Uncovering Small RNA-Mediated Responses to Cold Stress in a Wheat Thermosensitive Genic Male-Sterile Line by Deep Sequencing

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The male sterility of thermosensitive genic male sterile (TGMS) lines of wheat (Triticum aestivum) is strictly controlled by temperature. The early phase of anther development is especially susceptible to cold stress. MicroRNAs (miRNAs) play an important role in plant development and in responses to environmental stress. In this study, deep sequencing of small RNA (smRNA) libraries obtained from spike tissues of the TGMS line under cold and control conditions identified a total of 78 unique miRNA sequences from 30 families and trans-acting small interfering RNAs (tasiRNAs) derived from two TAS3 genes. To identify smRNA targets in the wheat TGMS line, we applied the degradome sequencing method, which globally and directly identifies the remnants of smRNA-directed target cleavage. We identified 26 targets of 16 miRNA families and three targets of tasiRNAs. Comparing smRNA sequencing data sets and TaqMan quantitative polymerase chain reaction results, we identified six miRNAs and one tasiRNA (tasiRNA-ARF for Auxin-Responsive Factor) as cold stress-responsive smRNAs in spike tissues of the TGMS line. We also determined the expression profiles of target genes that encode transcription factors in response to cold stress. Interestingly, the expression of cold stress-responsive smRNAs integrated in the auxin-signaling pathway and their target genes was largely noncorrelated. We investigated the tissue-specific expression of smRNAs using a tissue microarray approach. Our data indicated that miR167 and tasiRNA-ARF play roles in regulating the auxin-signaling pathway and possibly in the developmental response to cold stress. These data provide evidence that smRNA regulatory pathways are linked with male sterility in the TGMS line during cold stress.

In flowering plants, the male gametophyte plays an important role in plant fertility and crop production through the generation and delivery of male gametes to the embryo sac for double fertilization (Borg et al., 2009). The development of the male gametophyte involves an array of extraordinary events, including the differentiation of sporogenous cells, the transition from the sporophytic to the gametophytic generation, and the modification of cell division to produce microspores (Wilson and Yang, 2004). Plants are more vulnerable at the reproductive growth stage than the vegetative growth phase to many environmental stresses, including cold stress. Recently, the molecular basis underlying the developmental responses of the anther to environmental stresses has been a subject of intense research (Tang et al., 2011).

Because microRNAs (miRNAs) and other endogenous small silencing RNAs were discovered in plants (Llave et al., 2002; Mette et al., 2002; Park et al., 2002), these small RNA (smRNA)-based silencing systems have changed our understanding of the mechanisms of transcription, translation, and regulation of gene expression. In plants, miRNAs and small interfering RNAs (siRNAs) of 21 to 24 nucleotides are two broad categories of these regulatory RNA molecules, and both types function as negative regulators of gene expression (Voinnet, 2009). The major difference between these two categories lies in their genomic origin and biogenesis. miRNA primary transcripts arise from intergenic regions via the action of RNA polymerase II (Lee et al., 2004). miRNAs are processed from their precursors by the RNase III enzyme Dicer-Like1 (DCL1) or DCL4, which digests the imperfectly base-paired miRNA:miRNA* duplexes (Kim, 2005). The mature miRNAs of the duplexes combine with protein factors...
to form RNA-induced silencing complexes. Subsequently, miRNAs guide the RNA-induced silencing complexes to target mRNA molecules, where they regulate mRNAs primarily at the posttranscriptional level by directing mRNA cleavage via the endoribonuclease activity of the Argonaute protein (Baulcombe, 2004; Chapman and Carrington, 2007; Voinnet, 2009). In contrast, siRNAs are derived from long double-stranded RNA molecules generated by RNA-dependent RNA polymerases; this feature distinguishes them from miRNAs. Endogenous siRNAs can be further classified into transacting small interfering RNAs (tasiRNAs), natural antisense transcript-derived siRNAs, and heterochromatin siRNAs (Schwach et al., 2009). Most siRNAs target the same locus they were derived from, except for tasiRNAs, which target mRNAs from different loci, similar to miRNAs. tasiRNAs are phased 21-nucleotide RNA molecules whose production is triggered by miRNA-directed cleavage of the TAS transcripts (Allen et al., 2005; Axtell et al., 2006).

The pool of smRNAs in plants is extremely complex, consisting of a diverse set of miRNAs (Lu et al., 2005; Kasschau et al., 2007; Johnson et al., 2009). Extensive research has demonstrated the critical role of miRNAs in controlling developmental processes and organ identity (Jones-Rhoades et al., 2006; Mallory and Vaucheret, 2006; Chen, 2009). There is also evidence that miRNAs are associated with abiotic stress responses (Sunkar et al., 2007; Leung and Sharp, 2010; Sunkar, 2010). In keeping with this, many miRNAs target transcription factors with roles in developmental patterning and show unique tissue-specific, development-related, and stress-induced expression (Juarez et al., 2004; Sunkar and Zhu, 2004; Jones-Rhoades et al., 2006).

In Arabidopsis (Arabidopsis thaliana), the signaling pathway that mediates the response to the phytohormone auxin is particularly densely packed with miRNA regulation. In this pathway, miR393 targets mRNAs encoding TIR1 and other closely related F-box proteins (Jones-Rhoades and Bartel, 2004). These F-box proteins are auxin receptors that target repressors of the Auxin-Responsive Factor (ARF) for ubiquitin-mediated degradation in response to auxin. Interestingly, not only the receptors of auxin but also the transcripts of ARFs are either directly or indirectly regulated by miRNAs (Jones-Rhoades et al., 2006). miR160 targets ARF10, ARF16, and ARF17, and its regulation of these transcripts appears to be important in many aspects of shoot and root development (Mallory et al., 2005; Wang et al., 2005; Liu et al., 2007). ARF6 and ARF8, which are targeted by miR167, act redundantly to regulate ovule and anther development (Wu et al., 2006; Ru et al., 2006). miR390 directs the cleavage of TAS3, leading to the production of tasiRNAs that target ARF3 and ARF4 mRNAs. The regulation of ARF3 and ARF4 mediated by tasiRNA promotes the abaxial identity of lateral organs as well as the expression of adult vegetative traits in leaves (Adenot et al., 2006; Fahlgren et al., 2006; Garcia et al., 2006; Hunter et al., 2006). Additionally, several studies have investigated the expression patterns of miRNAs under cold conditions using deep sequencing and/or miRNA microarrays (Liu et al., 2008; Lee et al., 2010; An et al., 2011). That research identified the putative miRNAs and their targets, but the results require validation, and the overall pathways have yet to be determined.

The thermosensitive genic male sterile (TGMS) lines of wheat (Triticum aestivum) are hypersensitive to low temperature during the meiosis stage (Tang et al., 2011). Our previous study revealed that the development of the pollen mother cell (PMC) in a wheat TGMS line relies on the proper regulation of gene expression. In that transcriptome study, we identified thousands of differentially expressed genes in the anthers from PMC through meiosis stages (Tang et al., 2011). However, our knowledge about the gene regulatory link between anther development and the cold stress response is very limited. Because miRNAs play roles in both plant development and stress responses, it is plausible to assume that miRNAs have important roles in anther development in the TGMS line under cold conditions. However, to date, only 44 wheat-annotated miRNAs have been deposited in the miRBase database, and only a few miRNA targets have been validated experimentally. At present, it is unknown whether important regulators like miRNAs play a role in the function of PMCs during cold stress.

To fill this knowledge gap, we have sequenced smRNAs from seven independent libraries to obtain a large inventory of smRNA species in the TGMS line. We identified a total of 78 unique miRNAs belonging to 30 families and tasiRNAs derived from two TAS3 genes. To understand how miRNAs are integrated in diverse biological networks, it is necessary to confirm their target genes. To identify transcriptome-wide smRNA targets in the TGMS line of wheat, we generated two degradome sequencing libraries from spike tissues under cold or control conditions. A total of 26 target genes were confirmed as miRNA targets, and three ARF transcripts were identified as targets of TAS3-siRNA. Similar to coding mRNAs, it is essential to obtain accurate expression profiles of individual miRNAs to understand their function. To address this question, we examined the expression profiles of all candidate smRNAs using TaqMan quantitative PCR (qPCR) with two biological replicates. A total of six miRNAs and one tasiRNA (tasiRNA-ARF) showed significant changes in abundance under cold stress. The tissue-specific expression of smRNAs was investigated using a tissue microarray (TMA) approach. Comparing the expression profiles of smRNAs and their targets, we determined that smRNAs involved in the auxin signaling pathway may play a significant and specific role in the plant response to cold stress during spike development in the TGMS line. Changes in the abundance of these cold stress-responsive smRNAs mediate the abnormal activity of target ARFs that are important for anther development, causing...
male sterility of the TGMS line under cold conditions. The results presented in this study provide an insight into the regulatory role of smRNAs in the TGMS line in response to cold stress and provide valuable information about the cold-induced male sterility of the TGMS line.

RESULTS

Overview of smRNA Libraries from the Wheat TGMS Line at Various Spike Developmental Stages

We aimed to identify smRNAs from the wheat TGMS line that may be involved in the regulation of anther development and/or the response to cold stress. Therefore, we constructed seven smRNA libraries from spikes of plants that were treated or not with cold (10°C), which strongly affects anther development in the TGMS line during the fertility-sensitive stage (Tang et al., 2011). The seven smRNA libraries were sequenced using Solexa sequencing technology and yielded an average of 12.3 million raw reads per sample (Supplemental Table S1). Poor-quality reads, those without inserts, and those with inserts smaller than 18 nucleotides were excluded from further analysis. Finally, we obtained an average of 4.5 million nonredundant reads per sample, ranging from 18 to 27 nucleotides (Supplemental Table S1).

The composition of smRNAs often reflects the roles of different categories of smRNAs in a particular tissue or species, in different physiological conditions, and in various biogenesis machineries (Wei et al., 2009). In our study, the majority of the smRNAs were 20 to 24 nucleotides long, which is the typical size range for DCL-derived products. Consistent with previous reports on plants, the 24- and 21-nucleotide smRNAs were the most abundant smRNA species (Supplemental Fig. S1A; Moxon et al., 2008; Hsieh et al., 2009). The distribution of redundant sequences in different size classes was similar in spikes from cold-stressed and control plants (Supplemental Fig. S1A). When the unique read signatures were examined, the patterns of the seven libraries were also nearly identical (Supplemental Fig. S1B). As generally described, the 24-nucleotide smRNA population was clearly the most diverse. As expected, the 21-nucleotide class showed the highest redundancy because a relatively small number of nonredundant sequences were expressed at high levels (Supplemental Fig. S1).

Conserved miRNAs in Spikes of the Wheat TGMS Line

Conserved families of miRNAs are found in many plant species and have important functions in plant development and stress responses (Jones-Rhoades et al., 2006; Chen, 2009; Rubio-Somoza et al., 2009). To identify conserved miRNAs, we analyzed the smRNAs by BLAST searches against the known noncoding RNAs (tRNA, rRNA, snRNA, and small nucleolar RNA) deposited in the RNA family database and National Center for Biotechnology Information (NCBI) database. An average of 45,203 distinct smRNAs per cDNA library belonging to these categories were filtered to avoid degradation contamination. The remaining reads were analyzed to identify conserved and novel miRNAs.

We compared the entire sets of unique smRNAs with the miRBase database (version 17). The search criteria were more rigorous, requiring smRNAs to display a perfect or nearly perfect match (mismatch < 2) to published miRNAs, and the mismatch was required to be outside the “seed” region. Based on these criteria, a total of 553 sequences from seven independent libraries showing less than two mismatches or deletions in comparison with a registered miRNA were identified, corresponding to 2,388,039 reads (3.08% of the total reads). These smRNAs belong to 30 miRNA families, containing 78 miRNAs described previously in other plant species (Table I). Some miRNA* in addition to the miRNAs were also identified for the miR156, miR160, miR166, miR390, miR396, and miR399 families (Table I). Only nine of the Triticum aestivum (tae)-miRNAs found in wheat tissues (pooled sample of leaves, roots, and spikes) by Yao et al. (2007) were retrieved in our libraries, possibly because of low expression levels, tissue-specific expression in the TGMS spikes, or still unsaturated miRNA sequencing due to the large wheat genome.

As expected, we identified members of almost all conserved miRNA families (18 of 24 families conserved in plants) in the seven cDNA libraries (Table I; Jones-Rhoades et al., 2006; Sunkar and Jagadeeswaran, 2008). Sequence analysis revealed that 13 conserved miRNA families were represented by more than one member in our libraries (Table I). Among these conserved families, the miR166 family was the largest, including 11 miRNA members and three miRNA*. The second largest family was miR167, containing six miRNA members. The expression levels of a few miRNA families were similarly high (miR166 and miR167) or low (miR160, miR399, miR408, and miR444; Supplemental Table S2), indicating that expression varies greatly among the different miRNA families in spike tissues of the wheat TGMS line. In other families, only one particular member showed high abundance in all libraries (Supplemental Table S2). This result highlights that some specific miRNA isoforms show differential expression patterns in spike tissues of the wheat TGMS line.

In addition to the broadly conserved miRNAs, there are other known miRNAs that are not conserved but are found in only one or a few plant species (Jones-Rhoades et al., 2006). Several nonconserved miRNAs from nine families were present with very low abundance in our data sets, except for tae-miR528 and tae-miR894, which showed high read numbers (Table I). Interestingly, in a previous study, it was speculated that tae-miR894 might be specific to moss (Fattash et al., 2007). The miR2118 family is conserved in rice (Oryza sativa) and maize (Zea mays), and its members...
Table I. Known miRNAs present in spikes of the wheat TGMS line

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<th>Length</th>
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are almost exclusively expressed in the inflorescence; therefore, members of this family were proposed to play an important role in the development of reproductive structures in both cereals (Johnson et al., 2009). However, in our seven independent smRNA cDNA libraries, tae-miR2118 was present with low abundance in spike tissues (Supplemental Table S2). Our quantitative reverse transcription-PCR also showed that tae-miR2118 only could be detected with extended PCR cycle numbers to 40 in optimized PCR performance (small nuclear RNA U6 with a cycle threshold value of 18.5). These results indicated that data on smRNAs from more species are necessary to understand the evolution of these weakly conserved miRNAs.

To further characterize the sequenced miRNA candidates, we searched for putative precursors within the genomic trace file archive and EST databases. We found seven conserved miRNA precursors among the ESTs and whole-genome shotgun sequences (Supplemental Fig. S2).

### Identification of tasiRNAs in Spikes of the Wheat TGMS Line

The tasiRNAs constitute a small class of phased smRNAs that are also capable of altering gene expression. Although tasiRNAs play an important role in developmental timing and patterning in Arabidopsis (Peragine et al., 2004; Vazquez et al., 2004; Fahlgren et al., 2006), their expression has not been reported to change under cold stress. In our study, we analyzed the smRNA sequencing data sets to identify clusters of smRNAs that exactly matched some transcripts in a phased manner, which clearly revealed the presence of tasiRNAs. We detected phased smRNAs from transcripts that are homologous with previously described TAS genes (TAS3a and TAS3b) in Arabidopsis (Axtell et al., 2006; Shen et al., 2009), having approximately four to nine group-phased smRNAs matching their transcripts in both strands (Supplemental Fig. S3). The most abundant phased siRNAs were generated from the 5′D6(+) and 5′D2(−) positions in TAS3a and TAS3b, respectively (Supplemental Fig. S3). However, the TAS3 homolog (accession no. BQ171265) predicted by Allen et al. (2005) in wheat was not detected in our phased smRNA data set. Both TAS3a and TAS3b transcripts are first cleaved by miR390 to initiate the generation of phased siRNAs. As we described above, tae-miR390 was present as a highly abundant read in our smRNA data sets. These results indicated that the TAS3-miR390 tasiRNA pathway was operating in spikes of the wheat TGMS line.

### Construction of Degradome Libraries, Sequencing, and Sequence Analysis

To identify transcriptome-wide smRNA targets in spikes of the wheat TGMS line, we applied the recently developed high-throughput technology of degradome library sequencing (Addo-Quaye et al., 2008; German et al., 2009; Li et al., 2010; Pantaleo et al., 2010). We obtained a total of 26,387,851 and 35,477,509 sequencing reads that represented the 5′ ends of uncapped and polyadenylated RNAs from cold-stressed spikes and controls, respectively (Supplemental Table S1). After initial processing, we obtained 11,132,526 and 14,363,576 unique signatures from cold-stressed spikes and controls, respectively. Using BLASTN searches against the Rfam database, we were able to exclude the known

### Table I. (Continued from previous page.)

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*“Ta” at the start of the name indicates homologous miRNA. *Underlined nucleotides represent nonconserved nucleotides among wheat and other plant species. A dash indicates the deletion of a nonconserved nucleotide. The relative number of reads was obtained by normalizing read counts of each miRNA in each smRNA cDNA library to TPM (Supplemental Table S2). Reads only encompass defined mature miRNAs. The maximum TPM of each miRNA in the seven smRNA cDNA libraries is shown.
noncoding RNAs (rRNA, tRNA, snRNA, and small nucleolar RNA), which were represented at an average rate of 0.27% in our two unique signature data sets. Similarly, we removed signatures corresponding to transposable elements from the unique data sets by performing searches against the repeat sequence database (http://www.repeatmasker.org/). The remaining reads were mapped to the wheat unigene data set. In total, 11,132,526 (65.9%) and 14,363,576 (64.1%) unique reads from cold-stressed spikes and controls were mapped to the wheat unigene data set, representing 12,713 unigenes. Previous degradome analyses reported that reads composed of poly(A) fragments are another source of noise in the degradome sequencing library (German et al., 2008; Li et al., 2010). However, only an average of 0.2% of the unique reads ended with four or more A residues in our two degradome sequencing libraries. Taken together, these results indicated the high quality of the degradome sequencing libraries obtained in this study. The reads that mapped to wheat unigenes were subjected to further analysis.

Identification of Targets for Annotated miRNAs and tasiRNAs

In plants, miRNAs degrade their mRNA targets by slicing precisely between the 10th and 11th nucleotides from the 5′ end of the miRNA in the complementary region of the target transcript. Therefore, the sliced mRNA should have distinct peaks of degradome sequence tags at the predicted cleavage site relative to other regions of the transcript (German et al., 2008, 2009). In this study, we applied the recently developed CleaveLand technology to identify sliced targets for miRNAs that were annotated as described above (Addo-Quaye et al., 2009a). For the miRNAs shown in Table I, we identified a total of 26 target mRNAs (Table II). We also analyzed the phased siRNAs shown in Supplemental Figure S3 to identify their targets. Only the tasiRNAs derived from the 5′D6(+) position in TAS3a and the 5′D4(+) position in TAS3b targeted to three ARF genes (Table II). Based on the sequenced tags at the sliced site and all along the region of the transcript, the cleaved target transcripts were categorized into three classes (class I, II, or III) as described previously in other degradome analyses (Addo-Quaye et al., 2008, 2009b). Among the 29 mRNAs targeted by miRNAs and tasiRNAs, 21 fall into the class I category (Fig. 1A; Table II), where the degradome tags corresponding to the expected miRNA-mediated cleavage site were the most abundant tags matching the transcript. In the class II category, the abundance of the cleavage signatures was greater than the median number of signatures on the transcripts but less than the maximum number. In our degradome data sets, five target miRNAs were identified to belong to this category (Fig. 1B; Table II). The remaining targets, with a low abundance of cleavage signatures, were grouped into the class III category. Only two transcripts were grouped into this category (Fig. 1C; Table II). Because the abundance of miRNA-guided cleavage remnants in class I and class II categories was much higher than those of other signatures, targets in these two categories could show low false discovery rates and, therefore, could be more accurate. Most of the identified targets are members of different families of transcription factors, such as the ARF, Growth-Regulating Factor (GRF), GAMYB, and Squamosa Promoter-Binding families (Table II), which play important roles in anther development (Chen, 2009; Wilson and Zhang, 2009).

We identified target mRNAs for 16 out of 21 conserved miRNA families. Among the five conserved miRNA families without any identified targets, miRNAs of three conserved families (miR395, miR399, and miR444) were expressed at very low levels (less than 10 tag counts per million total tags [TPM]; Table I), which may explain the absence of cleaved targets. However, in a few cases, miRNAs belonging to the miR168 and miR164 families were expressed at high levels without the targets being identified in the degradome libraries. The degradome sequencing also revealed that one tasiRNA precursor (TAS3b) was cleaved at a 3′ miR390 complementary site (Fig. 1D), which then initiated the production of phased siRNA from the cleaved end (Supplemental Fig. S3). Furthermore, the abundance of tags associated with 488 transcripts that are not targeted by known miRNAs (all Viridiplantae miRNAs in miRBase) showed distinct peaks of degradome sequencing tags, suggesting a high number of novel and nonconserved miRNAs in wheat or a high rate of turnover of these transcripts through uncapping and 5′–to-3′ exosome-mediated pathways (Li et al., 2010).

Expression Profiles of smRNAs throughout Spike Development under Cold Stress

In our study, the challenge for measuring miRNA levels arises from the existence of miRNA families, such as miR166 and miR167 families (Table I), whose members differ by as little as one nucleotide but nevertheless show different expression patterns. Sequencing of smRNA by Solexa technology allowed the rapid discovery of miRNAs in spikes of the TGMS line under cold and control conditions. However, digital gene expression of smRNAs has recently been demonstrated to generate inherent biases (Linsen et al., 2009; Hafner et al., 2011). This highlights the importance of further quantitative analysis to confirm the relative abundance of smRNAs. TaqMan qPCR is often considered as the “gold standard” for detecting and quantifying miRNA (Chen et al., 2005). Therefore, to complement the smRNA sequencing analysis and build a comprehensive repository of information on cold-responsive miRNAs in spikes of the TGMS line, we measured the expression levels of smRNAs by TaqMan qPCR.
We used two criteria to select miRNAs for validation by qPCR: (1) miRNAs must target transcription factors in our degradome data set or must be predicted to target transcription factors in miRBase; and (2) the average number of sequencing tags of miRNAs must be greater than 10 TPM in our smRNA sequencing data sets. Except for tae-miR171a, whose TaqMan probe could not be designed, a total of 19 miRNAs and one tasiRNA [TAS3a-5’D6(+); tasiRNA-ARF] were selected for further analysis with TaqMan qPCR, using two biological repeats (Supplemental Fig. S4). With the exception of tae-miR160, tae-miR164, and tae-miR167, all of the other miRNAs were identified in other species by homology searches. All were readily detected by TaqMan qPCR (Supplemental Fig. S4), which indicated that these miRNAs are authentic miRNAs. Analysis of the overall correlation between smRNA sequencing data and TaqMan qPCR data showed that they were slightly inconsistent, with a Pearson $r^2$ close to 0.62 (Supplemental Fig. S5). In contrast, the results of TaqMan qPCR for each biological replicate were highly reproducible ($r^2 = 0.86$; Supplemental Fig. S5). Therefore, the mean expression value for each miRNA at each time point was used for further analysis.

As described above, the miR166 and miR167 families showed the highest levels of expression among the miRNAs in our smRNA sequencing data set. Our TaqMan qPCR results demonstrated that members of the miR166 family did not show significant changes in expression levels between cold and control conditions (Fig. 2). In addition, the expression patterns of these miRNAs were similar in cold and control conditions (Fig. 2). These observations indicated that the miR166 family might play a redundant role in spike development. However, in the miR167 family, four miRNAs showed distinctive expression patterns; although tae-miR167 and tae-miR167e were highly expressed and unaffected by cold stress in spike tissues, their expression patterns were slightly reversed (Fig. 2). tae-miR167d and tae-miR167c were dramatically repressed at the L1.5 stage during cold stress, but tae-miR167c was up-regulated at the L3.0 stage (Fig. 2). This result indicates that miRNAs in the miR167 family might play different roles in spike development.

<table>
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<th>Category</th>
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$^a$Contig sequences were assembled from wheat ESTs (shown in Supplemental File S1). Other identifiers are NCBI EST accession numbers. Boldface entries were independently confirmed by RNA ligase-mediated 5’ RACE.
at the L1.5 stage, which is consistent with the slight reduction in the accumulation of tae-miR390 at the L1.5 stage (Fig. 2). Furthermore, the expression levels of tae-miR172a, tae-miR393, tae-miR396a, and tae-miR444c.1 were lower at an early stage of the cold treatment (Fig. 2). The other miRNAs, including tae-miR156a, tae-miR160, tae-miR164, tae-miR169a, and tae-miR319, did not show significant changes in their expression levels between cold and control conditions (Fig. 2).

The differentially expressed miRNAs with greater than 1.5-fold or less than 0.5-fold relative change between cold and control conditions were selected as candidate miRNAs. A total of seven smRNAs [tae-miR167c, tae-miR167d, tae-miR172a, tae-miR393, tae-miR396a, tae-miR444c.1, and TAS3a-5’D6(+) ] met these criteria. Among these smRNAs, homologs of tae-miR167c and tae-miR444c.1 target ARFs in Citrus (Song et al., 2009) and a MADS box gene in rice (Sunkar et al., 2005; Lu et al., 2008a), respectively. However, the targets of tae-miR167c and tae-miR444c.1 could not be identified in the degradome sequencing data set by the CleaveLand pipeline, which might be a limitation of the wheat unigene data set. Therefore, tae-miR167d, tae-miR172a, tae-miR393, tae-miR396a, and TAS3a-5’D6(+) were selected as candidates for further analysis. Among these miRNAs, tae-miR167d, tae-miR172a, and tae-miR396a, and TAS3a-5’D6(+) are involved in auxin signaling pathways.

Expression Patterns of smRNA-Targeted Genes in Response to Cold Stress

To assess whether the cold stress-responsive smRNAs identified in spikes of the TGMS play an important role in spike development, we further analyzed the expression patterns of all target genes that encode transcription factors (Table II).

In the auxin-signaling pathway, the genes TIR1 and TIR1-like were targeted by tae-miR393 (Table II). Compared with its expression in control conditions, the expression of tae-miR393 was slightly depressed...
under cold stress, but there was no significant change in the expression of its target genes (Fig. 3). tae-miR160, tae-miR167, tae-miR167d, and TAS3a-5’D6(+) target different ARFs (Table II). Among these nine ARFs, the transcript levels of ARFs that were targeted by tae-miR160 and tae-miR167 were not significantly altered by cold stress (Fig. 3), which is consistent with the expression patterns of tae-miR160 and tae-miR167 as described above. These results suggest that tae-miR160 and tae-miR167 do not act downstream of these ARF genes. tae-miR167d targets one ARF gene (EST Contig9875; Table II). The expression level of this ARF was induced at the L1.5 stage under cold conditions but was unchanged at the L2.2 and L3.0 stages (Fig. 3). Among the nine ARFs, the transcript levels of ARFs that were targeted by tae-miR160 and tae-miR167 were not significantly altered by cold stress (Fig. 3), which is consistent with the expression patterns of tae-miR160 and tae-miR167 as described above. These results suggest that tae-miR160 and tae-miR167 do not act downstream of these ARF genes. tae-miR167d targets one ARF gene (EST Contig9875; Table II). The expression level of this ARF was induced at the L1.5 stage under cold conditions but was unchanged at the L2.2 and L3.0 stages (Fig. 3). The ARF genes located at EST Contig28378, Contig4296, and Contig1892 were targeted by TAS3a-5’D6(+) (Table II). Compared with its expression in control conditions, there was no significant change in the expression of EST Contig28378 under cold stress (Fig. 3). However, the ARF genes located at EST Contig4296 and Contig1892 showed increased expression at the L1.5 stage and then dramatically repressed expression at the L2.2 stage (Fig. 3). Taken together, these analyses revealed that the expression

**Figure 2.** Differentially expressed smRNAs in response to cold stress in spikes of the wheat TGMS line. Relative fold change (FC) values greater than 1.5 or less than 0.5 are highlighted in red. The asterisks indicate significant differences between the cold stress and control samples (* P < 0.01, ** P < 0.05).
levels of tae-miR167d and TAS3a-5’D6(+) and their target ARFs were largely noncorrelated at the transcript levels and raised the possibility that the auxin-signaling pathway, via tae-miR167d and TAS3a-5’D6(+) may play an important role in spike development in the TGMS line.

To better understand the molecular mechanisms by which tae-miR167d and TAS3a-5’D6(+) regulate the cold stress response in spikes of the TGMS line, we investigated the global expression pattern in spike tissues in cold and control conditions using the Digital Gene Expression (DGE) technique. The self-organizing map analysis indicated that the cluster including the ARF gene located at EST Contig1892 showed different expression patterns in cold and control conditions (Fig. 4A). This expression cluster comprised transcripts with markedly increased abundance at the L1.5 stage and then decreased abundance at the L2.2 stage; however, these transcripts also showed slight changes in abundance in the controls (Fig. 4A). The transcript cluster in Figure 4B, including ARF genes located at EST Contig9875 and Contig4296, showed increased expression from the L1.5 to L2.2 stages. Compared with their expression during cold treatment, these genes showed slight changes in transcript levels in controls (Fig. 4B).

In addition, tae-miR396a targeted two GRFs (Table II). Although tae-miR396a showed depressed expression at the L1.5 and L2.2 stages (Fig. 2), only one GRF (NCBI EST accession no. CJ645826) showed slight changes in expression at the L1.5 and L2.2 stages (Fig. 3). In contrast, the GRF gene C0347544 showed no significant changes in expression levels under cold stress (Fig. 3). The APETALA2 gene located at EST Contig803, which was targeted by tae-miR172a (Table II), showed similar expression patterns in cold and control conditions (Supplemental Fig. S6). As described above, tae-miR156a, tae-miR169a, and tae-miR319 showed no significant changes in expression under cold stress, compared with their respective expression levels in the control (Fig. 2). Consistent with their expression profiles, their target genes also showed no changes in transcript levels during cold stress (Supplemental Fig. S6).

In summary, we observed changes in the expression patterns of tae-miR167d and TAS3a-5’D6(+) and their targeted ARFs in the control of cold-induced male sterility in the TGMS line. Taking into consideration the observation that the activities of ARFs are also regulated by the ubiquitin-mediated degradation pathway in response to endogenous auxin (Vanneste and Friml, 2009; Sakata et al., 2010), we measured endogenous auxin levels in anthers of the TGMS line under both cold and control conditions. The levels of endogenous auxin did not change in response to cold stress compared with that in the control (Supplemental Fig. S7). The endogenous auxin accumulated normally throughout the developing anthers, from rachis cells around vascular bundles to epidermal and sporogenous cells (Supplemental Fig. 3).

Figure 3. qPCR analysis of target genes in spikes from the TGMS line in cold and control conditions. Error bars indicate sn.
This result suggests that the cold-induced change in fertility as controlled by miR167/tasiRNA-ARF was largely mediated by ARFs in the TGMS line.

Differentially Expressed smRNAs Are Associated with Anther Development

To understand the precise role of smRNAs in biological processes, we investigated the spatial and temporal patterns of smRNA accumulation by in situ hybridization. In our study, we conducted anther TMA to interpret the tissue-specific expression of miRNAs/tasiRNA-ARFs. Using this method, the entire cohort representing tissues from hundreds of anthers is treated in an identical manner, which provides for rigorous statistical analysis (Supplemental Fig. S8).

We selected three differentially expressed miRNAs (tae-miR167d, tae-miR172a, and tae-miR396a) and one differentially expressed tasiRNA-ARF [TAS3a-5′D6(+)] for in situ hybridization. The in situ hybridization results showed that tae-miR172a and tae-miR396a showed similar expression patterns in anther tissues of the TGMS line (Fig. 5, A–L). tae-miR172a and tae-miR396a were mainly localized in the tapetum and microsporocytes during anther development (Fig. 5, A–L). However, tae-miR167d and TAS3a-5′D6(+) showed similar expression patterns and were expressed in different zones and in broader regions than those in which other miRNAs were expressed. tae-miR167d and TAS3a-5′D6(+) were expressed in all of the anther tissues at an earlier stage of anther development (Fig. 5, M–X). As a consequence, tae-miR167d and TAS3a-5′D6(+) showed the highest expression levels in vascular bundles, the middle layer, the tapetum, and microsporocytes (Fig. 5, M–X). Based on rigorous statistical analysis of the TMA, the expression levels of miRNAs/tasiRNA-ARF in TMA was consistent with their expression profiles as described in Figure 2.

Spatial localization of miRNA/tasiRNA-ARF expression showed that all of them were expressed in the microsporocytes. In our previous study, we reported that cold stress contributed to the abnormal development of microsporocytes in the TGMS line (Tang et al., 2011). Therefore, miRNA/tasiRNA-ARF-mediated regulation in response to cold stress may be linked to male sterility of the TGMS line.

DISCUSSION

The TGMS line is very important for the utilization of heterosis in wheat breeding. The sterile male phenotype is heritable in the TGMS line but is strictly regulated by an appropriate temperature (Li et al., 2006; Tang et al., 2011). Our previous study demonstrated that cold stress contributes to the abnormal development of PMCs during meiosis (Tang et al., 2011); however, the regulation of the developmental transition underlying the histological changes remains poorly understood. In terms of their biological roles, miRNAs are predominantly associated with plant development, but they also play an important role in stress responses (Jones-Rhoades et al., 2006; Mallory and Vaucheret, 2006; Sunkar et al., 2007; Chen, 2009; Leung and Sharp, 2010; Sunkar, 2010). Realizing that anther development in the TGMS line is controlled by cold stress, identification of miRNAs and elucidation of their functions in anther development will help us understand the regulation of male sterility by cold stress.

Known miRNAs and tasiRNAs in Spike Tissues of the TGMS Line

As posttranscriptional regulators of gene expression, miRNAs are widely distributed in plants, animals, and...
some viruses (Mallory and Vaucheret, 2006; He et al., 2008; Siomi and Siomi, 2010). There are 232 and 491 annotated miRNAs in Arabidopsis and rice, respectively, according to the miRBase database (version 17; www.mirbase.org). Wheat is an ancient polyploid crop with a larger and more complex genome than those of Arabidopsis and rice (Chalupska et al., 2008; Matsuoka, 2011). Although 44 wheat miRNAs were identified in previous studies, the number of annotated miRNAs in wheat is still very limited, and there are considerably fewer annotated in wheat than in Arabidopsis and rice. Recent innovations in sequencing technology and the accumulation of known miRNAs in the miRBase have allowed extensive surveys of smallRNA populations in crop plants, notably in species for which the genome has not been fully sequenced. Here, we show that miRNAs are a diverse component of the smallRNA transcriptome during spike development in the wheat TGMS line. We identified a total 78 miRNAs belonging to 30 miRNA families by deep sequencing. Although most of these miRNAs were identified by homology searches in other species, at least 19 miRNAs were validated by TaqMan qPCR as genuine mature miRNAs. According to the recent criteria for plant miRNAs (Meyers et al., 2008), seven of the conserved miRNA genes can be considered as genuine miRNA precursors (Supplemental Fig. S2), based on the detection of both the miRNA and miRNA* in our smallRNA sequencing libraries. When the sequencing of the wheat genome is completed in the near future, biological research on miRNA will be greatly advanced.

In the analysis of miRNAs, we found that miR390 was abundantly present in spike tissues of the TGMS line. The noncoding TAS3 transcripts are the targets of miR390, and cleaved TAS3 gives rise to tasiRNAs that target transcripts in the ARF family (Allen et al., 2005; Axtell et al., 2006). It will be interesting to see whether TAS3 or other TAS-like genes are involved in the cold stress response in spike tissues of the wheat TGMS line. We predicted that phased siRNAs were derived from two TAS3 loci that are targeted by tae-miR390 (Supplemental Fig. S3), which is consistent with a previous report that AtTAS3 homologs are present in diverse seed plants (Allen et al., 2005). The successful prediction of TAS3-like genes was validated experimentally. This is clear evidence that both the miRNA pathway and the tasiRNA pathway are functioning during spike development.

Furthermore, we emphasize that the high-throughput degradome sequencing data set was especially useful for the global identification of targets of miRNA in the TGMS line. Functional characterization of the

Figure 5. Differential accumulation of miRNA/tasiRNA-ARF in anthers of the wheat TGMS line. Cross-sections of anthers in anther tissue microarray were hybridized with 5’ and 3’ double-labeled locked nucleic acid-modified oligonucleotides detecting tae-miR172a (A–F), tae-miR396a (G–L), tae-miR167d (M–R), and TAS3a-5’D6(+) (S–X). Anther developmental stage and treatment conditions (L, cold stress; C, control condition) are shown in parentheses. The locked nucleic acid probe complementary to Caenorhabditis elegans let-7 served as a negative control. Brown staining shows probe localization. ML, Middle layer; Ta, tapetum; V, vascular bundles. Bars = 50 μm.
miRNA targets is essential to provide deep biological insights into certain miRNA-mediated pathways in spike development of the TGMS line under cold stress. In previous studies, the majority of miRNA targets in wheat were predicted using bioinformatics approaches, and only a few targets were validated experimentally. Computational analysis of the degradome data set confirmed 29 miRNAs as genuine targets for wheat miRNAs/tasiRNAs. Our previous transcriptomic study showed that many transcription factors exhibit dynamic gene expression changes during anther development under cold stress and that miRNAs target transcription factors that are well represented in our degradome sequencing data set. Given the presence of miRNAs targeting a transcription factor family, such as ARF (miR160, miR167, and tasiRNA-ARF), HD-ZIP (miR165 and miR166), GRF (miR396), and AP2 (miR172), there should be no doubt that miRNAs modulate the expression of many transcription factors during spike development under cold stress and control conditions.

miRNAs/tasiRNA-ARF Responses to Cold Stress in Spikes of the TGMS Line

In this study, we used the TaqMan qPCR technique to investigate the expression profiles of 19 miRNAs and one tasiRNA-ARF. Among these miRNAs, the miR169, miR319, miR393, miR160, and miR167 families are known to show differential expression during abiotic stress, such as cold, drought, salt, oxidative stress, and hormone signaling (Sunkar and Zhu, 2004; Ding et al., 2009; Zhang et al., 2009b; Lee et al., 2010; An et al., 2011). We selected miR156, miR166, tasiRNA-ARF, miR172, and miR396 to assess whether miRNAs reported to be involved in plant primary developmental processes, such as phase transitions, pattern formation, and morphogenesis, are affected by cold stress during spike development in the TGMS line (Jones-Rhoades et al., 2006; Axtell and Bowman, 2008; Chen, 2009; Nogueira et al., 2009).

TaqMan qPCR analyses showed that six miRNAs and one tasiRNA-ARF were differentially expressed during cold stress, compared with control conditions (Fig. 2). Surprisingly, all of them were significantly repressed at the early stage of the cold treatment (Fig. 2). Several studies have focused on the regulatory roles of miRNAs in response to abiotic stress (Phillips et al., 2007; Sunkar et al., 2007). miR393 is responsive to all tested stresses in Arabidopsis (Sunkar et al., 2007). In addition, several cold- or low temperature-responsive miRNAs have been identified by many platforms (Sunkar and Zhu, 2004; Lu et al., 2008b; Zhou et al., 2008; Zhang et al., 2009a; Lee et al., 2010; Lv et al., 2010; An et al., 2011). However, for several miRNAs, the expression profiles were inconsistent among plant species and even among different tissues of the same species (Supplemental Table S3). During cold stress, tae-miR393 was repressed in spike tissues of the TGMS line, the reverse of its expression pattern in Arabidopsis (Sunkar et al., 2007). However, the target genes of tae-miR393 did not show significant changes in expression levels during cold stress (Fig. 3). This result indicated that the 10°C treatment causes male sterility of the TGMS line but is less stressful than extreme cold conditions for common wheat lines. In Arabidopsis, low-ambient-temperature (16°C) treatment resulted in a decreased expression of miR172 during flowering. We obtained a similar result in this study: tae-miR172a was significantly repressed at the early stage of spike development during cold stress (Fig. 3). A previous study demonstrated that miR172 plays an important role in the regulation of flowering time under normal (nonstress) temperature conditions (Lee et al., 2010). The accumulation of miR172 contributes to complete flowering through the negative regulation of AP2-like transcription factors. We identified one AP2-like gene with a predicted miR172 target site in our degradome data set; however, the expression pattern of the target gene was not completely noncorrelated, as would have been expected (Supplemental Fig. S6). Previous studies demonstrated that miR172 mainly plays its regulatory role via translational repression rather than by transcript cleavage (Aukerman and Sakai, 2003; Chen, 2004). Although more research is required to identify the downstream target genes of miR172, these results indicated that miR172-mediated regulatory pathways in response to nonstress low temperature may be similar in Arabidopsis and the TGMS wheat line.

In addition, cold-responsive members of a miRNA family may be further differentiated by their temporal response to cold treatment. Thus, the functions of plant miRNAs can be dissimilar even if they share a high degree of sequence similarity and belong to the same family. In our smRNA data set, we identified nine members of the miR166 family. We selected the five most abundant of these miRNAs for TaqMan qPCR analyses and found that they showed similar expression patterns in cold stress and control conditions and were insensitive to cold stress in spike tissues of the TGMS line (Fig. 2). Two HD-ZIP transcription factors, which could not be detected by extended qPCR cycles, were completely repressed by members of the miR166 family (Table II). A previous study demonstrated that the miR166 family exhibits complex spatiotemporal patterns of expression in developing primordia (Nogueira et al., 2009). Furthermore, several studies showed that members of miR166 family are induced during cold stress (Supplemental Table S3). In contrast, our results could indicate that the miR166 family plays a redundant role during spike development, because its members showed no changes in expression levels during cold stress in the TGMS line. Members of the miR166 family may function in the formation or maintenance of floral organs after phase transition.

Members of the miR166 family showed different expression patterns (Fig. 2). tae-miR167 and tae-miR167e showed similar expression patterns in cold and control conditions (Fig. 2). Interestingly, tae-miR167 and tae-miR167e showed completely reversed expression profiles in spikes of the TGMS line (Fig. 2).
tasi-miR167c and tae-miR167d showed distinct expression patterns in response to cold stress in the TGMS line (Fig. 2). The miR167 family is strongly expressed in floral organs of Arabidopsis, and the target genes of the miR167 family are ARF genes (Reinhart et al., 2002; Ru et al., 2006; Wu et al., 2006; Fujioka et al., 2008). Additionally, individual miRNAs within the miR167 family target different ARF genes in spike tissues of the TGMS line (Table II). Taken together, these results suggest that each individual miRNA in the miR167 family has a unique function in spike development in the TGMS line via targeting specific ARF transcripts.

**ARF Signaling Pathways Activated by miR167 and tasiRNA-ARF in the TGMS Line during Cold Stress**

Cold stress-responsive miRNAs may be involved in signaling pathway(s) during spike development in the TGMS line. An interesting observation is that cold stress-responsive tae-miR167d and tasiRNA-ARF may affect auxin-signaling pathways. The expression of tae-miR167d and tasiRNA-ARF was dramatically depressed at an early stage of spike development (L1.5 stage) during cold stress (Fig. 2). The accumulation of tae-miR167d and tasiRNA-ARF was inversely correlated with the expression levels of three ARF transcripts (Fig. 2). Another striking observation is that one ARF targeted by tasiRNA-ARF was significantly induced (70-fold) during cold stress (Fig. 3).

Auxin regulates many important aspects of plant growth and development as well as responses to environmental stresses (Cecchetti et al., 2008; Sundberg and Østergaard, 2009; Vanneste and Friml, 2009; Sakata et al., 2010). Within the auxin-signaling pathway, ARFs function as core positive and negative regulators (Guilfoyle and Hagen, 2007; Lau et al., 2008). miR167 plays a role in regulating the auxin signal by cleaving atARF6 and atARF8 transcripts during anther development (Nagpal et al., 2005; Ru et al., 2006; Wu et al., 2006). miR167 was abundantly accumulated in floral organs during an early stage of anther development (Fujioka et al., 2008; An et al., 2011). In Arabidopsis, overexpression of miR167 causes male sterility (Ru et al., 2006; Wu et al., 2006). These observations indicated that miR167 plays an important role in the accurate regulation of anther development. Combined with our results, miR167 might also play a role in the cold stress response by affecting the auxin-signaling pathway, which possibly links cold stress with male sterility in the TGMS line.

The roles of tasiRNA-ARF in the spike remain obscure. However, tasiRNA-ARF regulates target genes, including ARF3/ETTIN mRNA, in a gradient across the leaf primordium. Interestingly, modeling of a polarized maize leaf suggests that morphogen-like movement of TAS3a tasiRNAs through the negative regulation of ARF3 mRNA (Fahlgren et al., 2006). Late stages of flower development in Arabidopsis, rice, and *Lotus japonicus* rely on regulated ARF3 function (Hunter et al., 2006; Liu et al., 2007; Yan et al., 2010). Furthermore, recent data indicate that tasiRNAs are also involved in environmental stress responses (Moldovan et al., 2010). Therefore, it is tempting to speculate that during cold stress, the abnormal decline of tasiRNA-ARF levels contributes to the aberrant development of the anther in the TGMS line through the negative regulation of ARFs.

Because the activity of ARFs is known to be controlled by endogenous auxin levels, we investigated auxin levels in the anther in cold and control conditions. The auxin levels in the anther did not change during cold stress (Supplemental Fig. S7), suggesting that the abnormally repressed miR167 and tasiRNA-ARF directly contribute to the up-regulation of three ARFs, and, as a consequence, cause male sterility in the TGMS line during cold stress.

A previous study showed that miR167 accumulated in the vasculature of the anther (Válóczi et al., 2006). However, we detected the expression of miR167 throughout the entire anther, including the vascular bundle, PMC, tapetum, and middle layer; thus, the expression of miR167 showed a similar spatiotemporal pattern to that of tasiRNA-ARF (Fig. 5). This difference suggests that the expression patterns of members of the miR167 family may differ in different developmental stages of the anther, or that miR167 may move between cells like tasiRNA-ARF, as described elsewhere (Nogueira et al., 2009). Both miR167 and tasiRNA-ARF showed depressed expression in PMCs during cold stress (Fig. 5), which is consistent with our previous finding that PMCs are more vulnerable than sporogenous cells during anther development (Tang et al., 2011).

**CONCLUSION**

In summary, we have presented an extensive survey of the smRNAs showing differential expression profiles in response to cold stress in a TGMS line of wheat. These cold-responsive smRNAs and their target genes are possibly involved in anther development and the regulation of adaptive responses to cold stress. Research on the miR167 family and tasiRNA-ARF target regulation may increase our understanding of the biological role of these smRNAs and their contribution to cold-induced male sterility in wheat TGMS lines. Our work has opened a new avenue for functional studies on smRNA-mediated gene regulation in response to cold stress in wheat TGMS lines.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

The wheat (*Triticum aestivum*) TGMS line BS366 was used in this study (Tang et al., 2011). As described in our previous report, the cold treatment was initiated when plants were at the flag leaf stage (anther length of...
approximately 1.5 mm), when the flag leaf had half-emerged from the collar of the penultimate leaf (Tang et al., 2011). During the cold treatment, plants of BS366 were grown at 10°C with a 12-h-light/12-h-dark photoperiod for 5 d. Under control conditions, plants of BS366 were grown at 20°C with a 12-h-light/12-h-dark photoperiod. The reproductive growth of individual plants was well synchronized under the controlled conditions in the growth phytotron (Kotio).

Sample Collection and RNA Isolation

During the cold treatment, the anthers of the TGMS line developed from the PMC stage to the meiosis stage. We analyzed spikes from anthers at three developmental stages (including the T0 stage): 1.5-mm anthers in which secondary sporogenous cells were formed; 2.2-mm anthers in which all cell layers were present and mitosis had ceased; and 3.0-mm anthers at the meiotic division stage (Tang et al., 2011). To maximize the morphological synchronicity of samples at each individual stage, approximately 20 spikes were collected from primary stems at each stage. The spikes at corresponding stages were also harvested from the stems of plants kept in control conditions, using leaf age index and anther length as guides. Total RNAs were isolated from spike tissues using Trizol reagent (Invitrogen) according to the manufacturer’s instructions.

smRNA Library, DGE Library and Degradome Library Construction

After total RNA isolation, size-selected smRNAs 16 to 30 nucleotides in length were obtained from total RNA by size fractionation. The smRNA libraries were constructed following the instructions of the manufacturer. The 3' tag DGE libraries were constructed with Illumina’s DGE Tag Profiling kit according to the manufacturer’s protocol. The degradome libraries were constructed as described previously by Addo-Quaye et al. (2008) and German et al. (2008). smRNA reads, DGE tags, and degradome reads were all generated by an Illumina Genome Analyzer (Beijing Genomics Institute).

Bioinformatic Analysis of Sequencing Data

The raw sequencing data sets were preprocessed by the Fastx-toolkit pipeline to filter low-quality reads and to trim adapters (http://hannonlab.cshl.edu/fastx_toolkit/index.html). The ESTs from the NCBI public and private wheat EST databases were assembled by CAP3 into wheat unigenes for bioinformatic analysis (Huang and Madan, 1999; Yang et al., 2009). For the smRNA libraries, smRNAs ranging from 18 to 28 nucleotides were collected and mapped to the wheat unigene data set using SOAP2 (Li et al., 2009). smRNA reads showing sequences identical to those of known miRNAs in the miRBase were selected as the miRNA data set of the wheat TGC line (Griffiths-Jones, 2006). We modified the perl scripts described previously by Chen et al. (2007) to identify smRNAs phased in 21-nucleotide increments that could represent potential TAS genes. Before annotation of DGE tags, a preprocessed database of all possible CATG + 17-nucleotide tag sequences was created from the assembled wheat unigenes. The DGE tags were aligned against the preprocessed database using megabLAST with a size of 12 and filtering of low-complexity regions turned off (Morgulis et al., 2008). Only DGE tags that perfectly matched to tags in the preprocessed database without mismatches and gaps were considered. DGE tags mapping to unigenes with multiple homologous family members were excluded from our analysis. When there were multiple types of DGE tags aligned to different locations of the same unigene, the unigene expression levels were represented by the sum of all. The expression of smRNAs and unigenes was normalized to TPM in each lane of a flow cell.

For the degradome libraries, we used the CleaveLand pipeline to find sliced miRNA targets using the wheat unigenes. Viridiplantae miRBase 15.0 mature miRNAs, and miRNAs identified in our study as inputs (Addo-Quaye et al., 2009a). All alignments with scores up to 7 and no mismatches at the cleavage site (between the 10th and 11th nucleotides of mature miRNA) were considered as candidate miRNA targets. All smRNA reads and degradome tags obtained in this study have been deposited in the Gene Expression Omnibus database with the accession numbers GSE68687 and GSE37134, respectively.

smRNAs in Response to Cold Stress in a TGMS Line

TAGMan miRNA Assay and Real-Time PCR for miRNA Quantification

TAGMan miRNA assays were used to quantify miRNAs in this study, each with two independent biological replicates, as described previously (Chen et al., 2005). Briefly, 5 ng of total RNA was incubated with 1.5 μL of 10× reaction buffer, 0.15 μL of deoxyribonucleotide triphosphates (100 μM), 0.19 μL of RNase inhibitor, 1 μL of reverse transcriptionase, and 3 μL of stem-loop reverse transcription primer (Applied Biosystems) in a 15-μL reaction mixture. The real-time PCR for each assay was set up as a 20-μL reaction mixture containing 1.5 μL of cdNA, 10 μL of TaqMan 2× Universal PCR master mix, and 1 μL of 20× TaqMan assay mix including miRNA-specific primers and the TaqMan probe. The reaction was incubated in an Applied Biosystems 7900HT Fast Real-Time PCR System on 384-well plates at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Normalization was performed with small nuclear RNA U6 (NCBI accession no. X63066). Comparative real-time PCR was performed in triplicate, including template-free reactions.

For miRNAs, qPCR was performed with a MiniOpticon (Bio-Rad) using Maxima SYBR Green qPCR master mix (Fermentas). TaqGAPDH was used as the internal standard to reduce systematic and biological variance. Three replicates were analyzed for each sample along with template-free reactions as negative controls.

The relative expression ratio was calculated using the method of Livak and Schmittgen (2001). We used t tests to detect significant differences (P < 0.05) in expression between two samples.

RNA Ligase-Mediated 5' RACE

Total RNAs from spikes were used to purify mRNA using the Oligotex kit (Qiagen). 5' RACE analysis was carried out using the poly(A) plus fraction and the GeneRacer kit (Invitrogen). The final PCR products were detected by gel electrophoresis and cloned for sequencing. Primers used in RNA ligase-mediated 5' RACE are listed in Supplemental Table S4.

Construction of TMAs

The formalin-fixed and paraffin-embedded anthers from cold-stressed or control plants of the TGMS line were used to construct TMAs. The entire paraffin-embedded anthers as tissue cores were transferred to the recipient tissue microarray blocks using a precision instrument (model ATA-100; Chemicon International). For each anther developmental stage, 20 individual anthers were placed side-by-side on the recipient tissue microarray block. The block was then cut into 6-μm slices with a microtome (Leica). The cross-sections were placed on positively charged slides and then heated to 40°C for 30 min. After leveling paraffin and tissues, the TMA was cooled to 4°C for 15 min.

smRNA in Situ Hybridization

We used anther TMAs for smRNA in situ hybridization as described by Kidner and Timmermans (2006). Locked nucleic acid probes with sequences complementary to those of smRNAs were synthesized by TaKaRa and digoxigenin labeled with the DIG Oligonucleotide 3'-End Labeling kit (Roche Applied Science). Ten picomoles of each probe were used for each TMA slide, and hybridization and washing steps were performed at 55°C. Two TMA slides were prepared for each probe.

Supplemental Data

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

Supplemental Figure S1. Size distribution of redundant and nonredundant small RNA.

Supplemental Figure S2. Predicted RNA hairpin structures of conserved miRNA precursors.

Supplemental Figure S3. Diagrammatic representation of 21-nucleotide phased siRNA formation from TAS3a and TAS3b.

Supplemental Figure S4. Amplification plot of 19 miRNAs and one tasiRNA.
Supplemental Figure S5. Correlation between Solexa sequencing and Taqman qPCR.

Supplemental Figure S6. qPCR analysis of expression profiles of target genes in spikes.

Supplemental Figure S7. Endogenous auxin levels in anthers of the TGMS line.

Supplemental Figure S8. Overview of anther tissue microarrays.

Supplemental Table S1. Summary of small RNA and degradome sequencing data sets from spike tissues.

Supplemental Table S2. Abundant miRNAs present in seven small RNA libraries.

Supplemental Table S3. Low temperature- and cold stress-responsive miRNA families in different plant species.

Supplemental Table S4. Primers were used in RLM-5′ RACE.

Supplemental File S1. Contig sequences were assembled from wheat EST.

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LITERATURE CITED


smRNAs in Response to Cold Stress in a TGMS Line


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