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Identification and Characterization of Small RNAs from Vernalized *Arabidopsis thaliana*

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MicroRNAs (miRNAs) and small interfering RNAs (siRNAs) are two major classes of small non-coding RNAs with important roles in the regulation of gene expression, such as mRNA degradation and translational repression, heterochromatin formation, genome defense against transposons and viruses in eukaryotes. MiRNA- and siRNA-directed processes have emerged as a regulatory mechanism for growth and development in both animals and plants. To identify small RNAs that might be involved in vernalization, a process accelerating flowering brought on by a long period of cold, we generated a library of small RNAs from Arabidopsis that had been subject to vernalization. From the analysis of the library, 277 small RNAs were identified. They were distributed throughout all the five chromosomes. While the vast majority of small RNA genes locate on intergenic regions, others locate on repeat-rich regions, centromeric regions, transposon-related genes, and protein-coding genes. Five of them were mapped to convergent overlapping gene pairs. Two-hundred and forty of them were novel endogenous small RNAs that have not been cloned yet from plants grown under normal conditions and other environmental stresses. Seven putative miRNAs were up- or down-regulated by vernalization. In conclusion, many small RNAs were identified from vernalized Arabidopsis and some of these identified small RNAs may play roles in plant responses to vernalization.

Key words; microRNA, small interference RNA, vernalization

A number of multicelluar eukaryotes possess RNA-based silencing mechanisms that function at the transcriptional or posttranscriptional level to suppress a variety of endogenous and exogenous genetic elements, including genes, transposons and retroelements, viruses, and transgenes (Baulcombe, 2004; Bartel, 2004; Tomari and Zamore, 2005). The core silencing mechanism involves a large number of small non-coding RNAs including microRNAs (miRNAs) and diverse endogenous small interfering RNAs (siRNAs) that are 21 to 24 nucleotides (nts) in length (Aravin and Tuschl, 2005; Jones-Rhoades et al., 2006). MiRNAs are generated from self-complementary foldbacks, whereas siRNAs are generated from long double-stranded RNAs made by RNA-dependent RNA polymerase (RDR) (Meister and Tuschl, 2004; Baulcombe, 2004). The formation of small RNA duplexes from self-complementary foldbacks or dsRNA precursors is catalyzed by complexes containing the Ribonuclease III-like enzyme DICER or DICER-LIKE (DCL) proteins (Denli and Hannon, 2003). One strand of the duplex is then predominantly incorporated into an RNase H-like ARGONAUTE (AGO, PAZ/PIWI domain)-containing RNA-induced Silencing Complex (RISC) for sequence-specific translational inhibition (in the case of imperfect complementarity) or transcript cleavage (in the case of perfect or near perfect complementarity) (Fagard et al., 2000; Sigova et al., 2004; Voinnet, 2001; Waterhouse et al., 2001; Vaucheret et al., 2004) or a nuclear RNA-induced Initiation of Transcriptional Silencing (RITS) complex for the repression of gene expression through DNA methylation and chromatin modification (Verdel et al., 2004; Mette et al., 2000; Aufsatz et al., 2002; Gong et al., 2002; Hamilton et al., 2002; Volpe et al., 2002; Zilberman et al., 2003).

In plants, several classes of endogenous siRNAs exist. The most abundant heterochromatin-associated siRNA class, primarily 24 nt siRNAs, is generated from DNA repeats, such as retrotranselements and transposons, consistent with the idea that RNAi functions in silencing transposon expression and propagation (Sontheimer and Carthew, 2005). Another class of siRNAs, natural *cis*-antisense transcript-derived siRNAs (nat-siRNAs), were derived from bidirectionally oriented transcript pairs (Borsani et al., 2005). This class was processed to 21 and 24 nt nat-siRNAs by DCL1 and DCL2, respectively. Finally, *trans*-acting siRNAs (ta-siRNAs) are derived from long non-coding single stranded RNAs (ssRNAs) that are cleaved by miRNAs to produce truncated RNAs (Allen et al., 2005; Gasciolli et al., 2005; Peragine et al., 2004; Vazquez et al., 2004; Yoshikawa et al., 2005; Xie et al., 2005). Mature ta-siRNAs, derived from both strands, cleave *trans* endogenous mRNAs, similar to miRNAs (Peragine et al., 2004; Vazquez et al., 2004; Allen et al., 2005),

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and ta-siRNAs, derived from a positive strand, can also act *in cis* by guiding the cleavage of their precursor RNAs; in this way, they participate in feedback regulation of the pathway.

MiRNAs have been discovered using three approaches: direct cloning, forward genetics, and bioinformatic predictions followed by experimental validation (Jones-Rhoades et al., 2006). Unlike miRNAs, computational methods to predict endogenous siRNAs are currently unavailable. Consequently, their identification requires empirical approaches (Sunkar et al., 2005b). In Arabidopsis, it is known that highly diversified small RNAs (miRNAs, ta-siRNAs, nat-siRNAs and heterochromatin-associated siRNA) have roles in diverse biological processes, including developmental gene expression, response to environmental stresses, genome maintenance, adaptive protection against viruses, or genome defense against mobile DNA elements (reviewed in Xie et al., 2004; Jones-Rhoades et al., 2006; Bonnet et al., 2006, Vaucheret, 2006). Several Arabidopsis miRNAs and siRNAs have been reported to control flowering time (Achard et al., 2004; Aukerman and Sakai, 2003; Schwab et al., 2005; Swiezewski et al., 2007). For example, miR159 delays flowering under short days through the suppression of *LEAFY* expression whereas miR172 accelerates flowering through the suppression of floral repressors, *TOE1* and *2* (*TARGET OF EAT1*) (Aukerman and Sakai, 2003; Achard et al., 2004). It has also been reported that weak activity of *FLOWERING LOCUS C* (*FLC*), a strong floral repressor, in Lansberg *erecta* (Ler) ecotype is due to the suppression by siRNAs targeting a transposon naturally inserted in the second intron of *FLC* (Liu et al., 2004). Recently Swiezewski et al. (2007) reported that small RNA-mediated chromatin silencing directed to the 3′ region of *FLC* is an important regulatory mechanism. However, the presence of small RNAs mediating vernalization, a process accelerating flowering brought on by a long period of cold, has not been reported yet. Thus, we focused on the discovery of the endogenous small RNAs that are involved in the vernalization process for flowering.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The plant we used for the construction of a small RNA library is *FRIGIDA* (*FRI*) containing the Columbia (Col) isogenic line (described in Michaels and Amasino, 1999), which is named *FRI*-Col. In preparation for vernalization treatment, the seeds were incubated at 4°C for eight weeks under short day photoperiod (8 h light/16 h dark) conditions and were then returned to a warm temperature (22°C) for 2 days under long day photoperiod (16 h light/8 h dark) conditions. Arabidopsis mutants, *hen1-1, dcl1-14* (SALK_056143) and *dcl3-1* (SALK_005512) were provided by Dr. J. Carrington. The growth conditions of mutants, *hen1, dcl1-14,* and *dcl3-1,* were described by Kim and Lee (2006).

Cloning of Small RNAs from Arabidopsis

Total RNA was isolated from vernalized *FRI*-Col seedlings using TRI reagent (Sigma). The cloning of small RNAs was performed as described previously (Sunkar and Zhu, 2004). In short, about 600 μg of total RNA was resolved through four lanes on a denaturing 15% polyacrylamide gel containing 8M urea in TBE buffer (45 mM Tris-borate, pH8.0, and 1.0 mM EDTA). A gel fragment spanning the size range 15~26 nts was excised, and RNA was eluted overnight with 0.4M NaCl at 4°C. Labeled Decade Marker RNA (Decade Marker system; Ambion) was used as the size standard during electrophoresis. The RNA was recovered by ethanol precipitation, then dephosphorylated, and recovered again by ethanol precipitation. The small RNAs obtained were ligated sequentially to 5′ and 3′ RNA/DNA chimeric oligonucleotide adapters. The 3′ adapter oligonucleotide (5′-pUUaaccgcgaattccagX-3′; uppercase, RNA; lowercase, DNA; p, phosphate; x, inverted deoxythymidine) was then ligated to the dephosphorylated small RNAs. The ligated product was recovered from the gel, 5′ phosphorylated, then recovered by ethanol precipitation. Following that, the 5′ adapter (5′-acggaattcctcactAAA-3′; uppercase, RNA; lowercase, DNA) was ligated to the phosphorylated ligation product, as described above. The ligates were excised and eluted from the gel. Reverse transcription reaction was performed using the RT primer (5′-gactagctg**gaattc**gcggttaaa-3′; bold, *Eco*RI site) and Superscript II reverse transcriptase (Bethesda Research Laboratories Life Technologies). This was followed by PCR using the RT primer (5′-gactagctg**gaattc**gcggttaaa-3′; bold, *Eco*RI site) and forward primers (5′-cagccaacg**gaattc**ctcactaaa-3′; bold, *Eco*RI site). The PCR product was purified by phenol/chloroform extraction, precipitated with ethanol, and digested with *Eco*RI restriction enzyme. After additional phenol/chloroform extraction and ethanol precipitation, the digested products were concatemerized with T4 DNA ligase at a concentration of 40,000 units/mL (NEB). Concatemerization approaches were used to produce longer inserts (Lau et al., 2001). A gel fragment spanning the size range of 200~600bp was excised, and concatemers were eluted with QIAquick(R) Nucleotide Removal kit (Qiagen). This was followed by PCR using Taq polymerase with the reverse and forward primers. The concatamers were ligated into the T/A site of a pGEM-T Easy vector (Promega) using T4 DNA ligase (Promega). Positive colonies were identified by PCR using the M13F and M13R primers and colonies with inserts bigger than 200 bp in length were selected. The inserts from individual colonies were then sequenced.

RNA Gel Blot Analysis

Total RNA was isolated using TRI reagent (Sigma) from *FRI*-Col seedlings treated with (+V) or without (-V) vernalization or recovered in warm temperature for 2 days after vernalization ($+V^{RT}$). Low molecular weight RNA was isolated by polyethylene glycol precipitation from total RNA (Hamilton and Baulcombe, 1999). For the detection of low molecular weight RNA, ~50 μg of total RNA was precipitated with ethanol and dissolved in 300 μL DEPC-water. High molecular weight RNA was selectively precipitated from the total RNA by the addition of one volume of 20% PEG-1M NaCl. The resulting low-molecular weight-enriched RNA was then separated by 15% denaturing polyacrylamide gels containing 8M urea in TBE buffer and electrically transferred to Hybond-N^+ membranes. Synthetic oligonucleotides of length

21 and 24 nts were used as size standards during electrophoresis. Ethidium bromide staining and visualization of the 5S rRNA/tRNA bands in small RNA gels were used to monitor the loading of RNA samples. Low-molecular weight-RNA blots were probed with DNA oligonucleotides complementary to small RNA sequences (smRNA101, 5′-gtttcttctttctggcgatatc-3′; smRNA104, 5′-agcgaaaacgacgtcgttttattt-3′; smRNA107, 5′-tctgataactttaaaacggat-3′; smRNA115, 5′-cctctacgcctaggacaccagaata-3′; smRNA117, 5′-gtaaacatatcataaataagatca-3′; smRNA125, 5′-gtcaaagggtgtggcctgcaaatt-3′; smRNA128, 5′-ctattgaactacttacccac-3′). The probes were end-labeled with γ-^{32}P-ATP using T4 kinase (NEB). Unincorporated nucle-

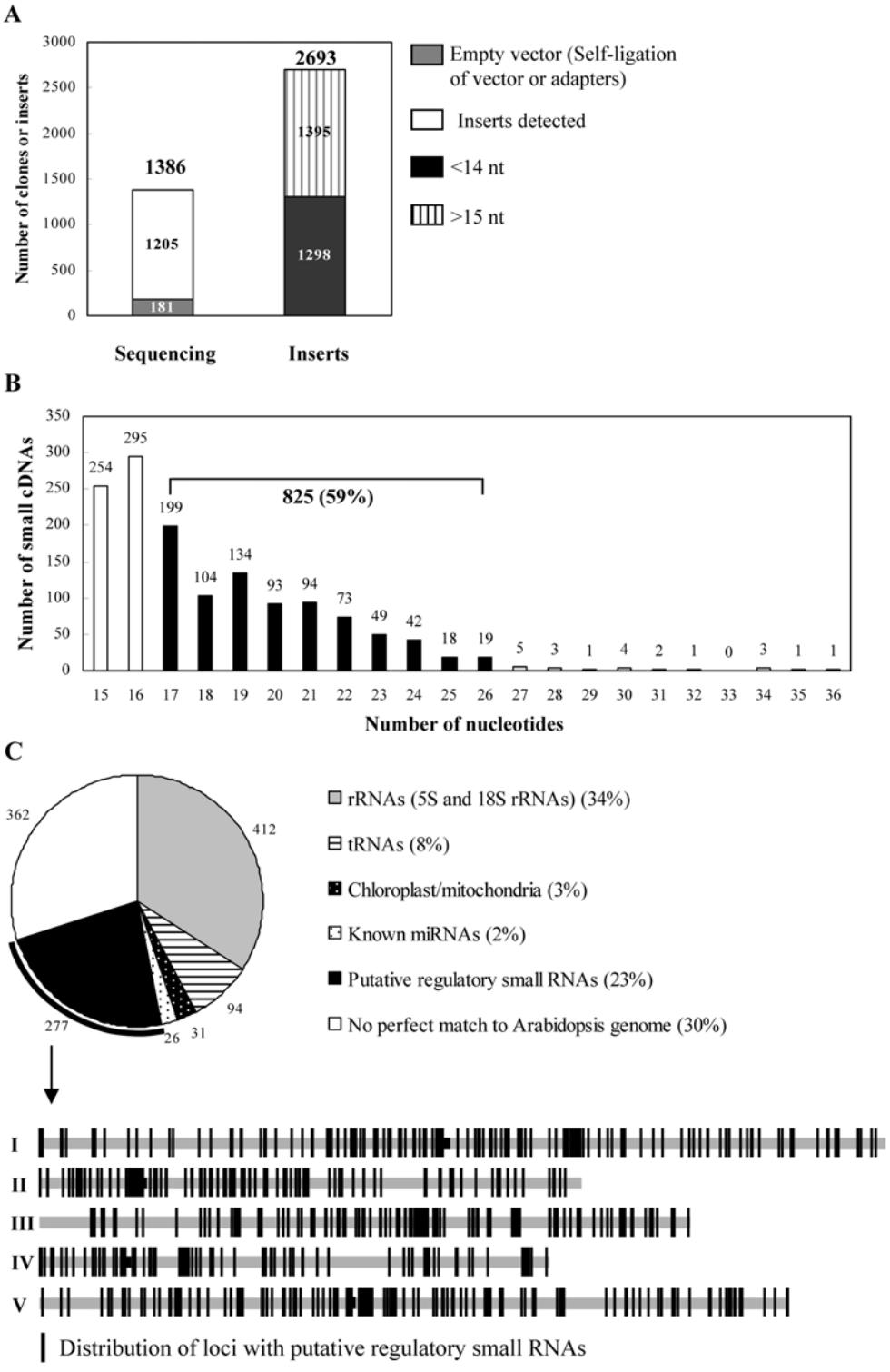

Figure 1. Summary of the small RNA library obtained from vernalized Arabidopsis.
(**A**) The number of sequenced clones and inserts (cDNA sequences), (**B**) The size distribution of cDNA sequences in the library, (**C**) Classification of the library of small RNAs and distribution of chromosomal loci for putative small RNAs.

otides were removed using QIAquick(R) Nucleotide Removal kit (Qiagen) following the manufacturer's instructions. The blots were prehybridized for at least 1 hr and hybridized overnight at 38°C using Perfect Hyb Plus buffer (Sigma). The blots were washed twice in 1X SSC and 0.1% SDS for 30 min at 45°C. The membranes were exposed to X-ray films.

RESULTS AND DISCUSSION

Cloning of small RNA library and sequence analysis

To identify endogenous small RNAs that might be involved in vernalization, a cloning approach was employed. A cDNA library of endogenous small RNAs was generated from *FRI*-Col seedlings which were subjected to a cold period of 8 weeks during germination and were then returned to warm temperature conditions (22°C) for 2 days. A total of 1386 positive clones was randomly selected from the library and sequenced. Among them, 181 clones were identified as self-ligated vectors or adapters (Figure 1A). A total of 2693 small cDNA sequences (referred as small cDNA below) were yielded by the 1205 clones (approximately 2~3 inserts in each clone) (Figure 1A). The length of the small cDNAs ranged from 4 to 36 nucleotides (nts), and almost half of all the small cDNAs (1298 of 2693 small cDNAs) were smaller than 14 nts in length (Figure 1A and 1B). Presumably, these sequences were formed as a result of the random breakdown of cellular RNA and were not excluded from the library during the size fractionation of total RNA. Thus, we excluded sequences less than 14 nts (<14 nts) from further analysis. Among the remaining small cDNAs >15 nts, a large proportion was distributed between 15 and 17 nts (Figure 1B). Only 59% of the small cDNAs was in the range of the average size of miRNAs and siRNAs (between 17 and 26 nts) (Figure 1B).

The 1395 small cDNAs larger than 15 nts in length were used to BLAST search against the Arabidopsis database. This process allowed the removal of most rRNA, tRNA, small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA) sequences from the library. The results of the BLAST search revealed that 70% of these sequences have at least one match in the Arabidopsis genome sequences. The small cDNAs derived from rRNAs and tRNAs were approximately 34% and 8% respectively (Figure 1C), which were slightly lower than the previous reports that had >80% of small cDNAs matched with these sequences (Sunkar and Zhu, 2004; Sunkar et al., 2005a and b). A small proportion of the sequences (3%) was also mapped to chloroplast or mitochondrial genomes (Figure 1C). This indicates that degradation of, or possibly regulatory products of, organellar RNA are included in this RNA pool, as previous reports have indicated (Llave et al., 2002; Sunkar and Zhu, 2004; Sunkar et al., 2005a and b). Thirty percent (362) of the small cDNAs did not show any matches to the Arabidopsis genome, and thus were not analyzed further. The lack of match in these sequences may be due to sequencing errors or may be due to their derivation from other microorganisms living with Arabidopsis. Twenty-six small cDNAs in the library were identical with 18 miRNA families previously reported from Arabidopsis (Figure 1C). These include miR156, miR157, miR158, miR159, miR160, miR161, miR165, miR167, miR168, miR169, miR171, miR173, miR319/miRJAW, miR389, miR393, miR397, miR398, miR408, and miR824 (Llave et al., 2002; Park et al., 2002; Reinhart et al., 2002; Palatnik et al., 2003; Sunkar and Zhu, 2004). Such a significant presence of reported miRNAs in our library strongly suggests the feasibility of our approach. Thus, we assumed that the remaining 23% of small cDNAs (307 small cDNAs representing 277 unique sequences), which show a perfect match against Arabidopsis genome sequences, is putative regulatory small RNAs. They were distributed all over the five chromosomes (Figure 1C).

Analysis of Putative Small RNAs in the Library

A total of 277 endogenous small RNA sequences identified in this study were heterogeneous in terms of size and origin. The size range was between 15 and 25 nts (Figure 2A). The majority was in the range of 15 to 17 nts and only 21% (59 of 277 putative small RNAs) was in the range of 21 to 24 nts, which is a typical size range for Dicer-derived products. Our analysis showed that most of the short sequences between 15 to 17 nts are derived from intergenic regions (IGR), and thus they were most likely to be small RNAs. This result is somewhat different from previous reports showing that the majority is in the range of 21 to 24 nts (Hamilton et al., 2002; Llave et al., 2002; Tang et al., 2003; Papp et al., 2003; Sunkar and Zhu, 2005; Sunkar et al., 2005b). Recently, the new miRNAs and siRNAs that might be regulated by various environmental stresses were reported by Sunkar and Zhu (2004). The size range of small RNAs in their library was also between 15 and 25 nts, although the majority is in the range of 20 to 24 nts and they identified small RNAs with 15 to 17nts in length as new abiotic stress regulated endogenous small RNAs. The short small RNAs they identified have not been found in the previously cloned small RNAs under normal growth conditions. Thus, many small RNAs with 15 to 17 nts in our library are likely to be accumulated by specific environmental signal, vernalization. However, we cannot completely exclude the possibility that some of the small RNA sequences between 15 to 17 nts in our library are formed as a result of the random breakdown of cellular RNAs or genomic DNA.

The nucleotide preference at 5′ end in small RNAs could be used as the criterion to determine the small RNA classes; 21 nt size miRNAs and endogenous ta-siRNAs produced by DCL1 and DCL4 respectively show a strong preference for U at 5′ end (Vazquez et al., 2004; Peragine et al., 2004; Borsani et al., 2005; Sunkar et al., 2005b), whereas 24 nt size endogenous siRNAs produced by DCL3 from repeat-rich heterochromatic regions show an A preference at 5′ end (Onodera et al., 2005; Xie et al., 2004; Pontes et al., 2006). By contrast, 21 nt siRNAs derived from transgenes do not show any preference for a specific nucleotide (Papp et al., 2003; Sunkar et al., 2005b). When we checked the nucleotide preference at 5′ end for the identified small RNAs, 106 among 277 small RNAs began with U, whereas the numbers of sequences that began with A, C, and G were 62, 54, and 55, respectively (Figure 2B). We also investigated nucleotide preference at 5′ end in small RNAs with 21 to 24 nts in size, which is the typical size range for

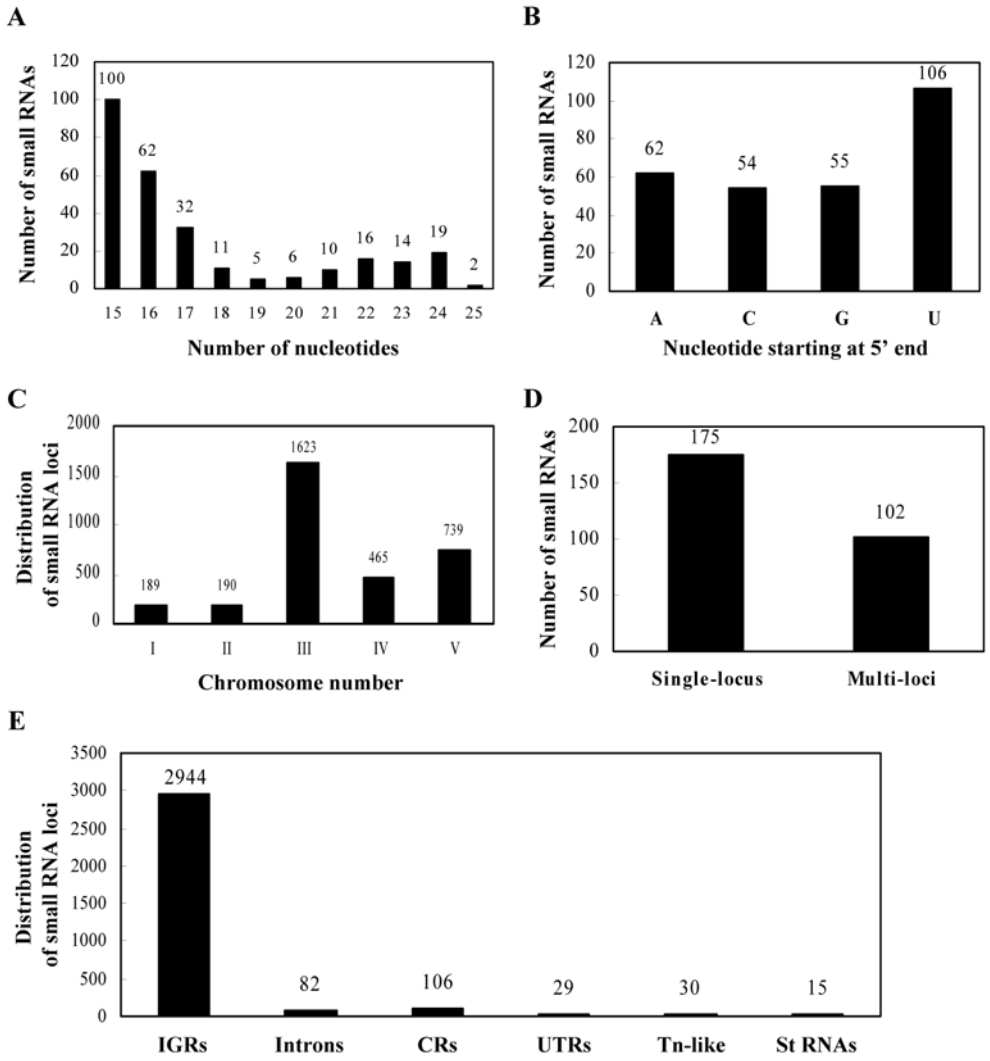

Figure 2. Analysis of endogenous small RNAs obtained in this study.
(A) Size distribution of putative small RNA sequences, (**B**) Nucleotide preference at the 5′ end of small RNAs, (**C**) The number of small RNA loci on each of the five chromosomes, (**D**) Comparison of the number of small RNAs mapped on a single locus and multiple loci, (**E**) The classification of small RNAs based on the origin. IGR; intergenic regions, introns, CRs; coding regions, UTRs; untranslated regions, Tn-like; transposon-like genes, St RNAs; structural RNA genes (tRNAs and snRNAs).

Dicer-derived products. Among 59 small RNAs with 21 to 24 nt size, 26 sequences start with U and 20 sequences start with A at 5′ end. Six and seven sequences start with C and G at 5′ end, respectively (data not shown). Most of them have 5′U (Bartel and Bartel, 2003; Bartel, 2004) and 5′A (Onodera et al., 2005; Xie et al., 2004) preference, that is similar to earlier reports. Thus, this result indicates that most of the small RNAs in range of 21 to 24 nts are probably either endogenous siRNA or miRNA processed by diverse DCLs.

The distribution of small RNA-generating loci was also analyzed. The 277 small RNAs were mapped to 3206 loci distributed throughout five chromosomes (Figure 1C, 2C). The 175 small RNAs were encoded by a single genomic locus, whereas the remaining 102 were encoded by multiple loci (2 to 429 loci) (Figure 2D). Interestingly, only 13 small RNAs mapped to 2661 genomic loci, which corresponds to 83% of the whole small RNA loci. The 13 small RNAs were scattered over several islands that contain highly repeated sequences in centromeric and pericentromeric regions (data not shown). For example, the sequences of a small RNA, smRNA120, were repeated 22, 56, 13, 229, and 109 times on centromeric and pericentromeric regions of chromosomes I to V, respectively. The number of small RNA loci among the five chromosomes was highly variable, namely 189 loci on chromosome I, 190 on chromosome II, 1623 on chromosome III, 465 on chromosome IV, and 739 on chromosome V (Figure 2C). Thus, the numbers of small RNA loci on chromosomes I and II were very low, whereas that on chromosome III was disproportionately high. The high frequency on chromosome III was mainly because 9 among the 13 small RNAs are present on chromosome III as highly repetitive sequences (mapped to 1507 loci) in the centromeric and pericentromeric regions. As an example, the sequences encoding small RNAs, smRNA110 and smRNA109, were repeated 273 and 261 times, respectively,

on chromosome III in both sense and antisense orientations. Our result is consistent with a previous report showing that the density of small RNA-homologous loci is highest in the centromeric and pericentromeric regions with highly repetitive sequences such as transposons and retroelements (Kasschau et al., 2007). However, in our study, a significant proportion of centromeric and pericentromeric small RNA loci were mapped to intergenic regions (~2600 loci), and only 12 of them corresponded to transposable elements in centromeric regions or nearby, thus, showing a subtle difference from the previous reports.

The origins of the identified small RNAs were classified. The small RNAs were derived from intergenic regions (IGRs), transposon-like genes, expressed genes, and structural RNA genes (Figure 2E). The vast majority of small RNAs (152 small RNAs mapped to 2944 loci) were derived from IGRs; 117 of them were derived from only an IGR position, whereas 35 were derived from multiple sites including protein-coding genes as well. Twenty-five small RNAs corresponding to multiple loci appeared to be derived from repeat-rich regions or centromeric regions (data not shown). The 129 small RNAs were mapped to genomic sequences of either sense or antisense polarity within a known or predicted protein-coding gene. Among them, 104 were mapped only to a protein-coding gene but 25 were mapped not only to a protein-coding gene but also to IGR. Among the 104 small RNAs, 66, 24 and 14 were mapped to protein coding regions, introns, and UTR, respectively. The 66 small RNAs were perfectly matched to the mRNAs of 77 protein coding genes, 37 in sense orientation and 40 in antisense orientation (data not shown).

Approximately 10% of Arabidopsis genes are in convergent overlapping gene pairs, also known as natural *cis*-antisense gene pairs (Boi et al., 2004; Wang et al., 2005; Jen et al., 2005). It has been reported that nat-siRNA formed from natural *cis*-antisense transcripts degrades the mRNA of one of the overlapping genes (Borsani et al., 2005). In our study, five small RNAs (smRNA112, smRNA113, smRNA127,

smRNA103	*A. thaliana*	UGGCUUUGGGCUUUUCCUGCGCA	
	Oryza sativa	UGGCUUUGGGC**C**UUUCCUGCGCA	
	Populus trichocarpa	UGGCUUUGGGC**C**UUUCCUGCGCA	
	Populus trichocarpa	UGGCUUUGGGC**CA**UUCCUGCGCA	
	Populus trichocarpa	UGGCUUUGGGC**C**UUUCCUCCGCA	
smRNA106	*A. thaliana*	AUAUUUUAAGUGUAAGAAGAC	
	A.thaliana	AUAUUUUAAGUGUA**G**GAAGAC	Chr. 4 (IGR)
	Lotus japonicus	AUAUUUUAAGUGUAAGAAG	
	Medicago truncatula	AUAUUUUAAGUGUAAGA	
smRNA115	*A. thaliana*	UAUUCUGGUGUCCUAGGCGUAGAGG	
	Medicago truncatula	UAUUCUGGUGUCCUAGGCGUAGAGG	
	Oryza sativa	UAUUCUGGUGUCCUAGGCGUAGAGG	
	Populus trichocarpa	UAUUCUGGUGUCCUAGGCGUAGAGG	
smRNA122	*A. thaliana*	UUUAUUAGUAGUAGAUUUGUUUUA	
	A. thaliana	UUUAUUAGUAGUAGAUUUUUUUU**C**	Chr. 2 (IGR)
	Oryza sativa	- - UAUUAGUAGUAGAUUUUUUUU	Chr. 7 (150554 bp)
	Medicago truncatula	- - UAUUAGUAGUAGAUUU**G**	
smRNA123	*A. thaliana*	UUGCUUUUUUAGTTATATGTATA	
	A. thaliana	UUGCUUUUU**C**AGTTATATGTATA	Chr. 4 (IGR)
	A. thaliana	UUGCUUUUUUUA**A**TTATATGTATA	Chr. 5 (IGR)
	Oryza sativa	UUGCUUU**A**UUAGTTATATG	Chr. 11 (43883 & 136853 bp)
	Lotus japonicus	- - GCUUUUUUAGTTATATGTAT	
smRNA114	*A. thaliana*	ACCGUUAACACUCCUUAACAGCGU	
	A. thaliana	ACCGUUAACACUCCUUAACAGCG**C**	Chr. 1 (IGR)
	A. thaliana	ACCGUUAACACUCCUUAACAGCG**C**	Chr. 4 (1st intron of At4g02320)
	A. thaliana	ACCGUUAACACUCCUUAACAGCG**C**	Chr. 4 (IGR)
	A. thaliana	ACCGUUAACACUCCUUAACAGCG**C**	Chr. 5 (IGR)
	A. thaliana	ACCGUUAACACU**U**CUUAACAGC**UC**	Chr. 1 (IGR)
	A. thaliana	ACCGUUAACACU**U**CUUAACAGC**UC**	Chr. 1 (IGR)
	A. thaliana	ACCGUUAACACU**U**CUUAACAGC**UC**	Chr. 2 (IGR)
smRNA117	*A. thaliana*	UGAUCUUAUUUAUGAUAUGUUUAC	
	A. thaliana	UGAUCUUAUUUAUGAUAUGUUUAC	Chr. 5 (IGR)
	A. thaliana	UGAUCUUAUUUAUGAUAU**A**UUUA**U**	Chr. 3 (IGR)
	A. thaliana	UGAUCUUAUUUAUGAUAU**A**UUUA**U**	Chr. 4 (IGR)
	A. thaliana	UGAUCUUAUUUAUGAUAU**A**UUUA**U**	Chr. 5 (IGR)
smRNA119	*A. thaliana*	GGUGUACGUCAUCUCGUACAC	
	A. thaliana	GGUGUAC**A**UCAUCUCGUACAC	Chr. 2 (IGR)
	A. thaliana	GGUGUAC**A**UCAUCUCGUACAC	Chr. 2 (IGR)
	A. thaliana	GGUGUAC**A**UCAUCUCGUACAC	Chr. 4 (IGR)
	A. thaliana	GGUGUAC**A**UCAUCUCGUACAC	Chr. 5 (IGR)
smRNA125	*A. thaliana*	AAUUUGCAGGCCACACCCUUUGAC	
	A. thaliana	AAUUUGCAGG**U**CACACCCUUUGAC	Chr. 4 (IGR)
	A. thaliana	AAUUUGCAGG**G**CACACCCUUUGAC	Chr. 5 (IGR)
	A. thaliana	AAUUUGCA**A**GCCACACCCUUUGA**T**	Chr. 5 (IGR)

Figure 3. Alignment of putative miRNA sequences identified in the library with predicted homologs in Arabidopsis and/or other plants. Identical sequences of putative miRNAs cloned in this study are labeled in gray letter. Black bold letters represent changes in nucleotide sequences in homologous sequences. The location of the putative miRNAs in Arabidopsis chromosomes is shown to the right of the sequences.

smRNA128, and smRNA131) were mapped to convergent overlapping gene pairs (data not shown). Several of these small RNAs may actually be processed into nat-siRNAs and degrade the mRNA of one of the overlapping genes.

Twenty eight small RNAs corresponded to 30 transposable elements that mostly exist in centromeric regions or nearby. Nine of them were derived from a unique transposable element, whereas 19 corresponded to multiple sites (also to IGR or protein-coding genes). These transposons included both class I, such as long terminal repeat (LTR), non-LTR retrotransposons and class II, such as MULE (Mutator-like elements), and CACTA-like transposons (class II) (data not shown).

The fold-back structure and sequence conservation are the most important criteria differentiating miRNA from siRNA. To identify new miRNAs from our library, 277 small RNA candidates were tested with fold-back (hairpin) secondary structure prediction using the MFOLD (Zuker, 2003). The prediction showed that 22 small RNAs (smRNA101, smRNA102, smRNA103, smRNA104, smRNA105, smRNA106, smRNA108, smRNA111, smRNA114, smRNA115, smRNA116, smRNA117, smRNA118, smRNA119, smRNA121, smRNA122, smRNA123, smRNA124, smRNA125, smRNA126, smRNA129, and smRNA131) could be derived from fold-back structure. For these 22 small RNAs, we checked sequence conservation among other plant species. To search homologous sequences, we allowed 1-2 nucleotide differences outside the so-called 'seed' sequences, the seven nucleotides at the 2-7 position in the mature miRNA, because the seed sequence is strictly conserved (Kim and Nam, 2006). Among 22 small RNAs predicted to have fold-back structure, 5 small RNAs (smRNA103, smRNA106, smRNA115, smRNA122, and smRNA123) have homologues in other species such as rice, lotus, Medicago, maize, and populus, whereas 4 small RNAs (smRNA114, smRNA117, smRNA119, and smRNA125) show homologous sequences in other regions of Arabidopsis (Figure 3). Such results indicate that the five putative miRNAs conduct important and perhaps conserved functions among plant species. Additionally, there is a growing recognition that significant numbers of miRNAs are not conserved in rice or poplar, and thus seem to have arisen during recent evolutionary process (Lindow and Krogh, 2005; Maher et al., 2006; Allen et al., 2004; Lu et al., 2005). The 4 putative miRNAs that exist as multiple members only in Arabidopsis may be the products of recent duplication. Among them, small RNA, smRNA114, is outstanding in that it is mapped

A. Alignment of the smRNA114 with the predicted homologs in Arabidopsis

(a)	ACCGUUAACACUCCUUAACAGCGT	
(b)	ACCGUUAACACUCCUUAACAGCG**C**	Chr. 1 (0.39kb downstream of At1g55280)
(c)	ACCGUUAACACUCCUUAACAGCG**C**	Chr. 4 (1st intron of At4g02320)
(d)	ACCGUUAACACUCCUUAACAGCG**C**	Chr. 4 (0.3kb downstream of At4g25320)
(e)	ACCGUUAACACUCCUUAACAGCG**C**	Chr. 5 (0.4kb downstream of At5g42430)
(f)	ACCGUUAACACU**U**CUUAACAGC**UC**	Chr. 1 (0.35kb upstream of At1g12370)
(g)	ACCGUUAACACU**U**CUUAACAGC**UC**	Chr. 1 (0.6kb upstream of At1g68340)
(h)	ACCGUUAACACU**U**CUUAACAGC**UC**	Chr. 2 (0.67kb downstream of At2g02610)

B. The predicted fold-back secondary structures

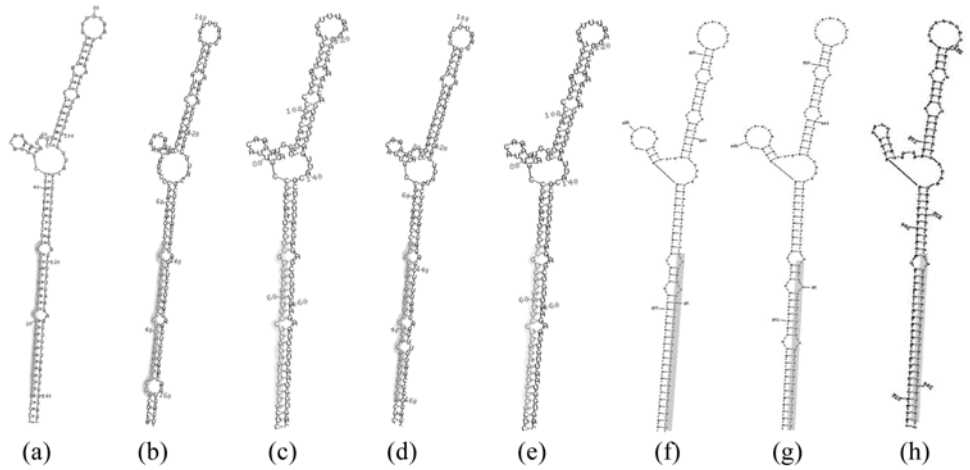

Figure 4. Alignment of a putative miRNA sequence, smRNA114, with predicted homologs and prediction of hairpin secondary structure of smRNA114 family members in Arabidopsis.
(**A**) Alignment of the putative miRNA, smRNA114, identified in the library with the predicted homologs in Arabidopsis, (**B**) The predicted hairpin secondary structures of smRNA114 family members. Identical sequences of smRNA114 are labeled in gray letters. Black bold letters represent changes in nucleotide sequences in homologs. The location of the putative miRNAs in Arabidopsis chromosomes is shown to the right of the sequences.

to only a single genomic locus but has the largest set of family members (Figure 4A). It has 7 family members; all except one are mapped to IGRs and 1 member mapped to intron in antisense orientation. Interestingly, all of the 7 family members showed similar fold-back structure prediction (Figure 4B). Thus, the putative miRNA, smRNA114, is likely to be one of the largest miRNA families in Arabidopsis and a unique Arabidopsis miRNA that is not conserved in other plants. Based on the hairpin structure and sequence conservation, some of the small RNAs identified are likely to be new miRNAs. However, it is yet to be confirmed if they are generated from a DCL1 involved biogenesis pathway.

The Expression of Endogenous Small RNAs Affected by Vernalization

To determine whether the expression of any endogenous small RNAs isolated in this study is regulated by vernalization, RNA gel blot analysis was performed. A low molecular weight RNA blot was prepared by using *FRI*-Col seedlings grown as vernalization untreated (–V), vernalization treated (+V), and recovered to warm temperature for 2 days after vernalization (+V^{RT}). The results of RNA gel blot analysis revealed that most of the small RNAs tested were not detected on the small RNA blots, presumably because expressions of these small RNAs are extremely low. However, signals at the appropriate sizes for seven small RNAs (smRNA101, smRNA104, smRNA107, smRNA115, smRNA117, smRNA125, and smRNA128) were detected (Figure 5). Five of them, smRNA101, smRNA104, smRNA115, smRNA117, and smRNA125 were predicted to have fold-back structure in the MFOLD analysis, and thus are very likely to be miRNAs. But two small RNAs, smRNA107, and smRNA128, failed to make fold-back structure, and therefore seem to be endogenous siRNAs. Hereafter, the small RNAs, smRNA101, smRNA104, smRNA115, smRNA117, and smRNA125, were called putative miRNAs and the small RNAs, smRNA107 and smRNA128, were called putative siRNAs. The putative miRNA, smRNA115, showed relatively strong expression, but the other small RNAs showed weak expression. Three putative miRNAs, smRNA101, smRNA115, and smRNA117, showed down-regulation by vernalization. In the case of smRNA115 and smRNA117, the reduced expression by vernalization was recovered during acclimation to the warm temperature (+V^{RT}). A putative miRNA, smRNA101, showed lower expression in +V^{RT} than –V, indicating the maintenance of suppression induced by vernalization. By contrast, two putative miRNAs, smRNA104, smRNA125, and two putative siRNAs, smRNA107, smRNA128, were up-regulated by vernalization. The expression patterns of three small RNAs, smRNA104, smRNA107, and smRNA125, were similar: stronger expression in +V and +V^{RT} than –V, but slightly lower expression in +V^{RT} than +V. The small RNA, smRNA128, that was mapped to convergent overlapping gene pairs, also showed higher expression in +V^{RT} than –V, suggesting up-regulation by vernalization. Vernalization promotes flowering primarily by repressing *FLC* expression (Michaels and Amasino, 1999; Sheldon et al., 2000; Michaels and Amasino, 2001). The repressed state of *FLC* by vernalization is maintained through mitotic cell divisions after a return to warm growing conditions (Michaels and Amasino, 1999). Likewise, small RNAs, smRNA101, smRNA104, smRNA107, smRNA125, smRNA128, maintained their expression, vernalization induced up- or down-regulation, after a return to warm temperature. Therefore, the result suggests that these small RNAs may be regulated by vernalization. However, unlike *FLC*, expression of the cold inducible genes return to the level before cold if the plants were transferred to warm temperature. In contrast, small RNAs, smRNA115 and smRNA117, showed the expression pattern of cold-inducible genes.

To examine if the expression of the newly predicted miR-

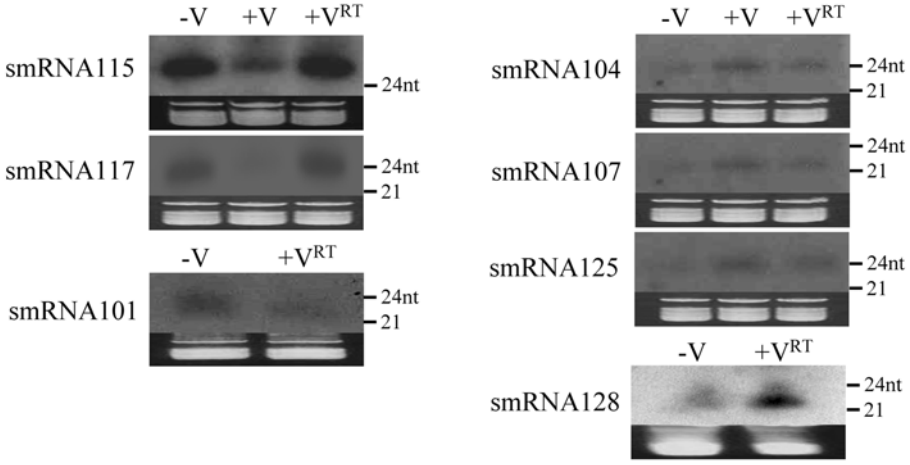

Figure 5. Expressions of seven small RNAs affected by vernalization.
The expressions of putative miRNAs, smRNA101, smRNA104, smRNA115, smRNA117, and smRNA125, and the putative siRNAs, smRNA107 and smRNA128, are shown. The expression of smRNA101, smRNA115, and smRNA117 in left panel were decreased by vernalization and the expression of smRNA104, smRNA107, smRNA125, and smRNA128 in right panel were increased by vernalization. Low molecular weight RNA was isolated from *FRI*-Col seedlings. –V, seedlings which were grown in the growth rooms at 22 for 6 days without vernalization treatment; +V, seedlings which were subjected to a cold period of 8 weeks while the seed was germinating; +V^{RT}; seedlings which were subjected to vernalization and transferred to the growth rooms at 22 for 2 days. The tRNA and 5S rRNA bands were visualized by ethidium bromide staining and serve as loading controls.

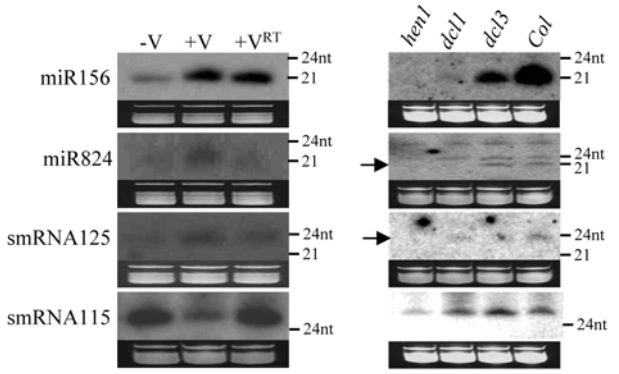

Figure 6. Accumulation of small RNAs in *hen1*, *dcl1*, and *dcl3* mutants.
Northern analysis of small RNAs from various small RNA processing mutants. One hundred mgs of low-molecular-weight RNA was loaded per lane. The blots hybridized with an oligonucleotide probe corresponding to the sequences of putative miRNAs, smRNA125 and smRNA115. The tRNA and 5S rRNA bands were visualized by ethidium bromide staining and serve as loading controls.

NAs, smRNA125 and smRNA115, are affected by the small RNA processing mutants, *hen1-1*, *dcl1-14*, and *dcl3-1*, RNA gel blot analysis was performed using RNA extracted from seedlings. We also checked the expression of two previously known miRNAs, miR156 and miR824 in our study, as controls, but their expression was not detected in *hen1-1* and *dcl1-14* (Figure 6) as expected (Park et al., 2002; Reinhart et al., 2002; Palatnik et al., 2003; Xie et al., 2004; Sunkar and Zhu, 2004, Bouche et al., 2006). On the other hand, miR824 accumulated normally, but miR156 showed reduced expression in *dcl3-1* mutant. The putative miRNA, smRNA125, was not detected in *hen1-1* and showed a reduced expression in *dcl1-14* and *dcl3-1* mutants. By contrast, the putative miRNA, smRNA115, showed reduced expression only in *hen1-1* mutant, but accumulated at near wild-type levels in *dcl1-14*, and *dcl3-1* mutants (Figure 6). Because HEN1 is a protein required for the efficient production of miRNAs and several siRNAs in Arabidopsis (Park et al., 2002), and DCL1 is involved in the processing of miRNA and nat-siRNA (Yu et al., 2005; Vazqiez et al., 2004; Borsani et al., 2005), our result suggests that smRNA125 is likely to be miRNA and smRNA115 is likely to be endogenous siRNA, although both of them are predicted to form a hairpin secondary structure.

In conclusion, we identified a large set of endogenous small RNAs from vernalized Arabidopsis using a direct cloning approach. A total of 277 unique putative small RNA sequences corresponding to 3206 genomic loci have been identified. Some of them were novel small RNAs that have not been cloned from plants grown under normal conditions or under other environmental stresses. Several small RNAs discovered in this study are either up- or down-regulated by vernalization, suggesting that they may be involved in vernalization-responsive gene expression. Further studies by generating transgenic plants overexpressing individual endogenous small RNAs or analyses of loss-of-function mutants may verify the roles of the identified small RNAs in the vernalization process. Eventually the discovery of many small RNAs and their targets, conditioned by epigenetic events such as vernalization, will provide an important opportunity to explore the potential roles of these molecules in gene regulation and genome maintenance.

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