supplemental Figures 4A and 4B). In summary, *miR156* levels are critical for HS memory.

As expected from previous reports on *miR156a-f* (reviewed in Huijser and Schmid, 2011), manipulating the levels of *miR156h* correlated with changes in growth and development. Reduction of active *miR156h* in *Pro35S:MIM156h* caused slightly early flowering with fewer leaves and a reduced leaf initiation rate (Supplemental Figure 4E and Supplemental Table 2). Overexpression of *MIR156h* caused an increased leaf initiation rate and very late flowering with respect to leaf number and days to flowering (Supplemental Figure 4E and Supplemental Table 2). Thus, *miR156a-f* and *miR156h* have largely overlapping functions during development and HS memory.

Repression of *SPL2* and *SPL11* by *miR156* Is Required for HS Memory

To test whether the effect of *miR156* on HS memory was mediated by downregulation of *SPL* function, we analyzed the HS memory of the *miR156*-resistant *ProSPL2:rSPL2* and *ProSPL11:rSPL11* lines. Indeed, HS memory was compromised in both lines similar to what was observed in the *miR156* knockdown lines (Figures 7A and 7B; Supplemental Figure 3C). The reduced HS memory of *ProSPL2:rSPL2* corresponded to a reduced maintenance of HS memory–related genes, particularly during the later stages after a priming HS (Figure 7C). Comparison of relative transcript levels at 48 and 72 h after HS indicated a 2- to 100-fold difference for *APX2*, *HSA32*, *HSFA2*, *HSP17.6A*, *HSP22.0*, and *DIN2*. Thus, *SPL2* and *SPL11* repression is required for HS memory and SPL2 directly or indirectly acts as a repressor of HS memory–related gene expression. SPL9, a close homolog of SPL2, can act as a transcriptional repressor in certain contexts by interfering with the assembly of activation complexes (Gou et al., 2011). A similar function could be envisioned for SPL2 and possibly SPL11 during the maintenance of acquired thermotolerance.

Expression of *miR156* after HS Is Critical for HS Memory

Because of the developmental effects in the *miR156*-manipulating lines, we sought to separate the functions of *miR156* during development and HS memory. To do so, we constructed transgenic lines that expressed either *MIR156h* or *MIM156h* under the control of the heat-inducible *HSP21* promoter (Supplemental Figure 5A). In those transgenic lines, manipulation of *miR156h* levels occurred only after HS as evidenced by quantification of *miR156h*, while *miR156a-f* levels were not or only very slightly affected (Figure 8G). However, we cannot rule out that *ProHSP21:MIM156h* also affects *miR156a-f*, especially since target miRNAs may be inactivated by sequestration with no effect on miRNA levels (Todesco et al., 2010). *ProHSP21:MIM156h* and *ProHSP21:miR156h* plants were phenotypically indistinguishable from nontransgenic plants under control conditions and after a priming HS (Figures 8A and 8D; Supplemental Figure 5B). However, *ProHSP21:MIM156h* plants showed growth defects if the priming HS was followed by a tester HS 2 or 3 d later, indicating that HS memory was reduced (Figures 8A to 8C). This was confirmed by quantifying seedling fresh weight and the fraction of seedlings in different damage classes (Figures 8B and 8C). Conversely, *ProHSP21:miR156h* plants showed better growth and survival under those conditions (Figures 8D to 8F), which was confirmed by quantifying seedling fresh weight and the fraction of seedlings in different damage classes (Figures 8E and 8F). Thus, induction or repression of *miR156h* is critical for HS memory only after the priming HS, and a modified shoot architecture (as seen in plants expressing the 35S-driven constructs) is not responsible for this effect.

To molecularly characterize the role of *miR156* in HS memory, we analyzed transcript levels of HS memory–related genes in *ProHSP21:miR156h* plants. Expression of memory-related genes was more sustained after HS in *ProHSP21:miR156h* than in control plants, particularly during the later stages (Figure 8H). Thus, the enhanced maintenance of acquired thermotolerance due to *MIR156h* overexpression is likely caused by the increased expression of HS memory–related genes. Taken together, our findings suggest that by increasing memory-related gene expression and modifying development and its timing *miR156* enhances HS memory.

Figure 6. (continued).

(C) Overexpression of *miR156h* enhances HS memory, assayed by applying a tester HS two (+2) or three (+3) days after a short ACC (37°C, 60'). Photographs were taken 14 d after ACC.

(D) Quantification of data shown in **(C)**; the percentage of seedlings in different phenotypic classes was determined for different genotypes and heat treatments. Gray arrows indicate the corresponding plates from **(C)**. Panels are (top to bottom): short ACC, short ACC + HS d+2 60', and short ACC + HS d+3 45'. Damage classes are described below panels and in Figure 2C.

(E) Quantification of **(C)**; average seedling fresh weight was determined from 27 ± 1 seedlings per genotype and treatment.

All plants of one treatment were grown on the same plate. The experiments were repeated at least five times with at least two independent transgenic lines with similar results. Asterisks indicate significantly different distribution compared with Col-0 (Fisher's exact test, *$P < 0.05$; **$P < 0.005$).

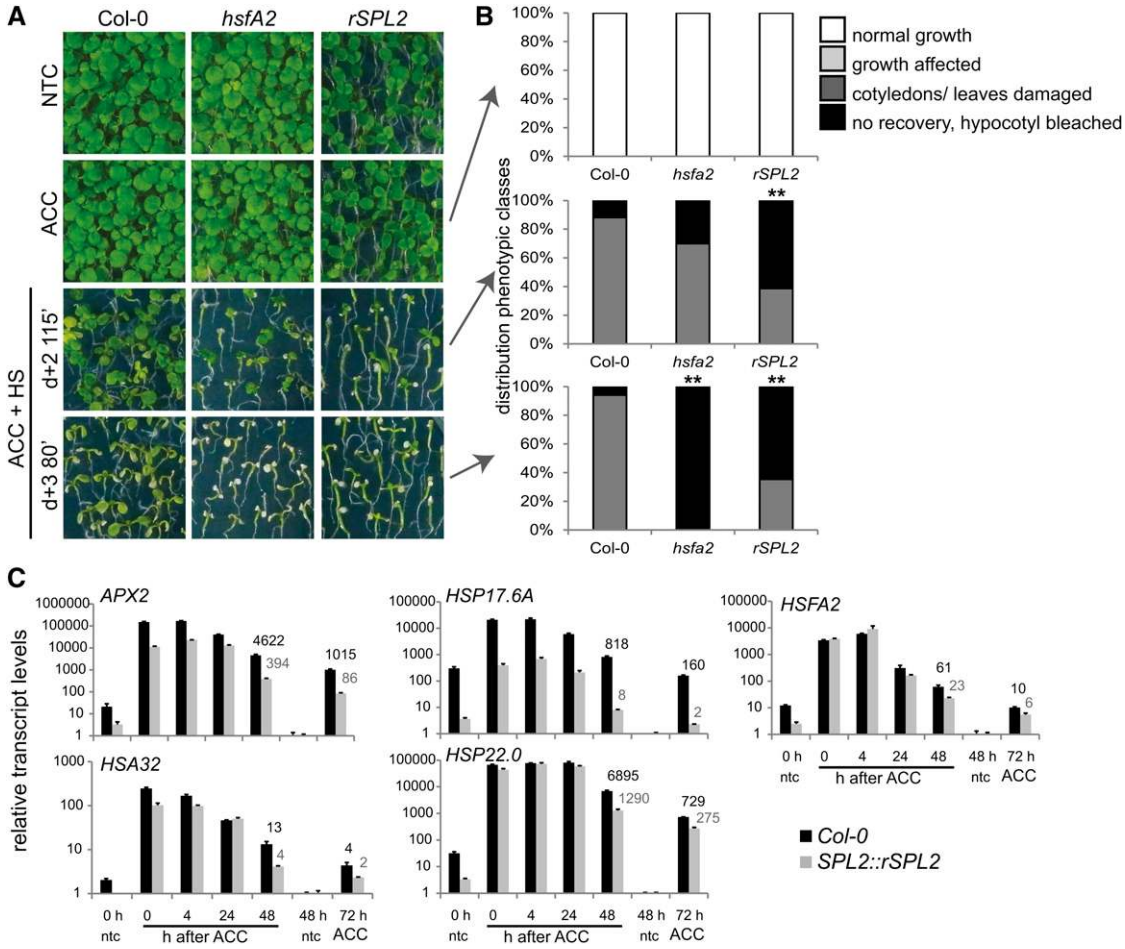

Figure 7. Downregulation of *SPL2* Is Required for the Maintenance of Acquired Thermotolerance and Sustained Expression of HS Memory–Related Genes.

(A) *ProSPL2:rSPL2* seedlings were assayed for the maintenance of acquired thermotolerance by applying a tester HS (HS) two (+2) or three (+3) days after a priming HS (ACC). Pictures were taken 14 d after ACC. All plants of one treatment were grown on the same plate. The experiments were repeated at least three times with two independent transgenic lines with similar results.

(B) Quantification of data shown in **(A)**; the percentage of seedlings in different phenotypic classes was determined for different genotypes and heat treatments. Gray arrows indicate the corresponding plates from **(A)**. Panels are (top to bottom): ACC, ACC + HS d+2 115', and ACC + HS d+3 80'. Damage classes are illustrated in Figure 2C. Asterisks indicate significantly different distribution compared with Col-0 (Fisher's exact test, $^{**}P < 0.005$).

(C) Expression of selected HS memory–related genes during maintenance of acquired thermotolerance determined by qRT-PCR in Col-0 (black) and *ProSPL2:rSPL2* (gray). Expression values were normalized to *TUB6* and 48 h ntc [$(GENE\ OF\ INTEREST/TUB6)_x/(GENE\ OF\ INTEREST/TUB6)_{48\ h\ ntc}$] and are displayed on a $\log_{10}$ axis for better resolution. Error bars are SE of the mean of three technical and two biological replicates.

DISCUSSION

While plant responses to acute stress are relatively well understood, those to chronic and recurring stress exposures are poorly characterized, despite their obvious importance for organisms in their natural environment. Here, we demonstrated that AGO1 acting through *miR156* and *SPL2* and *11* is an essential component of the HS memory in *Arabidopsis*. Despite not being the only miRNA induced after HS, *miR156* is not only necessary, but its overexpression is also sufficient to boost HS memory, indicating its central role. *miR156* is one of the most highly conserved miRNAs in plants, as it is found from higher plants to bryophytes (mosses), and its function in regulating the timing of developmental transitions appears to be conserved (Cho et al., 2012). Interestingly, *miR156* induction after HS has also been reported in field mustard (*Brassica rapa*) and wheat (*Triticum aestivum*; Xin et al., 2010; Yu et al., 2012), suggesting that not only its developmental functions but also its functions in HS memory may be conserved. This directly suggests a way to enhance HS memory in two economically important crop species.

MIR156h showed the strongest upregulation after HS in our experiments. *MIR156c* and *d* also showed significant induction that could account for the observed increase in *miR156a-f* after HS. The nucleotide substitutions in *miR156h* compared with *miR156a-f* may have occurred as an evolutionary adaptation to

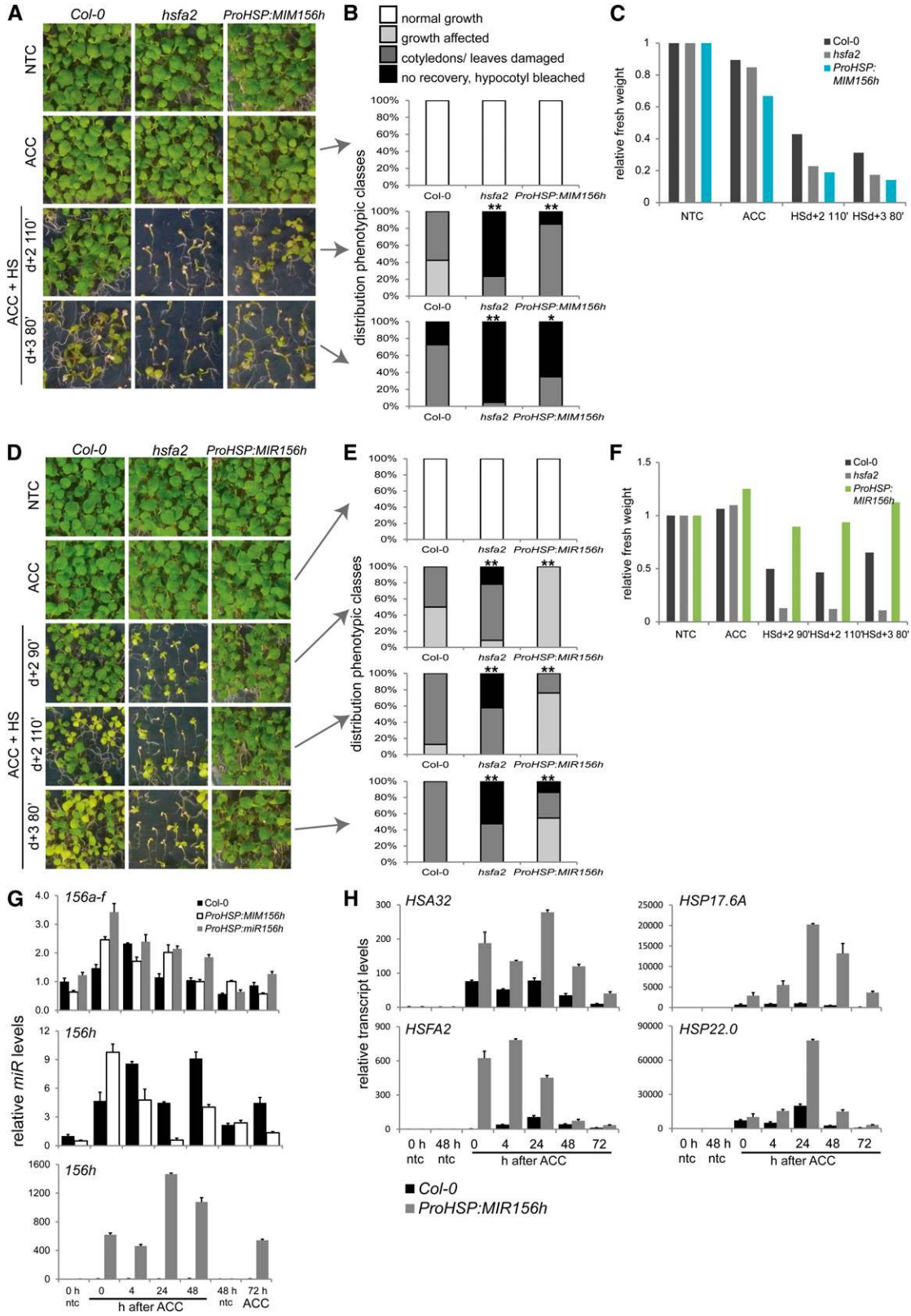

Figure 8. *miR156* Is Required after HS to Regulate the Maintenance of Acquired Thermotolerance.

functioning at elevated temperatures. It is conceivable that these substitutions may shift the balance of transcript cleavage and translational inhibition toward the latter, rendering the repression of target genes reversible and thus possibly better suited for HS conditions.

Similar to other stresses, HS transiently slows down growth and development including organ initiation (Skirycz and Inzé, 2010). As a predicted pectin methylesterase inhibitor, *At5g38610* activity may be required to loosen the cell walls at the sites of organ initiation at the shoot apical meristem (Peaucelle et al., 2008). Inhibiting this activity blocks organ initiation, suggesting that *At5g38610* acts similarly to SPL proteins to limit organ formation. It is conceivable that by promoting organ initiation shortly after HS (through repression of *SPL* and *At5g38610*), *miR156* counterbalances the negative effect of HS on growth and helps the plant to resume growth after it has subsided. A more direct effect of *miR156* appears to be the induction of HS memory–related genes, involving the HSFA2 transcription factor and release of SPL2-mediated repression. The SPL2-mediated repression of HS memory–related genes may be direct or indirect, as it has been shown that SPL9 can act as a transcriptional repressor (Gou et al., 2011). The genome-wide determination of direct SPL2 target genes during HS and development will be required for mechanistic insight into the extent to which the different functions involve the same or different downstream targets. It has been hypothesized that *miR156* target genes act as licensing factors for the transition to flowering (Wu et al., 2009). Thus, stress-mediated induction of *miR156* may delay the transition to flowering by prolonging the juvenile phase and serve to avoid flowering during a hot spell. The effect of a transient HS on the transition to flowering has not been systematically studied and may be different from the effect of chronic elevated ambient temperatures that promote the transition to flowering (Posé et al., 2013; Wigge, 2013). Because *miR156* expression was not controlled by one of the well-described flowering time pathways (Bäurle and Dean, 2006; Fornara et al., 2010) and was found to gradually decrease with plant age, *miR156* was grouped into the autonomous pathway and suspected to report the endogenous plant age for integration with other pathways. Recently, *MIR156* expression was reported to be repressed by sugars, suggesting a role in the integration with endogenous energy levels (Yang et al., 2013; Yu et al., 2013). *miR156* was also reported to delay flowering under cool ambient temperatures (Kim et al., 2012). Our findings add an additional dimension to this view in that plant age appears to be recorded not simply as chronological time, but rather as chronological time minus the time spent under adverse conditions and, thus, as "time well spent."

Taken together, *miR156* modulates plant development and response to HS. Several other miRNAs involved in plant development have also been found to be regulated by environmental stresses (Khraiwesh et al., 2012; Kruszka et al., 2012; Sunkar et al., 2012). Thus, a general pattern seems to emerge where plant development and its modulation in response to the environment is mediated by the same miRNAs. miRNAs may be particularly suitable for modulating longer term stress effects for two reasons: (1) regulation by a miRNA is reversible in the case of translational inhibition, and (2) miRNAs are relatively stable and are thus able to integrate signaling over longer periods of time. Interestingly, developmental timing in *Caenorhabditis elegans* is regulated by the miRNAs *lin-4* and *let-7*. The induction of dauer larvae depends on environmental cues and both miRNAs are involved in its regulation (Karp and Ambros, 2012). Thus, environmental modulation of development through miRNAs appears to be a general mechanism in plants and animals.

It cannot be ruled out that the function of AGO1 during HS memory is in part mediated by additional miRNAs, such as other heat-induced miRNAs identified in this study. The elucidation of this question is an interesting field of further study. Here, we report the functional role of a plant miRNA in the temporal

Figure 8. (continued).

All plants of one treatment were grown on the same plate. The experiments were repeated at least three times with two independent transgenic lines with similar results. Asterisks indicate significantly different distribution compared with Col-0 (Fisher's exact test, *P < 0.05; **P < 0.005).

(A) Heat-inducible knockdown of *miR156h* (*ProHSP21:MIM156h*) impairs the HS memory, assayed by applying a tester HS (HS) two (+2) or three (+3) days after a priming HS (ACC). Photographs were taken 14 d after ACC. For quantification, see **(B)** and **(C)**.

(B) Quantification of data shown in **(A)**; the percentage of seedlings in different phenotypic classes was determined for different genotypes and heat treatments. Gray arrows indicate the corresponding plates from **(A)**. Panels are (top to bottom): ACC, ACC + HS d+2 110', and ACC + HS d+3 80'. Damage classes are illustrated in Figure 2C.

(C) Quantification of **(A)**; average seedling fresh weight was determined from 32 ± 3 seedlings per genotype and treatment.

(D) Heat-inducible overexpression of *MIR156h* (*ProHSP21:MIR156h*) enhances the HS memory, assayed by applying a tester HS two (+2) or three (+3) days after a priming HS (ACC). Photographs were taken 14 d after ACC. For quantification, see **(E)** and **(F)**.

(E) Quantification of data shown in **(D)**; the percentage of seedlings in different phenotypic classes was determined for different genotypes and heat treatments. Gray arrows indicate the corresponding plates from **(D)**. Panels are (top to bottom): ACC, ACC + HS d+2 110', and short ACC + HS d+3 80'. Damage classes are illustrated in Figure 2C.

(F) Quantification of **(D)**; average seedling fresh weight was determined from 24 ± 3 seedlings per genotype and treatment.

(G) Mature miRNA levels of *miR156a-f* and *miR156h* in *ProHSP:MIR156h* and *ProHSP:MIM156h* determined by qRT-PCR relative to *TUB6* (*miR156h*/*TUB6*)$_x$. A several hundred fold induction of *miR156h* is observed in *ProHSP:MIR156h* plants after ACC, while no or only a very slight effect is observed for *miR156a-f*. Error bars are SE of the mean of three replicates. Three independent experiments were performed with one representative shown.

(H) Expression of HS memory–related genes determined by qRT-PCR is increased after a priming HS in *ProHSP:MIR156h*. Expression values were normalized to *TUB6* and 48 h ntc [(*GENE OF INTEREST*/*TUB6*)$_x$/(*GENE OF INTEREST*/*TUB6*)$_{48\ h\ ntc}$]. Error bars are SE of the mean of three replicates. Three independent experiments were performed with one representative shown.

modulation of abiotic stress adaptation. Studies in many different plant species have shown that *miR156* levels do not only change in response to heat stress but also after various other stresses such as cold, salt, and drought stress, as well as UV-B radiation, hypoxia, and biotic stress. The detailed analysis of the regulation of *miR156* remains an important research question, as it will be interesting to see whether *miR156* also integrates stress memory and development for other stresses.

METHODS

Plant Material, Growth, and Stress Treatment Conditions

Arabidopsis thaliana plants were grown in GM medium (1% [w/v] glucose) at 23°C/ 21°C in 16-h-light/8-h-dark cycles unless otherwise indicated. *ago1-25*, *ago1-27*, and *dcl1-9* were obtained from N. Baumberger and C. Dean, respectively. *ProSPL9:SPL9-GUS*, *ProSPL9:rSPL9-GUS*, and *suo-2* (Yang et al., 2012) were obtained from S. Poethig. *Pro35S:MIM156* and *Pro35S:MIMIPS1 Agrobacterium tumefaciens* stocks, *hsp101* (N566394), and *hsfa2* (N508978) were obtained from the Nottingham Arabidopsis Stock Centre.

HS treatments were performed in a water bath or incubator. For the priming HS (ACC), 4-d-old seedlings were subjected to a heat regime of 1 h, 37°C; 1.5 h, 23°C; and 45 min, 44°C, except in Figure 6C, where a short ACC was applied omitting the 44°C step. All priming treatments were started 6 h after dawn to ensure comparability and minimize diurnal effects. Nine hours after dawn, a tester HS (44°C only) was applied 2 d later for 90 to 115 min or 3 d later for 80 to 90 min, as indicated in the figures. After the short ACC (Figure 6C), the tester HS was applied 2 d later for 60 min or 3 d later for 45 min. For acquisition of thermotolerance, the regime was 1 h, 37°C; 1.5 h, 23°C; and 0 to 180 min, 44°C; basal thermotolerance was assayed by incubation for 0 to 60 min at 44°C without prior treatment at 37°C. After heat treatments, plants were returned to normal growth conditions until analysis. For analysis of gene expression during maintenance of acquired thermotolerance, 4-d-old seedlings were subjected to ACC or mock treatment and harvested at the indicated time points. Samples of time-course experiments were harvested at the same time of day throughout the experiment to minimize diurnal effects wherever possible.

Construction of Transgenic Lines

For *ProSPL2:rSPL2*, a 3.9-kb promoter fragment was amplified with primers (all primer sequences are given in Supplemental Table 3) introducing *Nco*I and *Sac*I restriction sites. The coding sequence and 3′ region (2.5 kb) was amplified, mutating the *miR156* binding site while preserving the amino acid sequence with primers introducing *Sac*I and *Xma*I sites (Wang et al., 2008). After sequencing, the *ProSPL2:rSPL2* construct was assembled in *pUC-ML939* and transferred via *Asc*I into *pBarMAP-ML516*. For *ProSPL11:rSPL11*, a 2.6-kb promoter fragment was amplified with primers introducing *Nco*I and *Sac*I restriction sites. The coding sequence and 3′ region (2.5 kb) was amplified, mutating the *miR156* binding site while preserving the amino acid sequence with primers introducing *Sac*I and *Xma*I sites. After sequencing, the *ProSPL11:rSPL11* construct was assembled in *pUC-ML939* and transferred via *Asc*I into *pBarMAP-ML516*. Transgenic plants were generated using the floral dip method (Clough and Bent, 1998). For *Pro35S:MIR156h*, a 0.5-kb fragment encompassing the *pri-miR156h* sequence was amplified introducing *Xho*I and *Bam*HI restriction sites and subcloned in *ML595*. *MIM156h* was generated using specific primer sequences analogous to *MIM156* (Franco-Zorrilla et al., 2007). *ProHSP21:MIR156h* and *PRoHSP21:MIM156h* were generated by replacing the *HSP21* coding sequence in a 2.8-kb genomic fragment with the *MIR156h* and *MIM156h* fragments described above. All experiments were performed with multiple independent homozygous lines carrying a single insertion (determined by segregation analysis).

RNA Preparation, Microarray Analysis, and qRT-PCR

RNA was extracted from seedlings using a Trizol-based protocol (Pant et al., 2009) or a hot-phenol RNA extraction protocol (Bäurle et al., 2007). For microarray analysis, RNA from three biological replicates per genotype and treatment (4 h after ACC, 52 h after ACC, and 4 h ntc) was purified over an RNeasy Plant RNA extraction column (Qiagen) and processed for hybridization of Affymetrix ATH1 GeneChips (NASC microarray facility). The times were chosen to harvest all samples at the same time of day. For qRT-PCR, total RNA was treated with TURBO DNA-free (Ambion), and 2 μg was reverse transcribed with SuperScript III (Invitrogen) according to the manufacturers' instructions. cDNA was diluted 1:100 into qPCR reactions with GoTaq qPCR Master Mix (Promega) and an Applied Biosystems AB7300 or Roche LightCycler 480 instrument. Expression was normalized to *TUBULIN6* using the comparative CT method. Primer sequences are listed in Supplemental Table 3. Time of sampling was chosen to match either the 0- or 4-h time point (all harvesting was done at the same time of day), without noticeable difference between experiments. Robust expression at low levels was detected in ntc controls for all HS-responsive genes in microarray and qRT-PCR experiments. Gene Ontology term enrichment was calculated with the AmiGO term enrichment tool (Boyle et al., 2004).

Primary miRNA Profiling, miRNA Target Prediction, and Mature miRNA Detection

The pri-miRNA profiling was performed as described (Pant et al., 2009). The miRNA target prediction was performed using online search tools with the default settings (Moxon et al., 2008; Ossowski et al., 2008; Alves et al., 2009; Dai and Zhao, 2011) and published degradome data (Addo-Quaye et al., 2008). For detection of mature miRNA, a modified stem-loop qRT-PCR protocol was used with SYBR Green as a dye (Chen et al., 2005; Pant et al., 2008).

MUG Assay

To quantify GUS protein level, MUG activity was determined with a fluorescence spectrophotometer according to standard procedures (Jefferson et al., 1987).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: *AGO1* (*AT1G48410*), *DCL1* (*AT1G01040*), *HSFA2* (*At2G26150*), *APX2* (*AT3G09640*), *HSA32* (*At4G21320*), *HSP17.6A* (*AT5G12030*), *HSP22.0* (*AT4G10250*), *HSP70* (*At3G12580*), *At5G38610*, *At5G40510*, *SPL2* (*AT5G43270*), *SPL9* (*AT2G42200*), *SPL11* (*AT1G27360*), *MIR156A* (*AT2G25095*), *MIR156B* (*AT4G30972*), *MIR156C* (*AT4G31877*), *MIR156D* (*AT5G10945*), *MIR156E* (*AT5G11977*), *MIR156F* (*AT5G26147*), *MIR156G* (*AT2G19425*), *MIR156H* (*AT5G55835*), *HSP101* (*AT1G74310*), *SUO* (*At3G48050*), and *HSP21* (*At4G15802*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Acquisition of Thermotolerance and Basal Thermotolerance in *ago1-25* and GO Term Frequency Analysis of ATH1 Transcriptome Profiling.

Supplemental Figure 2. *MIR156h* and *SPL2*, *9*, *11* Expression Levels (Additional Time Points) and Primer Positions.

Supplemental Figure 3. Expression Profile of New *miR156h* Target Genes after a Priming HS and Requirement of *SUO* and the miRNA Binding Site of *SPL11* for Maintenance of Acquired Thermotolerance.

Supplemental Figure 4. Further Characterization of *Pro35S:MIM156* and Pro*35S:miR156h*.

Supplemental Figure 5. Further Characterization of Heat-Inducible *miR156*-Manipulating Lines.

Supplemental Table 1. *miR156a-f* and *miR156h* Targeted Genes Predicted by at Least Two Search Tools and Their Gene Description and Expression.

Supplemental Table 2. Average Flowering Time (Days and Terminal Leaf Number) and Leaf Initiation Rate of *miR156*-Manipulating Transgenic Lines.

Supplemental Table 3. Sequences of Oligonucleotides Used in This Study.

Supplemental Data Set 1. Transcriptome Profiling in Col and *ago1-25* at 4 and 52 h after HS.

Supplemental Data Set 2. Profiling of Heat-Responsive Primary miRNAs.

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AUTHOR CONTRIBUTIONS

A.S., S.A., W.S., and I.B. designed research. A.S., S.A., K.H., B.D.P., and I.B. performed research. A.S., B.D.P., W.S., and I.B. analyzed data. A.S. and I.B. wrote the article.

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REFERENCES

Addo-Quaye, C., Eshoo, T.W., Bartel, D.P., and Axtell, M.J. (2008). Endogenous siRNA and miRNA targets identified by sequencing of the Arabidopsis degradome. Curr. Biol. **18:** 758–762.

Alves, L., Jr., Niemeier, S., Hauenschild, A., Rehmsmeier, M., and Merkle, T. (2009). Comprehensive prediction of novel microRNA targets in *Arabidopsis thaliana*. Nucleic Acids Res. **37:** 4010–4021.

Arazi, T., Talmor-Neiman, M., Stav, R., Riese, M., Huijser, P., and Baulcombe, D.C. (2005). Cloning and characterization of micro-RNAs from moss. Plant J. **43:** 837–848.

Axtell, M.J. (2013). Classification and comparison of small RNAs from plants. Annu. Rev. Plant Biol. **64:** 137–159.

Axtell, M.J., Snyder, J.A., and Bartel, D.P. (2007). Common functions for diverse small RNAs of land plants. Plant Cell **19:** 1750–1769.

Bäurle, I., and Dean, C. (2006). The timing of developmental transitions in plants. Cell **125:** 655–664.

Bäurle, I., Smith, L., Baulcombe, D.C., and Dean, C. (2007). Widespread role for the flowering-time regulators FCA and FPA in RNA-mediated chromatin silencing. Science **318:** 109–112.

Boyle, E.I., Weng, S., Gollub, J., Jin, H., Botstein, D., Cherry, J.M., and Sherlock, G. (2004). GO:TermFinder—open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. Bioinformatics **20:** 3710–3715.

Cardon, G., Höhmann, S., Klein, J., Nettesheim, K., Saedler, H., and Huijser, P. (1999). Molecular characterisation of the Arabidopsis SBP-box genes. Gene **237:** 91–104.

Charng, Y.Y., Liu, H.C., Liu, N.Y., Chi, W.T., Wang, C.N., Chang, S.H., and Wang, T.T. (2007). A heat-inducible transcription factor, HsfA2, is required for extension of acquired thermotolerance in Arabidopsis. Plant Physiol. **143:** 251–262.

Charng, Y.Y., Liu, H.C., Liu, N.Y., Hsu, F.C., and Ko, S.S. (2006). Arabidopsis Hsa32, a novel heat shock protein, is essential for acquired thermotolerance during long recovery after acclimation. Plant Physiol. **140:** 1297–1305.

Chen, C., et al. (2005). Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. **33:** e179.

Cho, S.H., Coruh, C., and Axtell, M.J. (2012). miR156 and miR390 regulate tasiRNA accumulation and developmental timing in *Physcomitrella patens*. Plant Cell **24:** 4837–4849.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J. **16:** 735–743.

Dai, X., and Zhao, P.X. (2011). psRNATarget: a plant small RNA target analysis server. Nucleic Acids Res. **39:** W155–W159.

Ding, Y., Fromm, M., and Avramova, Z. (2012). Multiple exposures to drought 'train' transcriptional responses in Arabidopsis. Nat. Commun. **3:** 740.

Fahlgren, N., Jogdeo, S., Kasschau, K.D., Sullivan, C.M., Chapman, E.J., Laubinger, S., Smith, L.M., Dasenko, M., Givan, S.A., Weigel, D., and Carrington, J.C. (2010). MicroRNA gene evolution in *Arabidopsis lyrata* and *Arabidopsis thaliana*. Plant Cell **22:** 1074–1089.

Fornara, F., de Montaigu, A., and Coupland, G. (2010). SnapShot: Control of flowering in Arabidopsis. Cell **141:** 550, e1–e2.

Franco-Zorrilla, J.M., Valli, A., Todesco, M., Mateos, I., Puga, M.I., Rubio-Somoza, I., Leyva, A., Weigel, D., García, J.A., and Paz-Ares, J. (2007). Target mimicry provides a new mechanism for regulation of microRNA activity. Nat. Genet. **39:** 1033–1037.

Fujii, H., Chiou, T.J., Lin, S.I., Aung, K., and Zhu, J.K. (2005). A miRNA involved in phosphate-starvation response in Arabidopsis. Curr. Biol. **15:** 2038–2043.

Gems, D., and Partridge, L. (2008). Stress-response hormesis and aging: "that which does not kill us makes us stronger." Cell Metab. **7:** 200–203.

Gou, J.Y., Felippes, F.F., Liu, C.J., Weigel, D., and Wang, J.W. (2011). Negative regulation of anthocyanin biosynthesis in Arabidopsis by a miR156-targeted SPL transcription factor. Plant Cell **23:** 1512–1522.

Huijser, P., and Schmid, M. (2011). The control of developmental phase transitions in plants. Development **138:** 4117–4129.

Ito, H., Gaubert, H., Bucher, E., Mirouze, M., Vaillant, I., and Paszkowski, J. (2011). An siRNA pathway prevents transgenerational retrotransposition in plants subjected to stress. Nature **472:** 115–119.

Jacobsen, S.E., Running, M.P., and Meyerowitz, E.M. (1999). Disruption of an RNA helicase/RNAse III gene in Arabidopsis causes unregulated cell division in floral meristems. Development **126:** 5231–5243.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6:** 3901–3907.

Jenks, M.A., and Hasegawa, P.M. (2005). Plant Abiotic Stress. (Oxford, UK: Blackwell).

Jones-Rhoades, M.W., and Bartel, D.P. (2004). Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Mol. Cell **14:** 787–799.

Jones-Rhoades, M.W., Bartel, D.P., and Bartel, B. (2006). MicroRNAS and their regulatory roles in plants. Annu. Rev. Plant Biol. **57:** 19–53.

Karp, X., and Ambros, V. (2012). Dauer larva quiescence alters the circuitry of microRNA pathways regulating cell fate progression in *C. elegans*. Development **139:** 2177–2186.

Khraiwesh, B., Zhu, J.K., and Zhu, J. (2012). Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. Biochim. Biophys. Acta **1819:** 137–148.

Kim, J.J., Lee, J.H., Kim, W., Jung, H.S., Huijser, P., and Ahn, J.H. (2012). The microRNA156-SQUAMOSA PROMOTER BINDING PROTEIN-LIKE3 module regulates ambient temperature-responsive flowering via FLOWERING LOCUS T in Arabidopsis. Plant Physiol. **159:** 461–478.

Kozomara, A., and Griffiths-Jones, S. (2011). miRBase: integrating microRNA annotation and deep-sequencing data. Nucleic Acids Res. **39:** D152–D157.

Kruszka, K., Pieczynski, M., Windels, D., Bielewicz, D., Jarmolowski, A., Szweykowska-Kulinska, Z., and Vazquez, F. (2012). Role of microRNAs and other sRNAs of plants in their changing environments. J. Plant Physiol. **169:** 1664–1672.

Larkindale, J., Mishkind, M., and Vierling, E. (2005). Plant responses to high temperature. In Plant Abiotic Stress, M.A. Jenks and P.M. Hasegawa, eds (Oxford, UK: Blackwell), pp. 100–144.

Le Masson, I., Jauvion, V., Bouteiller, N., Rivard, M., Elmayan, T., and Vaucheret, H. (2012). Mutations in the Arabidopsis H3K4me2/3 demethylase JMJ14 suppress posttranscriptional gene silencing by decreasing transgene transcription. Plant Cell **24:** 3603–3612.

Ma, Z., Coruh, C., and Axtell, M.J. (2010). *Arabidopsis lyrata* small RNAs: transient MIRNA and small interfering RNA loci within the *Arabidopsis* genus. Plant Cell **22:** 1090–1103.

Meiri, D., and Breiman, A. (2009). Arabidopsis ROF1 (FKBP62) modulates thermotolerance by interacting with HSP90.1 and affecting the accumulation of HsfA2-regulated sHSPs. Plant J. **59:** 387–399.

Mittler, R., Finka, A., and Goloubinoff, P. (2012). How do plants feel the heat? Trends Biochem. Sci. **37:** 118–125.

Morel, J.B., Godon, C., Mourrain, P., Béclin, C., Boutet, S., Feuerbach, F., Proux, F., and Vaucheret, H. (2002). Fertile hypomorphic ARGONAUTE (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance. Plant Cell **14:** 629–639.

Moxon, S., Schwach, F., Dalmay, T., Maclean, D., Studholme, D.J., and Moulton, V. (2008). A toolkit for analysing large-scale plant small RNA datasets. Bioinformatics **24:** 2252–2253.

Navarro, L., Jay, F., Nomura, K., He, S.Y., and Voinnet, O. (2008). Suppression of the microRNA pathway by bacterial effector proteins. Science **321:** 964–967.

Ossowski, S., Schwab, R., and Weigel, D. (2008). Gene silencing in plants using artificial microRNAs and other small RNAs. Plant J. **53:** 674–690.

Pant, B.D., Buhtz, A., Kehr, J., and Scheible, W.-R. (2008). MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. Plant J. **53:** 731–738.

Pant, B.D., Musialak-Lange, M., Nuc, P., May, P., Buhtz, A., Kehr, J., Walther, D., and Scheible, W.-R. (2009). Identification of nutrient-responsive Arabidopsis and rapeseed microRNAs by comprehensive real-time polymerase chain reaction profiling and small RNA sequencing. Plant Physiol. **150:** 1541–1555.

Peaucelle, A., Louvet, R., Johansen, J.N., Höfte, H., Laufs, P., Pelloux, J., and Mouille, G. (2008). Arabidopsis phyllotaxis is controlled by the methyl-esterification status of cell-wall pectins. Curr. Biol. **18:** 1943–1948.

Pecinka, A., Dinh, H.Q., Baubec, T., Rosa, M., Lettner, N., and Mittelsten Scheid, O. (2010). Epigenetic regulation of repetitive elements is attenuated by prolonged heat stress in Arabidopsis. Plant Cell **22:** 3118–3129.

Posé, D., Verhage, L., Ott, F., Yant, L., Mathieu, J., Angenent, G.C., Immink, R.G., and Schmid, M. (2013). Temperature-dependent regulation of flowering by antagonistic FLM variants. Nature **503:** 414–417.

Rhoades, M.W., Reinhart, B.J., Lim, L.P., Burge, C.B., Bartel, B., and Bartel, D.P. (2002). Prediction of plant microRNA targets. Cell **110:** 513–520.

Richter, K., Haslbeck, M., and Buchner, J. (2010). The heat shock response: life on the verge of death. Mol. Cell **40:** 253–266.

Rogers, K., and Chen, X. (2013). Biogenesis, turnover, and mode of action of plant microRNAs. Plant Cell **25:** 2383–2399.

Salisbury, F.B., and Spomer, G.G. (1964). Leaf temperatures of alpine plants in the field. Planta **60:** 479–505.

Sani, E., Herzyk, P., Perrella, G., Colot, V., and Amtmann, A. (2013). Hyperosmotic priming of Arabidopsis seedlings establishes a long-term somatic memory accompanied by specific changes of the epigenome. Genome Biol. **14:** R59.

Scharf, K.D., Berberich, T., Ebersberger, I., and Nover, L. (2012). The plant heat stress transcription factor (Hsf) family: structure, function and evolution. Biochim. Biophys. Acta **1819:** 104–119.

Skirycz, A., and Inzé, D. (2010). More from less: plant growth under limited water. Curr. Opin. Biotechnol. **21:** 197–203.

Sunkar, R., and Zhu, J.K. (2004). Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. Plant Cell **16:** 2001–2019.

Sunkar, R., Li, Y.F., and Jagadeeswaran, G. (2012). Functions of microRNAs in plant stress responses. Trends Plant Sci. **17:** 196–203.

Todesco, M., Rubio-Somoza, I., Paz-Ares, J., and Weigel, D. (2010). A collection of target mimics for comprehensive analysis of microRNA function in *Arabidopsis thaliana*. PLoS Genet. **6:** e1001031.

Voinnet, O. (2009). Origin, biogenesis, and activity of plant microRNAs. Cell **136:** 669–687.

Wang, J.W., Czech, B., and Weigel, D. (2009). miR156-regulated SPL transcription factors define an endogenous flowering pathway in *Arabidopsis thaliana*. Cell **138:** 738–749.

Wang, J.W., Schwab, R., Czech, B., Mica, E., and Weigel, D. (2008). Dual effects of miR156-targeted SPL genes and CYP78A5/KLUH on plastochron length and organ size in *Arabidopsis thaliana*. Plant Cell **20:** 1231–1243.

Wigge, P.A. (2013). Ambient temperature signalling in plants. Curr. Opin. Plant Biol. **16:** 661–666.

Wu, G., and Poethig, R.S. (2006). Temporal regulation of shoot development in *Arabidopsis thaliana* by miR156 and its target SPL3. Development **133:** 3539–3547.

Wu, G., Park, M.Y., Conway, S.R., Wang, J.W., Weigel, D., and Poethig, R.S. (2009). The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. Cell **138:** 750–759.

Xin, M., Wang, Y., Yao, Y., Xie, C., Peng, H., Ni, Z., and Sun, Q. (2010). Diverse set of microRNAs are responsive to powdery mildew infection and heat stress in wheat (*Triticum aestivum* L.). BMC Plant Biol. **10:** 123.

Yang, L., Wu, G., and Poethig, R.S. (2012). Mutations in the GW-repeat protein SUO reveal a developmental function for microRNA-mediated

translational repression in Arabidopsis. Proc. Natl. Acad. Sci. USA **109:** 315–320.

Yang, L., Xu, M., Koo, Y., He, J., and Poethig, R.S. (2013). Sugar promotes vegetative phase change in *Arabidopsis thaliana* by repressing the expression of MIR156A and MIR156C. Elife **2:** e00260.

Yeh, C.H., Kaplinsky, N.J., Hu, C., and Charng, Y.Y. (2012). Some like it hot, some like it warm: phenotyping to explore thermotolerance diversity. Plant Sci. **195:** 10–23.

Yu, S., Cao, L., Zhou, C.M., Zhang, T.Q., Lian, H., Sun, Y., Wu, J., Huang, J., Wang, G., and Wang, J.W. (2013). Sugar is an endogenous cue for juvenile-to-adult phase transition in plants. Elife **2:** e00269.

Yu, X., Wang, H., Lu, Y., de Ruiter, M., Cariaso, M., Prins, M., van Tunen, A., and He, Y. (2012). Identification of conserved and novel microRNAs that are responsive to heat stress in *Brassica rapa*. J. Exp. Bot. **63:** 1025–1038.