Stress-Induced Alternative Splicing Provides a Mechanism for the Regulation of MicroRNA Processing in Arabidopsis thaliana

Kang Yan,1 Peng Liu,1 Chang-Ai Wu,1 Guo-Dong Yang,1 Rui Xu,1 Qian-Huan Guo,1 Jin-Guang Huang,1 and Cheng-Chao Zheng1,2

1State Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University, Taian, Shandong 271018, P.R. China
*Correspondence: cczheng@sdau.edu.cn
http://dx.doi.org/10.1016/j.molcel.2012.08.032

SUMMARY

MicroRNAs (miRNAs) have emerged as a class of regulators of gene expression through posttranscriptional degradation or translational repression in living cells. Increasing evidence points to the important relationship between miRNAs and environmental stress responses, but the regulatory mechanisms in plants are poorly understood. Here, we found that Arabidopsis thaliana intronic miR400 was cotranscribed with its host gene (At1g32583) and downregulated by heat treatment. Intriguingly, an alternative splicing (AS) event that occurred in the intron (306 bp) where MIR400 was located was specifically induced by heat stress. A 100 bp fragment was excised, and the remaining 206 bp intron containing MIR400 transcripts was retained in the host gene. The stress-induced AS event thus resulted in greater accumulation of miR400 primary transcripts and a low level of mature miR400. Together, these results provide the direct evidence that AS acts as a regulatory mechanism linking miRNAs and environmental stress in plants.

INTRODUCTION

Environmental stress factors seriously influence plant growth and development. Extensive agricultural losses are attributed to temperature, often in combination with drought or other stresses (Zhu, 2002). A number of genes were proved to be involved in plant responses to various abiotic stresses (Achard et al., 2006; Baena-González et al., 2007). The regulation of gene expression is mainly controlled at the transcriptional level. Pre-miRNAs undergo splicing and are then translated into proteins. The introns in pre-miRNAs are removed, and the exons are joined to generate mature miRNAs (Moore and Proudfoot, 2009; Zhou et al., 2002). Alternative splicing (AS) generates two or more miRNAs from the same pre-mRNA with multiple introns by using different splice sites (SSs) (Brown and Simpson, 1998; Luco et al., 2011).

Previous studies have indicated that AS is common in plants and contributes to both transcriptome and proteome diversity (Filichkin et al., 2010; Ramani et al., 2011). Several reports have shown that various environmental stresses influence AS of pre-miRNAs, including some stress-response genes (Mach, 2009; Sugliani et al., 2010). For example, one study demonstrated that ultraviolet (UV) irradiation induced cell apoptosis by affecting the expression of apoptotic genes in an AS-dependent way, suggesting that transcriptional coupling due to AS is a key feature of the DNA damage response (Muñoz et al., 2009). Recently, it was reported that splicing variants of ABI3, a major regulator of seed maturation in plants, were influenced by a plant splicing factor (SUA) in seed germination, implying that AS participates in both ABA signaling control and plant responses to abiotic stresses (Sugliani et al., 2010). It was shown that the mutation of Rnu2-8, one of the mouse multicopy U2 snRNA genes and a conserved regulator that is essential for pre-mRNA splicing, caused ataxia and neurodegeneration (Jia et al., 2012). Also, some splice variants of serine/arginine-rich (SR) proteins, which are important splicing regulators, were identified in Arabidopsis thaliana under high- and low-temperature stress, and they might, in turn, alter the splicing of other pre-miRNAs (Barta et al., 2010; Reddy, 2007). These findings suggest that AS plays a role in regulating gene expression in both animal and plant stress responses.

Recently, miRNAs, a class of ~22 nt noncoding RNAs, have emerged as regulators of gene expression through posttranscriptional degradation or translational repression through base pairing to target miRNAs (Bartel, 2004, 2009; Voinnet, 2009). The discovery of large numbers of miRNAs and their diverse functions in both plants and animals has led to widespread agreement about their importance (Brodersen et al., 2008; Buchan and Parker, 2007). Increasing evidence has revealed that plant miRNAs play essential roles not only in basic physiological processes but also in stress responses (Wang et al., 2009; Wu et al., 2009). Several recent studies have shown that a number of stress-regulated genes encoding important transcription factors are targets of miRNAs (Reyes and Chua, 2007; Sunkar et al., 2006). However, the regulatory mechanisms linking miRNAs and environmental stress factors are rarely reported.

MicroRNAs (miRNAs) can be classified as either intergenic miRNAs or intronic miRNAs; the former are located in noncoding regions between genes and transcribed by their own promoters, while the latter are located within intronic regions and are derived from the introns of their host genes (Brown et al., 2008; Shomron and Levy, 2009). Generally, intronic miRNAs share the same
promoter with their host genes and are transcribed by RNA polymerase II as parts of their hosting transcription units at correlated levels (Liu et al., 2008b; Rodriguez et al., 2004). In animals, pre-mRNA splicing has been shown to participate in both intergenic and intronic miRNA processing (Berezikov et al., 2007; Morlango et al., 2008). In only a few cases, a distinct class of intronic miRNAs bypasses the Microprocessor via generation of mirtrons (short hairpin introns), implying that splicing activities may replace the function of the Microprocessor (Berezikov et al., 2007; Ruby et al., 2007). More recent studies in humans have shown that splicing signals play important roles connecting splicing activities and miRNA processing (Janas et al., 2011). To date, the mechanism of how splicing influences intronic miRNA biogenesis in plants remains unknown.

As of three plant miRNA primary transcripts (miR172b, miR163, and miR162a) has been conducted in previous studies (Aukerman and Sakai, 2003; Hirsch et al., 2006; Kurihara and Watanabe, 2004). Transcripts from the host gene containing miR162a displayed six different AS variants, and in some AS transcripts, pre-miR162a secondary structures were interrupted (Brown et al., 2008).

To our knowledge, no direct evidence of how stress-related AS regulates miRNA expression has been provided in previous research. In this study, intronic miR400 was cotranscribed with its host gene At1g32583 in various tissues of Arabidopsis. The first intron containing the miR400 hairpin experienced a specific AS event caused by heat stress, leading to a decrease of mature miR400. Based on these results, a mechanism of stress-induced AS affecting miRNA processing in plants is proposed. The findings of this study extend the current view about the regulatory mechanism of miRNAs.

RESULTS

Intronic miR400 Is Cotranscribed with the Host Gene in Various Tissues of Arabidopsis

Previously, we analyzed the effects of 117 miRNAs under high-salinity, drought, and low-temperature stress conditions with miRNA chips representing dozens of stress-related miRNAs in Arabidopsis (Liu et al., 2008a). Among them, the level of mature miR400 is obviously increased by environmental stress. miR400 (At1g32582) was detected for only one locus in Arabidopsis and Brassica rapa (Fahlgren et al., 2010; Yu et al., 2012), but no detailed analysis is available. Sequence analysis showed that miR400 derived from the first intron of At1g32583 in various tissues of Arabidopsis and Brassica rapa (Fahlgren et al., 2010; Yu et al., 2012), but no detailed analysis is available. Sequence analysis showed that miR400 derived from the first intron of At1g32583 in the 5’UTR, and the full-length At1g32583 contained 2,481 nucleotides, including three exons and three introns (Figure 1A). To understand the regulatory mechanism of the temperature affecting miR400 expression, we performed various transcript analyses. Quantitative real-time PCR results showed a similar expression pattern of MIR400 and its host gene (Figures 1B–1D). Both MIR400 and its host gene were expressed in various tissues of Arabidopsis; their expression was strongest in siliques; moderate in rosette leaves, flowers, and roots; and weakest in cauline leaves and stems (Figures 1B–1D). These results indicated that miR400 and its host gene might derive from a single primary transcript unit. Further reverse transcription analysis using total RNA with a gene-specific primer that was complementary to the downstream of the host gene showed that the full-length transcript of the host gene containing primary miR400 was amplified (see Figure S1 online), confirming that
miR400 was an intronic miRNA released from the 5'UTR of its host gene.

In order to investigate the detailed expression pattern of miR400 and its host gene, a 1 kb putative promoter sequence upstream from the predicted fold-back structure of miR400 precursor was isolated and fused to the coding region of β-glucuronidase (GUS) to generate promoter::GUS transgenic lines. Histochemical GUS staining showed that miR400 was constitutively highly expressed in leaf, root, silique, and floral tissues (Figure S2). Interestingly, with seedling growth, GUS staining in the root system was mainly restricted to the primary and lateral root tips and vascular tissues (Figure S2). The tissue pattern of GUS staining was consistent with qRT-RCR analysis. Taken together, these results show that miR400 was encoded in the intron of At1g32583 and cotranscribed with the host gene as a single unit in various tissues.

Heat Stress Suppresses Mature miR400 Expression but Upregulates MIR400 Transcripts

To investigate the relationship between miR400 expression and heat stress in Arabidopsis, we performed qRT-PCR and northern blot analysis using 2-week-old seedlings exposed to a temperature of 37°C for different lengths of time ranging from 0.5 to 12 hr. Surprisingly, expression of MIR400 and its host gene displayed opposite responses to heat stress. The level of host gene At1g32583 mRNA was slightly reduced, whereas MIR400 primary transcripts increased significantly under the same condition (Figure 2D). After 0.5 hr of heat stress, MIR400 transcripts were downregulated in accordance with the host gene. However, after 12 hr of treatment, MIR400 transcripts were upregulated approximately six times more than in the untreated condition. For mature miR400, expression was continuously downregulated by heat stress (Figures 2C and 2E). In addition, promoter::GUS analysis showed a similar expression pattern for the host gene’s response to heat treatment (Figures 2A and 2B).

The above results raised two interesting issues. First, under heat stress, the expression level of the host gene was in accordance with the promoter activity, so why did MIR400 from the same transcript unit show a different response? Second, why was the expression of mature miR400 significantly inhibited against the increased level of miR400 primary transcripts? To our knowledge, transcriptional regulation alone cannot address these two issues. Therefore, we suggest that, in addition to transcriptional inhibition, other regulation mechanisms in response to heat stress might play important roles.

A Heat-Induced Alternative Splicing Leads to an Accumulation of MIR400 Primary Transcripts

To gain clues about the accumulation of MIR400 primary transcripts under heat stress, we performed RT-PCR analysis using different primers flanking the miRNA region (intron 1 primers, Figure 3A and Figure S3). Intriguingly, pre-mRNAs underwent an AS event in the first intron of the host gene downstream of MIR400 transcripts under heat treatment (Figures 3A and 3C). A 100 bp excised fragment from the 3' end of the first intron was detected (Figures 3A–3C). To determine whether AS affected the excision of the first intron, we conducted RT-PCR analysis using different primers flanking the first intron region in the 5'UTR (UTR primers, Figure 3A and Figure S3). Surprisingly, the results showed that a new isoform was found, and the 206 bp fragments of the remaining region of the first intron were still retained in the 5'UTR, including the pri-miR400 sequence (Figures 3A...
Figure 3. Alternative Splicing Event of the miR400-Containing Intron in Response to Heat Stress

(A) Diagrammatic representation of the AS isoforms as deduced from sequencing products under heat treatment or untreated (intron 1 mock, intron 1 heat, UTR mock, and UTR heat). The arrow indicates the position of the RT-PCR primers which were used in RT-PCR experiments (intron 1 primers and UTR primers). Numbers beside each region indicate the length of fragment.

(B) Proposed splicing models of intron containing the miR400 hairpin. Different events (1–3) were kept separate by the curve. Event 1, Unspliced pre-mRNA hosting the intron; event 2, the intron spliced out and the primary miR400 released from the pre-mRNA; event 3, triggered by heat stress, part of intron containing miR400 hairpin retained instead. Events 1 and 2 occurred in any conditions, and event 3 only occurred in response to heat stress.

(C) Heat stress triggered the AS event specially. RT-PCR primer locations were shown in (A).

(D and E) RT-PCR analysis of temperature and time course of AS in response to heat stress. Tubulin was used as the loading control. A no-RT control (−) was run for each RNA sample to ensure that no DNA contamination was present.

(F) qRT-PCR analysis of the nascent or partially spliced transcripts containing primary miR400. Different RT primers were used to distinguish these transcripts, and error bars represent SE for three independent experiments. See also Figure S3.

and 3C, Figure S3). Sequence analysis of the AS products indicated that the host gene locus produced two isoforms under heat stress, which were generated by alternative 5' SS selection (Figure 3B). Thus, we suggest that the heat-triggered selection of downstream 5′ splice donor site interfered with the splicing of the remaining region of the first intron containing miR400, resulting in the accumulation of MIR400 transcripts.

However, as shown in Figures 3D and 3E, the original spliced mRNA (where the first intron was entirely spliced) was still detected by RT-PCR analysis, but its level gradually decreased during the heat treatment. qRT-PCR analysis also revealed that only the AS transcripts accumulated upon a decrease of original splicing events, whereas the levels of nascent pre-mRNA and hostgene transcripts remained almost unchanged (Figure 3F, Figure S3), suggesting that heat stress not only induced AS but also inhibited original splicing events. In addition, few nascent mRNA transcripts existed in the total RNA extracted from both heat-treated and untreated Arabidopsis plants. We therefore suggest an interpretation of the decreasing mature miR400 levels based on the accumulation of MIR400 transcripts under heat stress. In normal conditions, miR400 primary transcripts were released from the intron, which then generated mature miR400, whereas in heat stress conditions, AS affected the splicing of the miR400-containing intron by influencing miR400 processing (Figure 3B).

Additionally, other types of stress such as high salinity, drought, and cold could not trigger the AS event, indicating that AS might be triggered specifically by heat stress (Figure 3C).

Heat-Induced Alternative Splicing Event Inhibited Mature miR400 Expression

To further understand how AS influences intronic miR400 processing, we generated four intronic miR400 constructs to directly test the effects of different splicing isoforms in miR400 processing. A control reporter (MIR400 - hostgene) contained the genomic sequence of the full-length host gene carrying the miR400. A mutant reporter (MIR400 -AS- hostgene) was generated, and it contained the full-length sequence of the host gene, along with the deletion of the 100 bp intron fragments that appeared in the AS event to mimic the AS isoform under heat stress. Two other mutant reporters were constructed that only carried the first intron sequence containing the MIR400 sequence with or without the 100 bp excised fragments (MIR400 - intron and MIR400 -AS- intron, respectively) to mimic
the spliced intron (Figure 4A). The CaMV 35S promoter was used to drive four miR400 reporters for plant transformation. An Agrobacterium tumefaciens-mediated transient coexpression assay in Nicotiana benthamiana was performed using a construct containing MIR397a as an internal reference. The results showed that for the full-length host gene reporters, the deletion of heat-induced AS fragments resulted in a significantly decreased level of mature miR400, whereas for the separated first intron reporters, the 100 bp AS fragments did not affect mature miR400 processing (Figure 4B).

To confirm the transient expression results, we generated transgenic Arabidopsis plants to quantify mature miR400 expression in normal growth conditions and heat stress treatments. The northern blot and quantitative RT-PCR analysis results indicated that under normal growth conditions, in the mutant full-length reporter (MIR400 -AS- host gene) transgenic lines, miR400 expression was remarkably inhibited. In contrast, in the separated first intron reporter lines, no obvious difference in mature miR400 levels were observed with or without the 100 bp fragments (Figures 4C and 4D). Under heat stress conditions, in the full-length reporter (MIR400 - host gene) transgenic lines, the levels of mature miR400 were significantly reduced compared to those under normal conditions, whereas no differences were detected in other reporter lines (Figures 4C and 4D). Thus, the mature miR400 expression was inhibited in the heat-induced AS isoform, indicating that AS-mediated regulation resulted in decreased miR400 level by holding the 206 bp retained intron with the primary miR400 hairpin in the 5′UTR of the host genes. We concluded that the occurrence of heat-induced AS event inhibited the miR400 expression by affecting the normal processing of mature miR400 in Arabidopsis.

Accurate Recognition of the Splicing Signals Likely Mediates Normal Processing of miR400

The above results showed that the secondary structure of primiR400 was not interrupted in the heat-induced AS event, which could be folded to enable processing of the miRNA when the holding intron is spliced out of the pre-mRNA (Figures 4B–4D). In RNA splicing, an intron usually contains a clear signal that helps it to be recognized for splicing, and a masked splicing signal always results in a AS event. This raises the question of which splicing signals changed that led to inhibiting the miR400 expression under heat stress.

To further understand how AS suppresses miR400 expression, we performed a detailed sequence analysis of the first intron in the host gene. Sequence analysis revealed that two
isoforms were generated by alternative 5′ SS selection, and two 5′ donor sites (5′ SS and 5′ AS) competed with each other in response to heat stress. Moreover, a putative branchpoint (BP) was identified within the 100 bp excised AS fragments (Figure 5A).

To directly test the effects of these splicing signals on miR400 biogenesis, we introduced point mutations in the consensus sequences of the 5′ SS, 5′ AS, BP, and polypyrimidine tract in miR400-containing intron, respectively. qRT-PCR results showed these mutations have different effects on the mature miR400 levels in transfected Nicotiana benthamiana leaves. Interestingly, either mutations in 5′ SS or BP (BP-1) resulted in a significantly decreased level of mature miR400 under normal conditions or heat treatment (Figure 5B). In contrast, mutations in 5′ AS did not affect the miR400 production under normal conditions but resulted in the miR400 accumulation under heat stress, indicating that the 5′ AS points is a major factor influencing miR400 expression in response to heat stress (Figure 5B). In addition, mutations in polypyrimidine tract (BP-3) modestly reduced miR400 levels, while mutations to other BP (BP-2) basically had no effect on miR400 level (Figure 5B).

Moreover, we introduced 5′ SS+BP-1 or 5′ AS+BP-1 mutations to detect the relationship between these signals in miR400 processing. The results revealed that the combined mutations reduced miR400 expression to a similar level as the single mutation of 5′ SS or BP, indicating that these splicing signals are independent of each other (Figure 5B). The recognition of the pre-mRNA splicing sites and associated sequences is essential for normal processing of miR400. This finding further supports our hypothesis that AS is the major factor for inhibiting mature miR400 expression under heat stress.

**35S::MIR400 Plants Are More Sensitive to Heat Stress**

To further characterize the biological function of miR400 in heat stress, we generated transgenic Arabidopsis plants overexpressing miR400 under control of the constitutive CaMV 35S promoter (Figure S4). No phenotype differences were observed between the transgenic and wild-type plants under normal growth conditions. However, under heat stress, 35S::MIR400 seeds had a lower germination percentage and germinated more slowly compared with the wild-type seeds (Figures 6A and 6B). And the less hypocotyl elongation of 35S::MIR400 seedlings indicated a defect in acquired thermotolerance (Figure 6C). Moreover, heat treatment also reduced the root growth of 35S::MIR400 seedlings (Figure 6D). Together, these results revealed that overexpression of MIR400 made the plants more sensitive to heat stress, suggesting that miR400 might play a role in regulating the response to environmental stresses in plants.

**DISCUSSION**

**Alternative Splicing Acts as a Regulatory Mechanism Linking miRNA and Stress**

In eukaryotes, AS of pre-mRNAs contributes significantly to the proper expression of the genome and the diversity of the proteome (Jia et al., 2012; Yang et al., 2012). During the past 4 years, the estimated number of intron-containing genes undergoing AS in Arabidopsis increased 42% (Filichkin et al., 2010), suggesting that AS in plants is much more prevalent than previously thought. AS has been found to be an important regulatory process in different cell types and at different developmental stages, as well as in response to environmental cues (Luco et al., 2011;
Munoz et al., 2009). Recently, growing evidence has indicated the existence of a complex crosstalk between environmental stresses and miRNAs (Franco-Zorrilla et al., 2007; Reyes and Chua, 2007), but the regulatory mechanisms are still unclear. Here, we provide direct evidence that AS influences miRNA expression in response to stress in Arabidopsis. Heat stress suppressed intronic miR400 expression by triggering a specific AS event.

Intriguingly, we found that the heat-induced AS event occurred in the first intron where the miR400 hairpin was located, resulting in the rest of the intron carrying miR400 primary transcripts retained in the 5′UTR of the host gene, which affected miR400 processing. However, our data indicated that mature miR400 processing was unaffected when MIR400 transcripts were spliced out of the intron or when the whole intron containing miR400 was removed from the nascent mRNA (Figures 4B–4D). We therefore propose the existence of a regulatory pathway in which intronic miRNA processing in miRNA-located introns is excised after the splicing reaction and then generates mature miRNAs in plants.

It is also noteworthy that miR400 expression in the full-length mutant reporter transgenic lines (MIR400-AS; host gene) was at least twice as strong as it was in wild-type plants, suggesting that the unspliced intron did not completely block miRNA processing (Figures 4C and 4D). We cannot exclude the possibility that the unspliced intron may also participate in miRNA production. One can be sure that the efficient processing of intronic miRNAs depends on the splicing accuracy of the intron.

Thus, environmental stress may be an important factor for triggering the AS event as an efficient mechanism for miRNA expression. The miR400 case provides direct evidence of stress-induced and AS-involved miRNA-processing regulation.

To date, 17 intronic miRNAs have been discovered in Arabidopsis (Brown et al., 2008). Sequence analysis of the introns containing miRNAs showed that eight miRNAs have potential AS isoforms (Table S2), suggesting that the generation of other intronic miRNAs might be affected by AS events triggered under specific conditions in plants (as in the case of miR400). AS acts as a regulatory mechanism for miRNA expression in response to environmental cues, and we propose that the insights gained from the characterization of miR400 will be useful for studying many other miRNAs present in eukaryotic genomes. Future investigations are needed to determine whether AS might be involved in other intronic miRNA cropping in plants.

Phased Processing of Intronic miRNA in Plants May Differ from that in Animals

In animals, the miRNA pathway is initiated at transcription and generates a primary miRNA, and then a pri-miRNA is recognized and cleaved to a pre-miRNA by the Microprocessor complex, including the nuclear RNase III enzyme Drosha, its cofactor DGCR8, and other components. The pre-miRNA is exported from the nucleus to the cytoplasm, and the RNase III endonuclease Dicer further gives rise to mature miRNAs (Denli et al., 2004; Gregory et al., 2004).

Recently, two major pathways for the processing of miRNAs from introns in pre-miRNAs have been described. In one pathway, the most intronic miRNAs were processed from unspliced intron regions. The production of these miRNAs was not significantly affected by splicing, indicating that splicing is not a prerequisite for cropping (Kim and Kim, 2007; Shomron and Levy, 2009). In the other pathway, miRNAs were liberated from mirtrons (short hairpin introns) through the splicing reaction.
The Microprocessor complex was not required for processing these intronic miRNAs, which bypass the processing of the Microprocessor complex and generate mature miRNAs directly (Berezikov et al., 2007; Ruby et al., 2007). These findings suggest that although intronic miRNA biogenesis is complicated and diversified, intron splicing is not essential for most miRNA cropping and function in animals.

Based on the results of our study, we propose that the dominant pathways in plants for the processing of intronic miRNAs might be different from those in animals. In the case of intronic miR400 cropping, whether the miR400-containing intron was spliced out from the pre-mRNA is an important factor that affects miR400 production. After heat-induced AS, the complete miR400 hairpin retained in the 5′UTR of the host gene was barely able to generate mature miR400, indicating that the plant Microprocessor complex could not directly work with unspliced intronic miRNAs to accomplish miRNA processing. Thus, we propose that the splicing reaction of introns holding miRNA primary transcripts affects other intronic miRNA cropping in plants, and intronic miRNA biogenesis and splicing are interdependent and coordinated.

Two possible reasons may account for this. First, different splicing machinery exists in plants and animals due to differences in the characteristics of introns and the composition of spliceosomes. Introns in plant genes are much shorter in length and fewer in number compared with those in animal genes (Alexandrov et al., 2006; Zhou et al., 2002), suggesting that the SS recognition of introns differs in different kinds of organisms. Also, most conserved spliceosomal families are vastly expanded in plants with important proteins such as SRs, hnRNPs, and snRNPs (Jurica and Moore, 2003; Lorković et al., 2005; Wang and Brendel, 2004), indicating that specific splicing mechanisms might exist in plants. Alternatively, the plant Microprocessor complex has several functional components that are different from the animal Microprocessor complex (Schauer et al., 2002; Voinnet, 2009). For example, AGO and Dicer, respectively, play different roles in Arabidopsis miRNA processing (Vaucheret et al., 2004; Xie et al., 2005). Additionally, the homologs of Drosha and DGCR8, which are important components of the animal Microprocessor complex, have not yet been identified in plants.

Potential Roles of Splicing Signals in miRNA Cropping

Splicing signals are essential for recognition of the exon and intron boundary in pre-mRNAs. Alternative processing of the primary transcripts by recognition of different splicing signals could produce multiple mRNA isoforms from a single gene. It has been demonstrated that the short consensus sequences at the 5′ donor site, 3′ acceptor site, and BPs are the main splicing signals for pre-mRNA processing (Brett et al., 2002; Brown and Simpson, 1998; Lorković et al., 2000). However, the potential roles of splicing signals in miRNA cropping are largely unknown.

Based on our results and the preceding discussion, we present a regulatory model for the relationship between splicing signals and intronic miR400 processing (Figure 7). Normal intronic miRNA processing requires guiding of the initial cleavage of the miRNA-containing intron, and recognition of splicing signals in the intron sequence sets the phase for accurate processing. When plants are exposed to environmental stresses such as heat, the changed splicing signals could result in the occurrence of an AS event around the miRNA-containing intron, which in turn could affect the miRNA maturations of host gene expression. The accurate selection of the splicing sites in the miR400-containing intron would promote the release of miR400 primary transcripts and then fold into a secondary structure to enable miRNA cropping. We thereby conclude that AS is an important mechanism for affecting the efficiency of splicing and the accuracy of splice-site selection.

A recent study in animals showed that recognition of the 5′ SS promoted cropping of intronic miR-211, and in this process, U1 snRNP, a splicing-related protein of the spliceosome, was
involved (Janas et al., 2011). These findings strongly support our view, suggesting that recognition of the splicing signals by splicing factors plays an important role in intronic miRNA cropping in both plants and animals. Furthermore, previous studies have reported that three SR proteins (SR1/SR34, SR30, and SR34b), a family of highly conserved proteins that function as essential splicing factors in SS recognition and spliceosome assembly, are also responsive to heat stress (Barta et al., 2010; Reddy, 2007; Tanabe et al., 2007). We checked miR400 expression in these sr mutants (sr1, sr30, and sr34b) and found that the expression levels of both miR400 and miR400 were downregulated in sr30, indicating that heat-related SR30 likely plays a role in miR400 splicing event (Figure S5). But the heat-induced AS event of miR400 still occurred in these sr mutants, indicating that these SR proteins did not influence the occurrence of AS in the miR400 case directly. We suggest that some splicing factors might also affect the processing of intronic miRNAs in plants by interfering with the splicing reaction of miRNA-containing introns. To confirm the AS-regulated mechanism for intronic miRNA expression under stress conditions in plants, the identification of splicing-interacting proteins involved in miR400 AS events will be required in future work.

The last several years have spawned a vast amount of data on miRNA-mediated posttranscriptional regulations of stress-responsive genes in plants. However, very little is known about the regulation of miRNAs themselves in response to environmental stresses. In our study, strong evidence is provided that stress-related AS could be a regulatory mechanism modulating the expression of intronic miRNAs. It would be interesting to further investigate how widespread the mechanism is and whether processing of other intronic or intergenic miRNAs is governed by same or other pathways. Such investigation will be very helpful to gain more insights into the complex regulation mechanisms of miRNAs.

**EXPERIMENTAL PROCEDURES**

### Plant Material and Growth Conditions

*Arabidopsis thaliana*(Col-0) seeds were used in this study. The details for plant material and growth conditions are provided in the Supplemental Experimental Procedures.

### Transgenic Constructs

The details are provided in the Supplemental Experimental Procedures.

### Histochemical GUS Staining and Fluorometric GUS Assay

The promoter sequence of miR400 and its host gene were acquired from the TAIR database (http://www.arabidopsis.org/). The details are provided in the Supplemental Experimental Procedures.

### RNA Extraction

For RNA isolation, the plant tissues were harvested separately, frozen in liquid nitrogen, and stored at \(-80^\circ C\) until use. Total RNA was isolated from different *Arabidopsis thaliana* seedlings with TRIzol reagent (Invitrogen, Carlsbad, CA, USA).

### RT-PCR Analysis

Total RNA was extracted using TRIzol reagent from different tissues of *Arabidopsis*. Contaminated DNA was removed with RNase-free RNase I. First-strand cDNA synthesis was performed with 1 μg RNA using oligo (dT) primer or gene-specific primers and the QIAGEN one-step RT-PCR kit. Primers for amplifying genes were designed according to the sequences downloaded from the TAIR database (http://www.arabidopsis.org/). The RT-PCR experiment had been carried out at least three times under identical conditions using tubulin as an internal control. Details of primers were listed in Figure S3 and Table S1.

### Mature miRNA qRT-PCR Analysis

Small RNA for mature miRNA qRT-PCR was isolated with miRcute miRNA isolation kit (Transgen); poly(A) modification and first-strand cDNA synthesis were performed with miRcute miRNA first-strand cDNA synthesis kit (Transgen). Mature miRNA qRT-PCR analysis was performed with 0.5 μg small RNA and miRcute miRNA qRRT-PCR detection kit (Transgen). The experiments were carried out at least three times under identical conditions using U6 RNA as an internal control. Details of primers were listed in Figure S3 and Table S1.

### Small RNA Northern

Fifty micrograms of each RNA was subjected to electrophoresis on a 15% TBE-Urea Criterion gel (Bio-Rad) and electroblotted onto Hybond-N+ filter paper (Amersham) using a TransBolt-SD apparatus (Bio-Rad). The filter then was hybridized at 37°C in hybridization buffer (Clontech) with 32P-labeled probe. The filters were washed twice at 37°C in buffer containing 2× SSC (0.3 M NaCl and 0.03 M sodium citrate) and 0.5% SDS. Probe was made by end labeling an oligonucleotide that corresponded to either the sense or anti-sense strand of miR400 with T4 polynucleotide kinase (Takara) and T4P. Details of probes were listed in Table S1.

### Transient Expression in N. benthamiana

miR400-AS expression constructs were transformed into *Agrobacterium tumefaciens* strain 3101. The details are provided in the Supplemental Experimental Procedures.

### Analysis of Splicing Signals in Alternative Intron 1 Processing

Information about AS on miR400-containing intron was acquired from the Netgene2 database (http://www.cbs.dtu.dk/services/NetGene2/).

### ACCESSION NUMBERS

Sequence data from this article can be found in the *Arabidopsis* Genome Initiative or GenBank/EMBL databases under the following accession numbers: MI400 (At1g32582), host gene (At1g32583), SR1/SR34 (At1g28240), SR30 (At1g9140), SR34b (At4g02430), sr1 (SALK_106067), sr30 (SALK_132986), and sr34b (SALK_032344).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, two tables, and Supplemental Experimental Procedures and can be found with this article at http://dx.doi.org/10.1016/j.molcel.2012.08.032.

### ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation (grant numbers 31070240 and 30970225), the National Basic Research Program (grant number 2012CB114200), and the Genetically Modified Organisms Breeding Major Projects (grant number 2009ZX08009-092B) in China.

Received: March 3, 2012

Revised: June 19, 2012

Accepted: August 27, 2012

Published online: October 11, 2012

### REFERENCES

and consistent nomenclature for serine/arginine-rich protein splicing factors
Molecular Cell

Brodersen, P., Sakvarelidze-Achard, L., Bruun-Rasmussen, M., Dunoyer, P.,
Yamamoto, Y.Y., Sieburth, L., and Voinnet, O. (2008). Widespread transla-
miRNA. Science 317, 1877–1878.
Fahrenkrog, 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
Filichkin, S.A., Priest, H.D., Givan, S.A., Shen, R., Bryant, D.W., Fox, S.E.,
splicing in Arabidopsis thaliana. Genome Res. 20, 45–58.
Franco-Zorrila, J.M., Valli, A., Todesco, M., Mateos, I., Puga, M.I., Rubio-
Target mimicry provides a new mechanism for regulation of microRNA activity.
Gregory, R.L., Yan, K.P., Armutlu, G., Chengdivada, T., Doratotaj, B., Cooch,
N., and Shiekhattar, R. (2004). The Microprocessor complex mediates the
Hirsch, J., Lefort, V., Vankerschaver, M., Boualem, A., Lucas, A., Thermes, C.,
protein-coding mRNA genes in Arabidopsis, including the MIR162a-derived
Jana, M.M., Khaled, M., Schubert, S., Bernstein, J.G., Golan, D., Vugullima,
forward microprocessing and splicing activities at a microRNA-containing
1002330.
Jia, Y., Mu, J.C., and Ackerman, S.L. (2012). Mutation of a U2 snoRNA gene
causes global disruption of alternative splicing and neurodegeneration. Cell 149, 296–308.
Jurica, M.S., and Moore, M.J. (2003). Pre-mRNA splicing: awash in a sea of
26, 775–783.
(2009b). The evolution and functional diversification of animal microRNA
conservation of minor U12-type spliceosome between plants and humans.
RNA 11, 1095–1107.
Marcon, J. (2009). Alternative splicing produces a JAZ protein that is not broken
Morlando, M., Ballarin, M., Gromak, N., Pagano, F., Bozzi, I., and
Proudfoot, N.J. (2008). Primary microRNA transcripts are processed co-
Muñoz, M.J., Pérez Santangelo, M.S., Paronetto, M.P., de la Mata, M., Pelisch,
F., Boireau, S., Glover-Cutter, K., Ben-Dov, C., Bulaest, M., Lozano, J.J.,
et al. (2009). DNA damage regulates alternative splicing through inhibition of
Ramani, A.K., Kalacar, J.A., Pan, Q., Mavandadi, S., Wang, Y., Nelson, A.C.,
Genome-wide analysis of alternative splicing in Caeonohabditis elegans.
Genome Res. 21, 342–348.
Reyes, J.L., and Chua, N.H. (2007). ABA induction of miR159 controls tran-
scription levels of two MYB factors during Arabidopsis seed germination. Plant J. 49, 592–606.
Identification of mammalian microRNA host genes and transcription units.
Schauer, S.E., Jacobsen, S.E., Meinke, D.W., and Ray, A. (2002). DICER-
LIKE1: blind men and elephants in Arabidopsis development. Trends Plant
Sugliani, M., Brambilla, V., Clerkx, E.J., Koornneef, M., and Somme, W.J.
(2010). The conserved splicing factor SUA controls alternative splicing of the
two Cu/Zn superoxide dismutase genes in Arabidopsis is mediated by down-
regulation of miR398 and important for oxidative stress tolerance. Plant Cell 18, 2051–2065.
Differential expression of alternatively spliced mRNAs of Arabidopsis SR
protein homologs, atSR30 and atSR45a, in response to environmental stress.
Plant Cell Physiol. 48, 1036–1049.


