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*Ta*miR1123 originated from a family of miniature inverted-repeat transposable elements (MITE) including one inserted in the *Vrn-A1a* promoter in wheat

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ABSTRACT

More than half of spring wheat cultivars have a dominant *Vrn-A1a* allele that has an insertion of a miniature inverted-repeat transposable element (MITE) in its promoter. In this study, we found that the MITE present in the *Vrn-A1a* gene (MITE_VRN) is a nearly perfect palindrome and it can form highly stable hairpin loops when expressed as RNA. MITE_VRN also possessed sequences of a microRNA in *Triticum aestivum* (*Ta*miR1123). The P^{32} labeled *Ta*miR1123 probe detected two RNA molecules on a small RNA gel blot, one expected for MITE_VRN, and the other expected for *Ta*miR1123. These results demonstrated that MITE_VRN was expressed as RNAs and *Ta*miR1123 was originated from the MITE_VRN family. The isogenic line TDD carrying the dominant *Vrn-A1a* allele with MITE_VRN showed higher *Ta*miR1123 and *Vrn-A1a* transcript levels than the isogenic line TDE carrying the recessive *vrn-A1a* allele without MITE_VRN. *Ta*miR1123 were greatly up-regulated by plant age but slightly down-regulated by low temperature and short days. These findings have pointed to alternative regulatory mechanisms for plant development governed by *Vrn-A1a* in spring wheat.

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1. Introduction

Wheat is the most widely grown crop, occupying 17% of all cultivated land, providing approximately 55% of the world's carbohydrates, and serving as a major staple to approximately 35% of the world population [1]. Wheat cultivars are generally classified as two general types: winter wheat with variable vernalization requirement for a proper flowering time and thus successful grain reproduction and spring wheat without this requirement [2–4]. The decoding of vernalization genes can facilitate understanding of the vernalization phenomenon that extensively exists in the plant kingdom.

The cloning of a gene, *via* a positional cloning approach from hexaploid common wheat (*Triticum aestivum* L, $2n = 6x = 42$, AABBDD), seemed impossible a decade ago due to the large genome size (16,000 Mb), the complex structure of homoeologous genomes, and the highly repetitive genomic sequences of wheat (80%) [5–7]. Diploid wheat *Triticum monoccoccum* ($2n = 2x = 14$, A^mA^m) has a single genome and was selected to clone the first vernalization gene *VRN-A^{m}1*, in which its genetic effect segregated according to a one-gene model in a diploid wheat population [8]. *VRN-A^{m}1* was cloned [9], and it is an orthologue of the meristem identity gene *AP1* encoding a MADS-box protein for the initiation of the transition from vegetative to reproductive apices in Arabidopsis [10]. Allelic variation in *VRN-A^{m}1* between the dominant *Vrn-A^{m}1* allele for spring wheat and the recessive *vrn-A^{m}1* allele for winter wheat relies on mutations in the promoter in diploid wheat [11–14]. Mutations in the promoter region of the wild type *vrn-A^{m}1* in diploid winter wheat are believed to result in multiple spring *Vrn-A^{m}1* alleles varying in lengths of deletions (alleles *Vrn-A^{m}1a*, *Vrn-A^{m}1b*, *Vrn-A^{m}1g*, *Vrn-A^{m}1f*) involved in a so-called CArG-box recognition site [11,13,15].

The availability of these *VRN-A^{m}1* sequences has facilitated identifying allelic variation in orthologous *VRN1* genes in diverse wheat species, including two homoeologous genes *VRN-A1* and *VRN-B1* in tetraploid wheat *Triticum turgidum* ($2n = 4x = 28$, AABB), *VRN-A1* and *VRN-G1* in tetraploid wheat *Triticum timopheevii* ($2n = 4x = 28$, AAGG), and three homoeologous genes *VRN-A1*, *VRN-B1* and *VRN-D1* in hexaploid *T. aestivum*. The *Vrn-D1* gene in hexaploid wheat has a single form of mutations in intron one due to the presence of a large deletion [14]; the *Vrn-B1* gene has the deletion in intron one in tetraploid *T. turgidum* ssp. *durum* and hexaploid wheat [14–16]. In a recent study, it was found that the *Vrn-B1* gene has a 5.6 kb retrotransposable element (Retrotrans_VRN) in the 5′-untranslated region (UTR) in tetraploid wheat, which is prevalent among *T.*

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turgidum subsp. *carthlicum* [17]. In addition to small deletions in the promoter (*Vrn-A1d* and *Vrn-A1e*) observed in tetraploid wheat or a large deletion in intron one (*Vrn-A1c*) observed in hexaploid wheat, the *Vrn-A1* gene has a miniature inverted-repeat transposable element (MITE) that is inserted in its promoter (*Vrn-A1a*) (MITE_VRN, hereafter) in more than half of all hexaploid wheat varieties [16].

Without vernalization, the winter recessive *vrn-A1a* allele was not expressed and plant flowering time was delayed, whereas the spring dominant *Vrn-A1a* allele was expressed and the plant flowered [9,18–20]. A model was proposed to explain mechanisms underlying growth habit by *VRN-A1a*. In this model, without vernalization the winter *vrn-A1a* allele cannot be expressed due to the presence of repressors in the binding site in the *vrn-A1a* promoter, and the repressors can be removed by vernalization. Further studies have indicated that VRT2 [21] or TaFD1 [22] can bind to the *vrn-A1a* promoter. On the other hand, the spring *Vrn-A1a* allele is expressed without vernalization requirement, because the spring *Vrn-A1a* promoters are impaired due to the insertion of the MITE or retrotransposal elements or deletions; therefore, the flowering repressor cannot bind to the *Vrn-A1a* promoters. However, the previous model cannot explain why those plants that carry MITE_VRN produced more *Vrn-A1a* transcripts and flowered earlier than those plants that carry other mutant alleles with deletions in their promoters or intron 1 [16,23].

In our further analysis on MITE_VRN, we found that MITE_VRN possessed sequences of a microRNA in *T. aestivum* (*Ta*miR1123). This finding has encouraged us to test if there are alternative regulatory mechanisms underlying development through *Ta*miR1123 in spring wheat.

2. Materials and methods

2.1. Plant materials

We investigated the spring wheat near-isogenic Triple Dirk D line (TDD), which has a dominant *Vrn-A1a* allele with MITE_VRN but a recessive allele at each of *vrn-B1* and *vrn-D1*. As a control, we used the near-isogenic Triple Dirk E line (TDE), which has a recessive *vrn-A1a* allele without MITE_VRN but is also a spring type. The spring growth habit of the TDE line was determined by the dominant *Vrn-D1* gene [14]. These near-isogenic lines have provided a useful tool to study the effect of different *VRN1* genes in hexaploid wheat without the confounding effect of other genes affecting flowering time [2].

The TDD and TDE lines were initially grown in a greenhouse at 20–25 °C and with a long day (LD, 16/8 h light/dark). At the 3rd-leaf stage, the first set of the two lines were moved into a cold room with 4 °C and the same LD photoperiod, and the second set of the two lines were moved into a growth chamber with 20–25 °C but with a short day (SD, 8/16 h light/dark). After 5 days, these temperature-photoperiod treated plants and the 3rd set plants that were continuously kept in the greenhouse were collected for leaf samples for analyses of small RNA blot and *VRN-A1a* transcript levels.

2.2. RNA gel blot analysis with a P^{32} labeled Tami1123 probe

Small RNAs were isolated from leaves of the TDD and TDE plants growing under different conditions. Total RNA was extracted using Trizol regents (Invitrogen). RNA samples were size-fractioned on a denaturing 15% polyacrylamide gel and then electrophoretically transferred to a Hybond-N$^+$ membrane (Amersham Biosciences). The membrane was UV cross-linked for 1 min to fix RNA on the member in an incubator at 65 °C for 2 h. The small RNA membrane was hybridized with two probes. One probe was *Ta*miR1123 (5′-TCCGTGAGACCTGGTCTCATAGA-3′) that has the same sequence as observed in MITE_VRN. The other probe was U6 (5′-TCATCCTTGCGCAGGGGCCA-3′) that was used as a positive control to ensure that the membrane had small RNA molecules. The hybridization was performed using the protocol described previously [24]. Briefly, a DNA fragment was end-labeled with γ-^{32}P-ATP using T_4 polynucleotide kinase (New England Biolabs), which was used to test if the probe has sequence complementary to small RNA or micro RNA molecules that were size-fractionated on the small RNA blot. The blot membrane was pre-hybridized in PerfectHyb Plus buff (Sigma) for 2 h, and the ^{32}P probe was then added to hybridize with the membrane at 38 °C for 16 h. The hybridized membrane was washed with 2X SSC buffer plus 1% SDS for three times at 50 °C, each for 10 min. The filter was exposed for 24 h and images were taken using a scanner (Typhoon).

ImageJ 1.32 software (National institutions of Health, Bethesda, MD. http://rsb.info.nih.gov/ij) was used to quantify the expression of MITE_VRN or *Ta*mR1123 molecules in different samples on the same blot. The amounts of MITE_VRN or *Ta*mR1123 were converted to the percentage of its own signals over the signals of U6 in the same sample.

2.3. VRN-A1a expression pattern

The RNA samples from the TDD and TDE plants were treated with Deoxyribonuclease I, and first-strand cDNA was synthesized using a SuperScript™ II Reverse Transcriptase kit (Invitrogen). Primers vrn-A1-Exp-F1 (5′-GAATAAAGTTCTCCAGAAGGAACTCG-TG-3′) and vrn-A1-Exp-R2 (5′-GCATGAAGGAAGAAGATGAAGAGC-TG-3′) that are specific to *Vrn-A1a* [23] were used to determine its transcript levels in leaves of plants. Primers actin-F1 (5′-ATGGAAG-CTGCTGGAATCCAT-3′) and actin-R1 (5′-CCTTGCTCATACGGTCA-GCAATAC-3′) were used to amplify transcripts of *actin* as endogenous control. A quantitative RT-PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems) and iQ™ SYBR Green Supermix kit (BIO-RAD) and the Applied Biosystems 7500 Real-Time PCR Systems.

2.4. Sequence data analyses

It was reported that the TDD line has a duplicated copy including the promoter, exon 1 and partial intron 1 [16], but it was not known which copy is original or duplicated. The only difference observed between the two copies is the size of a MITE_VRN in the promoters. The MITE-VRN structure of each of two *Vrn-A1a* copies was predicted using DNA folding form program at http://mfold.rit.albany.edu/. The MITE-VRN sequences were used to search the miRNA database at http://www.mirbase.org.

The MITE_VRN sequences were used to search the wheat genomic sequence database at http://www.cerealsdb.uk.net to determine its copy number. The MITE_VRN flanking sequences of targeted fragments were searched in GenBank for potential genes. The *Ta*miR1123 sequences found in the sequences were also used to search in GenBank expressed sequence tags (EST) databases for potential target genes.

3. Results

3.1. The insertion of transposable elements in multiple Vrn-A1 genes

The previously reported foldback element (MITE_VRN) with a target site duplication (TSD = TTAAAAACC) in the dominant *Vrn-A1a* allele in the TDD line was inserted in a CG-rich region, where there are 14 G/C in 16 bp (CCTCCCCCCCTGCCGG) at the 3′ downstream of the TSD element (Fig. 1C). A member of the MITE_VRN family was

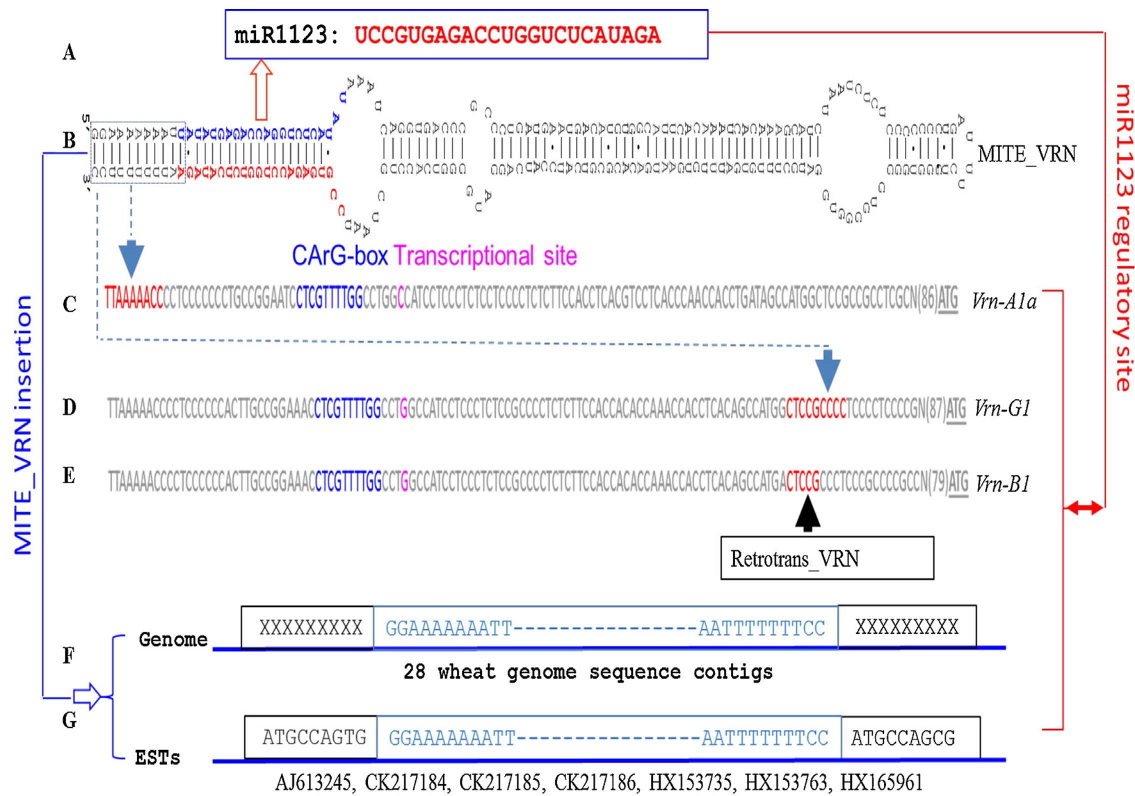

Fig. 1. MITE_VRN in wheat genomes. (A) *Ta*miR1123 is derived from MITE_VRN. (B) MITE_VRN forms a stem loop structure. (C) MITE_VRN is inserted in *Vrn-A1a* at TTAAAAACC. (D) MITE_VRN is inserted in *Vrn-G1* at CTCCGCCC. (E) Retrotrans_Vrn in Vrn-B1 is inserted at CTCCG in the same site as MITE_VRN in *Vrn-G1*. (C)–(E) CArG-box and transcriptional site in Vrn-A1a are indicated to show that MITE_VRN is in the Vrn-A1a promoter region, whereas MITE_VRN in Vrn-G1 and Retrotrans_VRN are in the 5′-UTR. (F) MITE_VRN is in 28 wheat genome sequence contigs with various host direct duplication (Table 1). (G) MITE_VRN is in 7 EST sequences with the same host direct duplication. A work model for the loop between MITE_VRN, miR1123, and targeted genes/genomic sites is diagrammed with lines.

found to insert in *Vrn-G1*, with a TSD (CTCCGCCC), where there are 9 G/C in 11 bp (TCCCCTCCCCG) at the 3′ downstream of the TSD element (Fig. 1D). Interestingly, a 5.6 kb retrotransposal element (Retrotrans_VRN) [17] was inserted in *Vrn-B1* at the exactly same site as *Vrn-G1*, though *Vrn-B1* has a different TSD sequence (CTCCG) (Fig. 1E).

These observations indicated that different MITE and TE inserted in *Vrn-A1* genes in the upstream region from the start codon ATG, where it is GC-rich. This is characteristic of a MITE or TE insertion preference [25]. The difference in the insertion site between *Vrn-A1a* and the other two *Vrn-1* genes is that MITE_VRN was placed on the upstream side of the transcriptional site (or the promoter region) in *Vrn-A1a* but MITE_VRN or Retrotrans_VRN was located on the downstream side of the transcriptional site or the 5′-UTR in the other two *Vrn-1* genes.

3.2. A nearly perfect palindrome of the MITE_VRN containing TamiR1123

A previous study reported that there were two copies of *Vrn-A1a* in the TDD line; one has the insertion of a 222-bp MITE and the other has a 131-bp MITE [16]. The two MITE_VRN sequences have the same inverted repeat sequences (GGAAAAAATT) but different lengths between the flanking sequences, suggesting that they were originated from a duplication event. Using DNA folding form program, it was predicted that the 222-bp MITE_VRN possessed a nearly perfect palindrome (Fig. 1B), whereas the shorter 131-bp MITE cannot form a perfect palindrome due to the absence of a 91-bp deletion (data not shown). It is likely that the 91-bp section in the duplicated *Vrn-A1a* copy lost during or after the gene duplication. Only the original *Vrn-A1a* containing MITE_VRN was further analyzed in this study.

The search of MITE_VRN sequences in the miRNA database at http://www.mirbase.org found that MITE_VRN possessed a 23 bp sequence, TCCGTGAGACCTGGTCTCATAGA (Fig. 1A), which was complementary to *Ta*miR1123 (Fig. 1A). The *Ta*miRNA1123 was a microRNA that was found expressed in wheat, but it was mistakenly named as miR507 [26]. There are 4 mismatches between *Ta*miR1123 and the element (TATATGAGACCAGGTCTCATATA) (Fig. 1B). These observations suggested that if MITE_VRN with highly stable hairpin loops was expressed as RNA molecules, it could be recognized by RNA interference enzymatic machinery and processed to form mature *Ta*miR1123.

3.3. The existence of MITE_VRN and TamiR1123 in small RNAs

To confirm that MITE_VRN was expressed as RNAs, a ^{32}P labeled *Ta*miR1123 probe was used to analyze a small RNA blot that was generated from the leaves of the TDD and TDE plants under different conditions. The ^{32}P labeled small RNA U6 probe was used as a control to show that small RNAs from each samples were transferred on blot membranes. As expected, the ^{32}P probes of both *Ta*miR1123 and U6 detected positive hybridization signals on the small RNA blot.

As shown in Fig. 2A, the U6 probe detected RNAs as a single band in all samples. When the same membrane hybridized with the U6 probe was washed and then hybridized with the *Ta*miR1123 probe, three bands appeared in each sample as shown in Fig. 2B. The highest band was from U6 signal rescues, and the middle band and the lowest band were two new signals that should be from the *Ta*miR1123 probe. When a brand new membrane was hybridized with the *Ta*miR1123 probe, only two bands with the same sized as shown for the middle band and the lowest band were observed

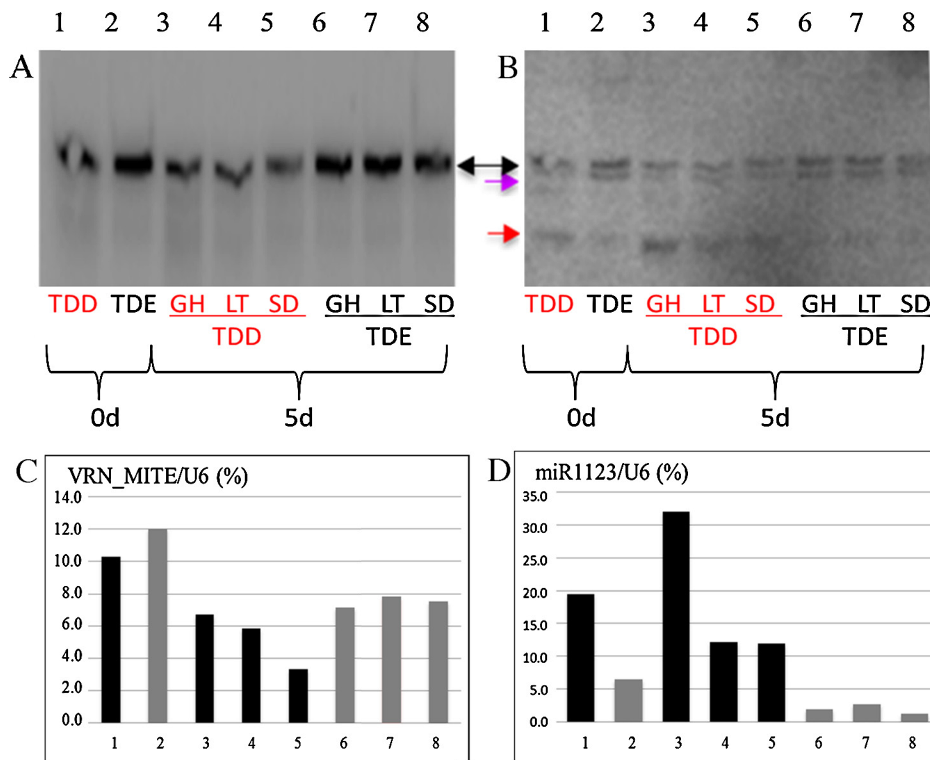

Fig. 2. Expression of MITE_VRN and *Ta*miR1123. (A) Expression patterns of micro RNA U6. (B) Expression patterns of *Ta*miR1123. RNA gel blots of small RNAs from isogenic TDD line with MITE_VRN at *Vrn-A1a* and the TDE line without MITE_VRN at *Vrn-A1a*. The blot was probed with labeled oligonucleotides. The small RNA samples were collected from leaves of the plants at the beginning of experiments (CK) and the plants grown at greenhouse (GH) or treated with low temperature (LT) or short days (SD) for 5 days. (C) Expression amount of MITE_VRN relative to U6. (D) Expression amount of *Ta*miR1123 relative to U6.

(data not shown), confirming that the *Ta*miR1123 probe detected the two small RNA molecules.

The middle band size (Fig. 2B) was expected for MITE_VRN, compared with the U6 band (250–300 bp), and the lowest band size was expected for *Ta*miR1123. These results demonstrated that both MITE_VRN and *Ta*miR1123 existed in the RNA samples. The appearance of the positive and discrete small RNA molecules on the small RNA blot indicated that they were not degradation products of RNAs. The expressed MITE_VRN with a hairpin structure was processed to mature *Ta*miR1123.

3.4. The regulation of MITE_VRN and TamiR1123 by internal and external factors

Both of the isogenic TDD line (lanes 1 and 3–5, Fig. 2B) and the isogenic TDE line (lanes 2 and 6–8, Fig. 2B) were detected to have the middle hybridization signals representing MITE_VRN and the lower signals representing *Ta*miR1123 molecules. Using the ImageJ software, the amounts of the MITE_VRN or *Ta*miR1123 molecules in each sample were converted to percentage of its own signals over U6 signals in the same sample. U6 was used as an endogenous control.

The MITE_VRN in the TDD line (graph 1, Fig. 2C) was not higher but slightly lower than that in the TDE line (graph 2, Fig. 2C). It was possible that the difference in MITE_VRN transcription between the TDD line and the TDE line was masked due to the presence of multiple MITE_VRN-like molecules in wheat, as shown in later results. In both TDD (graphs 3–5, Fig. 2C) and TDE (graphs 6–8, Fig. 2C), MITE_VRN signals were decreased with plant age and with treatments of low temperature and short days.

A significant difference in the *Ta*miR1123 molecules was detected between the two isogenic lines. The TDD line (graph 1, Fig. 2D) showed much more *Ta*miR1123 molecules than the TDE line (graph 2, Fig. 2D). When the TDD line was continuously kept in a greenhouse with long day and room temperature, the plants were observed to have more *Ta*miR1123 molecules with plant age (graph 3, Fig. 2D). When the plant was treated with low temperature (graphs 4, Fig. 2D) or short day (graph 5, Fig. 2B), *Ta*miR1123 molecules were significantly increased. The *Ta*miR1123 in the TDE line showed a low level under these conditions (graphs 6–8, Fig. 2D).

3.5. Association of TamiR1123 molecules with Vrn-A1a transcript levels

The *VRN-A1a* transcript levels in the TDD and TDE lines were determined using a quantitative RT-PCR (Fig. 3). The expression of dominant *Vrn-A1a* in the TDD line was detected in seedlings grown in the greenhouse (TDD-CK). The *Vrn-A1a* transcript level was 31.7 before the plants were treated with different conditions, and it was increased to 52.5 after 5 days (TDD-GH). *Vrn-A1a* transcript level was also increased in plants treated with low temperature (TDD-LT) or short day (TDD-SD) compared with TDD-CK but slightly decreased compared with the plants that were continuously kept in the greenhouse (TDD-GH). The *vrn-A1a* transcript level in the TDE seedlings grown in the greenhouse (TDE-CK) was 4.8, which was 40% in the TDE seedlings grown under the same condition after 5 days (Fig. 3). A similar result was shown in the TDE line treated with low temperature and short days as the TDD line. Overall, *Vrn-A1a* transcripts were 5.8 folds as *vrn-A1a* transcripts in the isogenic lines. Under any condition, the *Vrn-A1a* transcript level in the TDD line was significantly higher than the *vrn-A1a* transcript level in the TDE line.

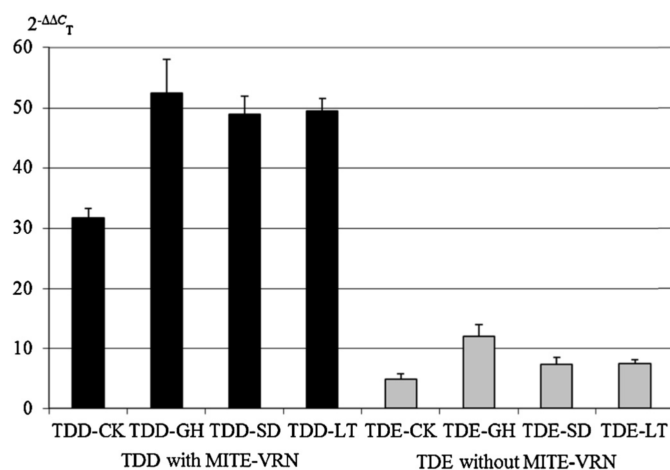

Fig. 3. Expression profiles of *Vrn-A1*. Transcript levels of *Vrn-A1a* in TDD and *vrn-A11a in* TDE are shown using the values calculated by the $2^{(-\Delta\Delta CT)}$ method, where CT is the threshold cycle, and *actin* was used as an endogenous control. RNA samples were collected from leaves of the plants at the beginning of experiments (CK) and the plants grown at greenhouse (GH) or treated with low temperature (LT) or short days (SD) for 5 days plants. The values represent mean expression levels ($n = 12$), and the bar indicates standard error.

3.6. The copy number of MITE_VRN in hexaploid wheat

The availability of 5-fold coverage of the whole-wheat genome (http://www.cerealsdb.uk.net) has facilitated identification of the copy number of MITE_VRN in the entire wheat genome. A total of 124 wheat genome contigs (as of March 30, 2013) were hit in 'Draft assembly of gene-rich regions' (*E* value < 1.0e−05). However, only 28 hits were found to have similar sequences in the complete MITE_VRN region (Fig. 1F), and the remaining hits included no or one end of the MITE_VRN. Further analyses showed that each member of the MITE_VRN family was inserted with a different host direct duplication of 3 or 7 bp sequences (Table 1). It was predicted to have approximately 375 copies of the MITE_VRN present in the tetraploid wheat genome based on the clone number in the Langdon BAC library [16]. The dramatic difference in the MITE-VRN copy number between hexaploid wheat and tetraploid wheat was because some positive BACs hit by the MITE_VRN probe should be false or not belong to the MITE_VRN family. In this study, only the genome sequence contigs that have intact MITE_VRN sequences were analyzed.

3.7. Target genes of MITE_VRN and TamiR1123

The search of MITE_VRN sequences in the wheat EST databases deposited in GenBank with BLAST algorithms found a total of 16 EST accessions (*E* < 0.01). After removing the EST sequences with more than 2 mismatches, seven EST accessions were found to have either MITE_VRN or *Ta*miR1123 or both in sequence and structure, including three from cultivar 'Norstar' (CK217184, CK217185, and CK217186), two from cultivar 'Halberd' HX153735 and HX153763), and the other two from 'Ofanto' (AJ613245 and 'Cranbrook' (HX165961). These ESTs were inserted with the same host direct repeat (ATGCCAGTG) (Fig. 1G). The MITE_VRN-like sequences from various cultivars showed more than 85% identity to each other (Fig. 4). The conservations of insertion sites of MITE_VRN-like sequences and their sequence identity suggested that these expressed ESTs belong to the same MITE_VRN family.

*Ta*miR1123 sequence alone was searched in the GenBank wheat EST databases. Expect for the seven ESTs that had MITE_VRN and thus *Ta*miR1123, no new wheat EST was found to have identical sequences to *Ta*miR1123.

4. Discussion

Many miRNA molecules have been found to exist in different plant species and they may play important roles in plant responses to abiotic and biotic stresses as well as signal transduction [27]. Tens of miRNA genes have been identified in wheat [26]. However, the origin and evolution of these non-coding regulatory sequences remain largely unknown. No miRNA has been characterized with a functional gene in wheat.

In this study, we demonstrated that *Ta*miR1123 was derived from a MITE that was inserted in the promoter of a dominant *Vrn-A1a* gene. First, MITE_VRN was nearly perfect palindromes. When expressed as RNA it can form highly stable hairpin loops. This structure can be processed to form mature *Ta*miR1123 sequences if MITE_VRN was recognized by the RNA interference enzymatic machinery as described in plants or animals [27–29]. It is possible that the MITE_VRN hairpin stem is cleaved and then degraded to produce a *Ta*miR1123. Second, the ^{32}P labeled *Ta*miR1123 detected RNAs in the same size as expected for MITE_VRN, indicating that the small RNA contained *Ta*miR1123. The probe also detected *Ta*miR1123 molecules, provided experimental evidence that *Ta*miR1123 could be released from MITE_VRN. The concomitant regulation of *Ta*miR1123 with *Vrn-A1a* at the transcript level in the TDD line suggesting the MITE_VRN in the *Vrn-A1a* promoter could be the direct target of *Ta*miR1123. However, the MITE_VRN in the *Vrn-A1a* promoter could be one of the targeted sites of

Table 1
The MITE_VRN family in wheat genomes.

Sequence source	Accession #	Host direct duplication sequences	MITE length (bp)	Sequence length (bp)
EST	HX153763	ATGCCAGTG	195	624
	CK217184	ATGCCAGTG	194	1049
	CK217185	ATGCCAGTG	195	1047
	CK217186	ATGCCAGTG	195	1027
Wheat genome sequence contig	Contig299482	CCAAATATAAG	219	1679
	Contig134865	ATGGTTTGAG	224	2194
	Contig80285	GTGTTTTTC	221	2554
	Contig3014060	CTATTATAC	214	525
	Contig859785	GCAGTTTAG	222	1093
	Contig311912	CATAATTAC	221	1663
	Contig4207044	CTGAATTTG	221	325
	Contig1137015	GCTCAACAC	220	955
	Contig279496	CTAGGATGC	220	1742
	Contig3124132	GCCAT	218	509
	Contig2088947	ATG	220	476

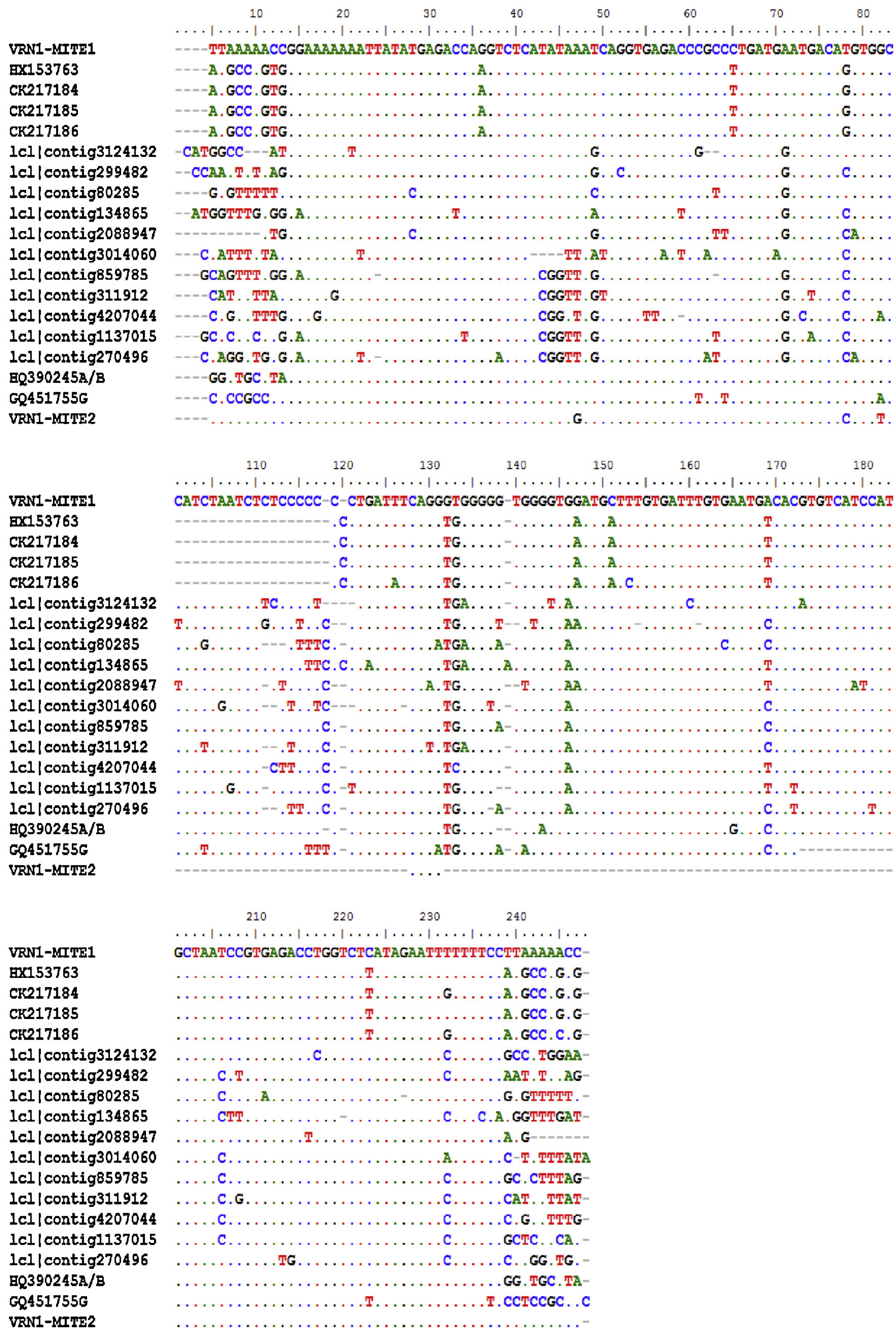

Fig. 4. Multiple sequence alignment of MITE_VRN. EST sequences are derived from GenBank. Wheat genome sequence contigs are derived at http://www.cerealsdb.uk.net. Except for host direct duplication, the sequences flanking MITE_VRN are included in the alignment.

*Ta*miR1123, since an average miRNA has approximately 100 target sites [30]. No other target site was found within the *Vrn-A1a* gene. The *Ta*miR1123 sequence in wheat was identified in the previous study [26], but its function is unknown. Our results clearly showed that the transcript level of *Ta*miR1123 was increased with plant age and down regulated by low temperature and short days. These observations provided a depth understanding of the MITE_VRN inserted in the promoter of the dominant *Vrn-A1a* allele in hexaploid wheat.

In this study, we provided experimental evidence that the transcript level of *Ta*miR1123 was positively correlated with the transcript level of *Vrn-A1a* in the TDD line; therefore, it is possible that *Vrn-A1a* was induced by *Ta*miR1123. However, it is also possible that *Vrn-A1a* was not induced directly by *Ta*miR1123 but *Ta*miR1123 could be a by-product of transcription of *Vrn-A1a*. Based on characteristics of the sequence and target site duplication of MITE_VRN, it is a Mutator-like element (MULE). A MULE can harbor the promoter of a gene for transcription [31,32], and the function of such a MULE was reported in the promoter of the hcf106-mum1 gene (Mutator transposons) [33]. The potential mechanism could explain why *Vrn-A1a* is linked to a much stronger expression than other *Vrn-A1* alleles. The two mechanisms presented in this study are different from the previous hypothesis that the insertion of MITE_VRN in the promoter of *Vrn-A1a* resulted in a damage of recognition site by a flowering repressor [9,16].

MITE_VRN in *Vrn-A1a* was expressed as RNA, which released *Ta*miR1123 that in return induced the expression by a direct or indirect manner. This formed a loop that regulated the expression of *Vrn-A1a* (Fig. 1). This loop could involve in plant development and other phenotypes through both MITE_VRN and *Ta*miR1123. On the one hand, the mobility of MITE_VRN enabled it to insert in a functional gene or a regulatory site like *Vrn-A1a* promoter. On the other hand, *Ta*miR1123 has many regulatory sites throughout the genome. The expression of these targeted genes could be regulated due to the insertion of MITE_VRN or recognition of their regulatory sites by *Ta*miR1123, which would form a dynamic gene regulatory network governed by *Vrn-A1a* in plant development. The *VRN1* region was reported to have association with multiple traits including vernalization, cold hardening, and the development of rosette [34]. This study provided a machinery explanation for the complex association among some of the multiple phenotypes.

The MITE_VRN derived *Ta*miR1123 characteristics can be applied in wheat breeding. The genomic and EST sequences flanking MITE_VRN-like fragment can be used to design specific primers to map members of the MITE_VRN family. The characteristics that such a MITE has a few hundred nucleotides will facilitate development of a PCR marker for mapping the MITE_VRN-like fragment dispersed throughout the genome. Any association of phenotypic variation with a marker for a MITE_VRN-like fragment can suggest that the phenotype could be linked with development controlled by *Vrn-A1a* or *Ta*miR1123. These potential applications of MITE_VRN and *Ta*miR1123 in wheat need to be investigated in the future studies.

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