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Development-associated microRNAs in grains of wheat (*Triticum aestivum* L.)

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Abstract

Background: MicroRNAs (miRNAs) are a class of regulatory small RNAs (sRNAs) that down-regulate target genes by mRNA degradation or translational repression. Numerous plant miRNAs have been identified. Evidence is increasing for their crucial roles during plant development. In the globally important crop of wheat (*Triticum aestivum* L.), the process by which grains are formed determines yield and end-use quality. However, little is known about miRNA-mediated developmental regulation of grain production. Here, we applied high-throughput sRNA sequencing and genome-wide mining to identify miRNAs potentially involved in the developmental regulation of wheat grains.

Results: Four sRNA libraries were generated and sequenced from developing grains sampled at 5, 15, 25, and 30 days after pollination (DAP). Through integrative analysis, we identified 605 miRNAs (representing 540 families) and found that 86 are possibly involved in the control of grain-filling. Additionally, 268 novel miRNAs (182 families) were identified, with 18 of them also potentially related to that maturation process. Our target predictions indicated that the 104 grain filling-associated miRNAs might target a set of wheat genes involved in various biological processes, including the metabolism of carbohydrates and proteins, transcription, cellular transport, cell organization and biogenesis, stress responses, signal transduction, and phytohormone signaling. Together, these results demonstrate that the developmental steps by which wheat grains are filled is correlated with miRNA-mediated gene regulatory networks.

Conclusions: We identified 605 conserved and 268 novel miRNAs from wheat grains. Of these, 104 are potentially involved in the regulation of grain-filling. Our dataset provides a useful resource for investigating miRNA-mediated regulatory mechanisms in cereal grains, and our results suggest that miRNAs contribute to this regulation during a crucial phase in determining grain yield and flour quality.

Keywords: MicroRNA, Grain development, Wheat (*Triticum aestivum* L.)

Background

Endogenous small RNAs (sRNAs) in plants, such as microRNAs (miRNAs) and short-interfering RNAs (siRNAs), were first reported in 2002 [1,2]. Since then, our knowledge about these regulatory molecules has been vastly improved [3]. Plant miRNAs are a class of short (~21-nt) sRNAs produced from non-coding, imperfectly complementary (stem-loop) RNA precursors

[4], which can be transcribed by RNA polymerase II [5]. The majority of currently known plant miRNAs was identified via size-selected cloning and sequencing [5-7]. Recently developed high-throughput sequencing strategies have greatly expanded the depth of miRNA cloning coverage [8,9]. In addition to model species, more sRNAs have been identified from crop plants such as rice [10-12], maize [13,14], and wheat [15,16]. Studies of plant miRNAs have indicated that this group of regulatory molecules plays crucial roles in numerous biological processes, e.g., general plant development [17,18] and responses to environmental signals [10,19-21]. Moreover, plant miRNAs and their roles in plant development have been extensively reviewed [3,22].

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Seed production is a unique transitional process during the life cycle of higher plants, providing a physical link between parental and progeny sporophytic generations [23]. It is likely that the roles of miRNAs in gene regulation are as critical in maturing seeds as they are in other tissues [24-26]. The millions of short sequence reads that have resulted from next-generation sequencing technologies make that technique explicitly suitable for the profiling of miRNAs. Because of high-throughput sequencing, researchers have been able to identify conserved and novel miRNAs in the seeds of rice [7,11], maize [27], barley [28], and *Brassica napus* [29]. Their presence in those species suggests that miRNA-mediated negative regulation has a crucial role during seed development.

Hexaploid wheat is one of the most valuable cereal crops, occupying 17% of all cultivated land and providing approximately 55% of the carbohydrates consumed by humans worldwide [30]. In addition to being a food source, these grains are used as livestock feed and industrial raw materials, which mainly exploits the endosperm reserves of starch and proteins that account for approximately 80% of a mature seed [31]. Hence, the process by which wheat grains develop directly determines yields and quality of the end product. To improve those traits, researchers must have a keen molecular understanding of the mechanisms that modulate those steps in plant growth. Because little is known about miRNA-mediated regulation in developing wheat grains, our study employed high-throughput sequencing to characterize the miRNAs that potentially participate.

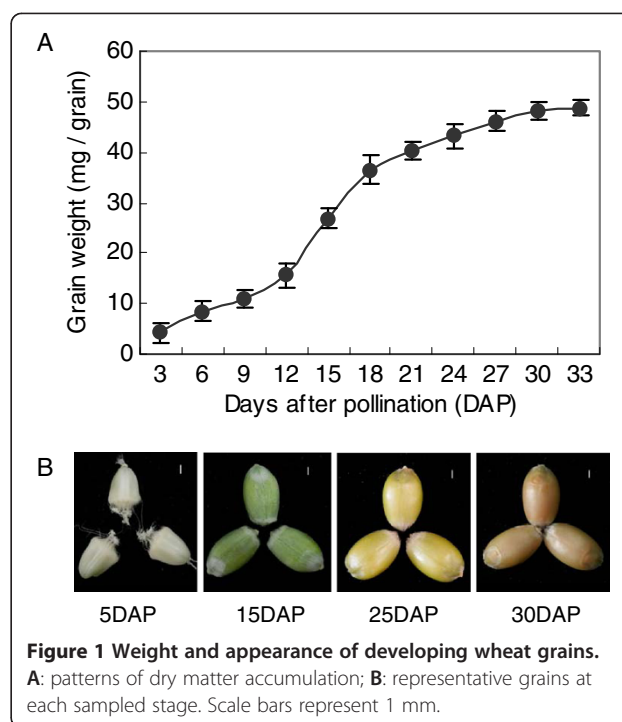
Results

Dry matter accumulation and appearance of maturing grain

To evaluate the developmental process for wheat grains, we monitored the pattern by which dry matter was accumulated in those tissues. Here, seed weights increased sharply between 12 and 18 days after pollination (DAP) and continued to rise until 30 DAP (Figure 1A). Based on the pattern we observed, we continued our focus mainly on activities between 15 and 25 DAP, the key period for grain-filling, as well as on grains sampled at both 5 DAP and 30 DAP so that we could compare the expression profiles of miRNAs near the beginning and end of that stage of formation. The size and appearance of developing caryopses at those time points are shown in Figure 1B.

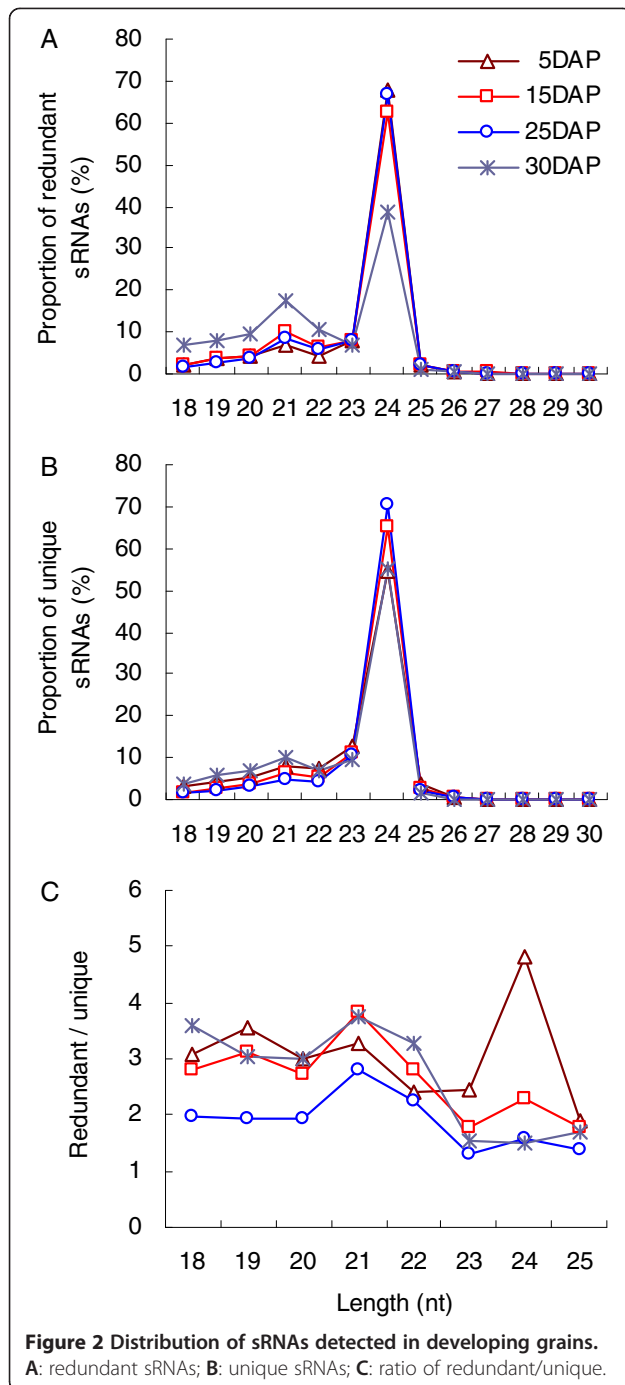
Deep-sequencing of sRNAs in developing grains

To investigate the enrichment of miRNAs, we generated four libraries from developing wheat grains sampled on four dates. After sequencing via the Illumina Genome Analyzer, we removed low-quality reads and corrupted adapter sequences (reads <18 nt or >30 nt long). In all, we obtained 13,525,513 reads (3,492,987 unique) representing



5 DAP, 14,460,398 reads (6,015,357 unique) for 15 DAP, 15,310,251 reads (9,243,757 unique) for 25 DAP, and 13,282,907 reads (6,200,456 unique) for 30 DAP. Our assessment of the size distributions demonstrated that approximately 98% of the detected sRNAs were 18 to 25 nt long, and that 24-nt sRNAs were prevailing in all stages whereas those that were 21 nt long were less abundant (Figure 2A). Further analysis revealed that the distribution of redundant sRNAs in various size classes was quite similar among the libraries from 5, 15, and 25 DAP grains. We found it interesting that the abundance of 24-nt redundant sRNAs was significantly decreased in 30 DAP grains while the proportion of those that were 18 nt to 22 nt were obviously increased (Figure 2A). The distribution patterns for unique sRNAs were similar among all four libraries (Figure 2B). The patterns of distribution between redundant and unique sRNAs indicated that the abundance of sRNAs fluctuated during the period of seed development. Therefore, we evaluated their relative abundance from 18 to 25 nt based on the ratio of redundant/unique and found that expression was generally altered over time (Figure 2C). Transcript levels for most sRNA sizes were obviously decreased in the 25 DAP samples. This was especially true for 24-nt sRNAs, which were gradually repressed. The canonical heterochromatic siRNAs are 24 nt long and these results suggested that siRNA-mediated gene regulation might be involved in the control of grain formation.

For annotation, we used the sRNA datasets to query the non-coding RNAs deposited in the NCBI GenBank (<http://www.ncbi.nlm.nih.gov>), Rfam database [32], and



miRBase (<http://www.mirbase.org/>). They were then classified into seven categories: miRNA, rRNA, siRNA, snRNA, snoRNA, tRNA, and those detected but without annotation (Additional file 1). The number of unique miRNAs increased gradually as the process of grain-filling continued (Figure 3A), implying that miRNA-mediated gene silencing is involved in developmental regulation. Moreover, the total read of miRNAs was relatively lower at 25 DAP (Figure 3B), leading to a lower ratio (redundant/unique) of

miRNAs at that time point (Figure 3C). Small RNAs were more abundant in the 25 DAP library than in any other (Figure 3D), suggesting that miRNA expression was generally down-regulated at that later stage of maturation.

Conserved miRNAs differentially expressed in developing grains

To identify the conserved miRNAs in developing grains, we aligned the sRNA sequences with known mature miRNAs from plants in the miRBase. In all, 605 miRNAs representing 540 families were identified (Additional file 2). In general, we found that 424 conserved miRNAs (382 families) were differentially expressed (p -values < 0.05) over time (Additional file 3). Their scatter plots illustrated the similarities during the stages of grain formation, as well as the broad relationships among miRNA expression profiles from 5 DAP through 30 DAP (Figure 4). However, some differences in profiles were apparent from this examination. When compared with values at 5 DAP, 231, 266, and 309 miRNAs were significantly up- or down-regulated ($p < 0.05$) at 15 DAP, 25 DAP, and 30 DAP, respectively. It is likely that the number of up-regulated miRNAs was gradually increased (114, 147, and 211 at 15 DAP, 25 DAP, and 30 DAP, respectively) over time, demonstrating that mechanisms for miRNA-mediated repression are involved in this developmental regulation.

Conserved miRNAs associated with grain-filling

Among those 424 differentially expressed miRNAs, expression was lower for 284 miRNAs, i.e., those with fewer than 20 TPMs (transcripts per million). For our investigation, we selected 140 miRNAs (125 families) highly expressed (at least has a TPM ≥ 20 in the four stages detected) in developing grains (Additional file 3). To find common expression patterns, we performed hierarchical clustering based on fold-changes in expression from a base line of 5 DAP. In all, 86 miRNAs could be sorted into eight clusters (Figure 5, Additional file 4) that we considered to be potentially involved in the regulation of grain-filling.

The first four clusters (I-IV) of conserved miRNAs generally included those that were up-regulated in the process of grain-filling. Clusters I and II comprised 16 miRNAs with expression that was very low at 5 DAP, but which greatly increased at 15 DAP, and remained high for most miRNAs through 30 DAP. Considering that 15 DAP and 25 DAP are crucial stages during which a complex gene regulatory network is involved in endosperm development, we concluded that those significantly induced miRNA candidates in Clusters I and II might have important regulatory roles. Expression of the miRNAs in Cluster III was gradually increased over time. For example, miR167a, miR156a, and miR156c, were quite abundant, with expression levels at 5 DAP of

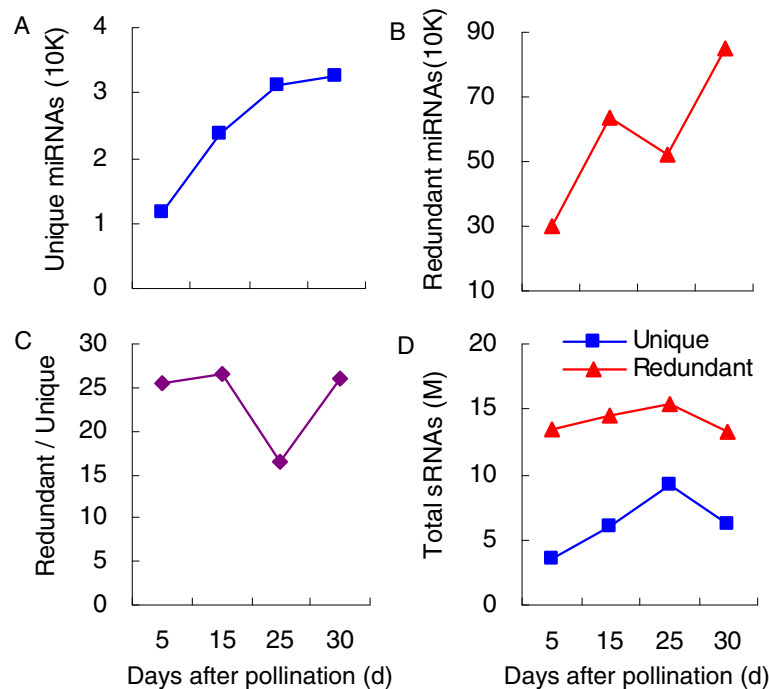


Figure 3 Enrichment of miRNAs in developing grains. **A:** unique miRNAs; **B:** redundant miRNAs; **C:** ratio of redundant/unique; **D:** total sRNAs.

1825.73, 845.74, and 212.12 TPM, respectively (Additional file 4). Although they were up-regulated by only a few fold at 15 DAP, they could have a greater regulatory effect because of their more abundant basis. Cluster IV had 11 miRNAs that were only slightly up-regulated. Of these, eight were more highly expressed, with TPM values of >100 at 15 DAP and 25 DAP. For example, the TPMs for miR168a were 7509.36 at 5 DAP and 21776.72 at 15 DAP.

Four clusters of miRNAs showed repression, including six members that were rapidly down-regulated in Cluster V. For example, although miR473 was highly expressed at 5 DAP (TPM = 150.97), its level decreased to 0.13 TPM at 25 DAP and was undetectable in 30 DAP grains (Additional file 4). Clusters VI and VII contained 3 and 9 miRNAs, respectively, all of which were significantly repressed at 15 DAP and/or 25 DAP. Finally, Cluster VIII had 20 members, including miR2628 and miR1146, were gradually repressed over time.

Novel miRNAs identified in wheat grains

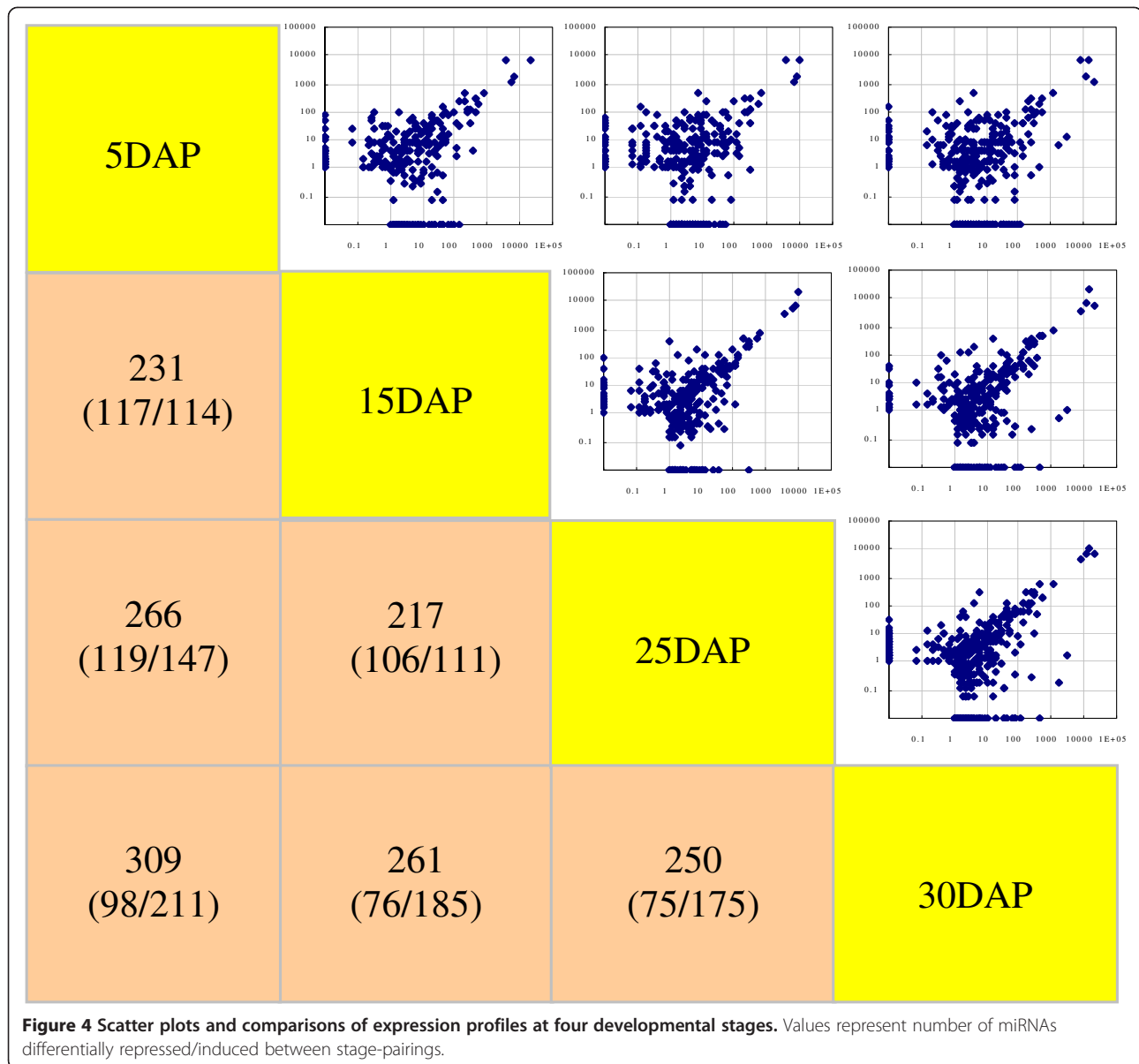
Novel miRNAs were predicted according to the characteristic hairpin structures of their precursors, which distinguishes them from other endogenous sRNAs [33,34]. A total of 268 putative novel miRNAs (182 families) were identified (Additional file 5, Additional file 6). Among them, 24 showed at least a relatively higher expression (TPM \geq 10) in developing grains (Table 1). We were interested to learn that Ta-miR034-3p and Ta-miR034-5p are homologous to complementary sequences in the same

stem-loop (Figure 6). Moreover, Ta-miR007-5p and Ta-miR007-3p were also located in the same stem-loop generated from a wheat EST (GB#: CJ832040).

Further analysis revealed that these 24 highly expressed novel miRNAs could be sorted into Clusters I through VI (Figure 7, Table 1). Cluster I contained four miRNAs for which expression rose sharply at 15 DAP and was maintained at a high level throughout the process of grain-filling. For example, Ta-miR021-1-5p was strongly up-regulated over time, peaking at 30 DAP (TPM = 7480.74). Expression of the five miRNAs within Cluster II was obviously increased at 15, 25, and 30 DAP when compared with levels detected at 5 DAP.

In Cluster III, Ta-miR042-3p, Ta-miR107-2-3p, and Ta-miR106-5p were highly expressed at 15 DAP, but not at any other developmental stages. By contrast, Ta-miR051-3p and especially Ta-miR154-5p, both in Cluster IV, were significantly up-regulated at 25 and 30 DAP. In Cluster V, four novel miRNAs were down-regulated over time. Ta-miR068-5p and Ta-miR057-1-3p were gradually repressed in developing grains whereas Ta-miR007-5p and Ta-miR007-3p were highly expressed at 5 and 15 DAP, but undetectable at 25 and 30 DAP. This contrast in up- and down-regulation among these 18 novel miRNAs from Clusters I through V demonstrated their important regulatory roles.

Finally, the novel miRNAs included in Cluster VI were either expressed only at 30 DAP (near the completion of the filling stage) or else their transcript abundance

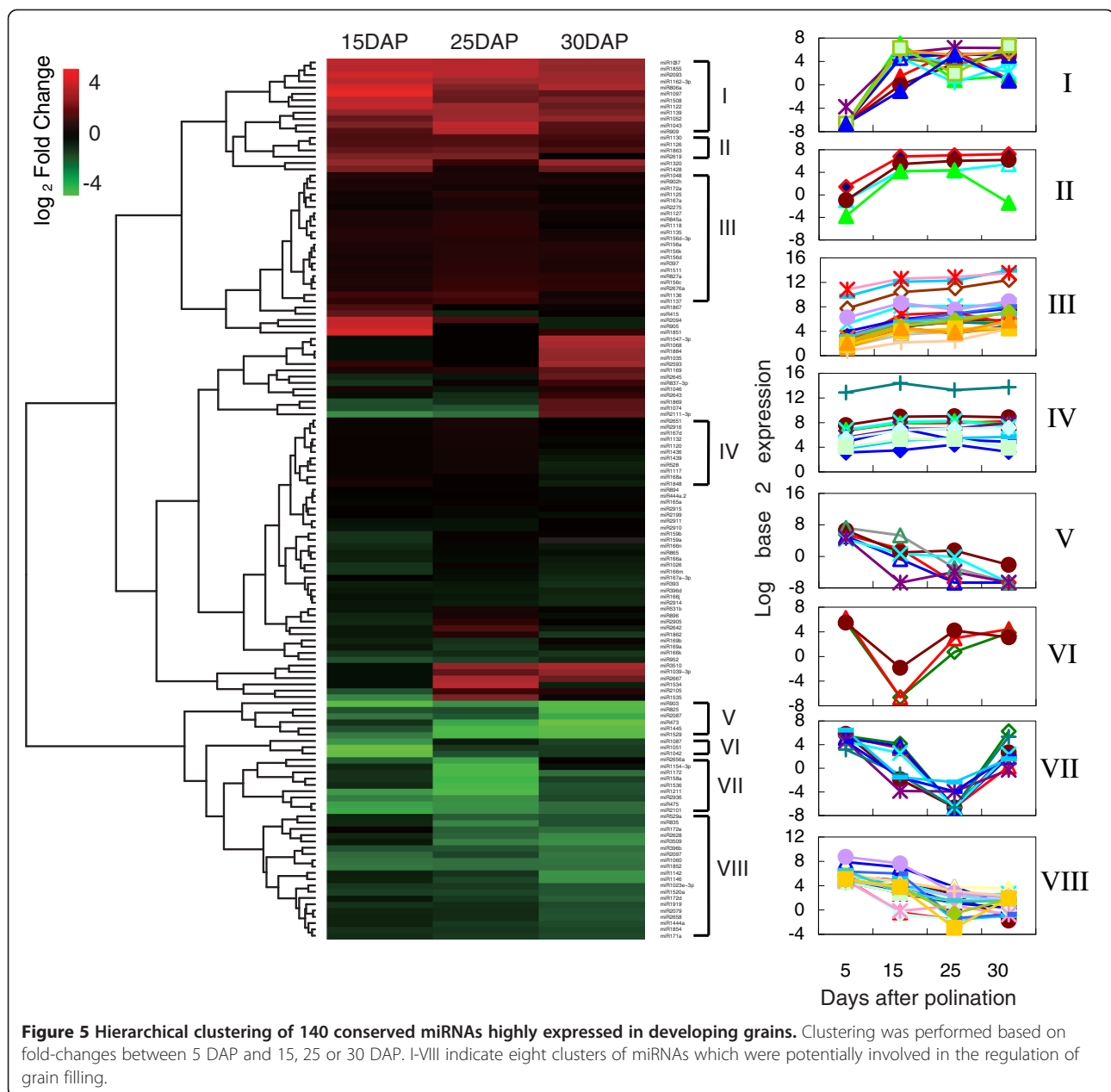


fluctuated over time. Therefore, their activity did not seem to be associated with this type of developmental regulation. The exception to this was Ta-miR034-5p, also in Cluster VI, which showed strong expression at all sampled time points.

Validation of miRNAs in developing grains

We conducted quantitative real time PCR (qRT-PCR) to validate the expression patterns of miRNAs identified via high-throughput sequencing. Based on their patterns of development-regulated expression, as determined by deep-sequencing, we selected eight miRNAs for examination. Seven of them -- miR167a, miR397, miR156a, Ta-miR021-1-5p, Ta-miR004-1-5p, Ta-miR044-1-3p, and miR827a --

were predicted to be induced while miR1852 was expected to be repressed. In fact, miR167a, miR397, miR156a, Ta-miR004-1-5p, Ta-miR044-1-3p, and miR827a were significantly up-regulated by 15 DAP and 25 DAP, with expression then peaking at 30 DAP (Figure 8). Their expression profiles were quite similar to those determined by high-throughput sequencing (Figures 5, 7). Although Ta-miR021-1-5p was up-regulated over time while miR1852 was down-regulated, the degree to which their expression was altered was not as dramatic as had been demonstrated with high-throughput sequencing (Additional file 4, Table 1). Those responses may have been affected by the relative abundance of the other sRNAs. Nevertheless, our qRT-PCR results were generally consistent with the data obtained



from our high-throughput sequencing, thereby indicating that it is possible to create a set of grain filling-associated miRNAs through deep-sequencing of wheat.

Targets of grain filling-associated miRNAs

The potential targets of 86 grain filling-associated conserved miRNAs (Additional file 4), as well as 24 highly expressed novel miRNAs (Table 1), were computationally predicted using the *Triticum aestivum* (wheat) unigene library, DFCI Gene Index (TAGI; version 12), and the psRNATarget program (<http://plantgrn.noble.org/psRNATarget/>) [35]. All of the targets predicted for those conserved and novel miRNAs are shown in Additional file 7 and Additional

file 8. Functional annotations of those target genes were performed by BLAST analysis with NCBI and it is found that these grain filling-associated miRNAs potentially target to multiple wheat genes, which including transcription factors, proteins involved in the membrane transporting, ubiquitin-mediated proteolysis, carbohydrate metabolism, responding to stress, signal transduction and phytohormone signaling.

To validate the expression patterns of potential targets for grain filling-associated miRNAs, we selected six target genes (TC383723, TC384445, TC370322, TC400547, DR734203 and TC370450) of candidate miRNAs, which were significantly up-regulated with the development of

Table 1 Novel miRNAs highly expressed in developing wheat grains

miRNA	Sequence 5' to 3'	Length (nt)	Abundance (TPM)				Target description (Accession No.)
			5DAP	15DAP	25DAP	30DAP	
Cluster I							
Ta-miR023b-5p	UCGCAAAUAAUGGUGGCCUUCG	22	–	6.09	9.21	12.05	Uncharacterized protein (GW667699; TC402735)
Ta-miR128-5p	GUGGAUGAUGAGAUACAAGUAA	23	–	21.58	29.78	23.56	DRG1 (TC386119); Glycolipid transfer protein (CA648678); Polyol transporter 5 (CA681870).
Ta-miR113-5p	UGGCUACUCCUUUCCUUGCC	22	–	22.68	13.52	14.08	bZIP transcription factor (TC448705); Phytosulfokine receptor 1 (TC443080).
Ta-miR021-1-5p	UCUGGCGAGGGACAUCACUGU	22	1.26	61.89	1771.23	7480.74	Protein Rf1 (DR736808); F-box protein PP2-A13-like (TC381152).
Cluster II							
Ta-miR018-5p	UCUGUAAACAAUGUAGGACG	21	2.22	24.55	8.69	59.25	CBL-interacting protein kinase (TC376281); P450 reductase (CK211052); Putative protein kinase (TC404251).
Ta-miR004-1-5p	UCACAAUUAAGAUGUUCU	20	2.96	12.72	6.73	16.64	Sucrose-phosphate synthase (TC410332); Nectarin-3 (CA728499); CTD-phosphatase (TC373796); TIF3 (TC398757); receptor-like kinase (TC369729); Lipoyl synthase (TC458824).
Ta-miR034-3p	AGGGGGCAAUCACCUCAAC	21	6.21	14.31	8.16	27.18	Serpin-Z2B (BQ243327)
Ta-miR036-3p	UUCCGAAAGGCUUGAAGCAAU	22	8.87	55.60	21.03	24.99	Light-induced protein (TC387010); SH3 domain protein (DR732608); Aquaporin TIP3-2 (TC390755).
Ta-miR044-1-3p	UGAGAAGGUAGAUCAUAAUAGC	22	3174.59	5490.93	3890.60	9689.90	Mla-like protein (TC368609); NBS-LRR resistance protein (GH723128).
Cluster III							
Ta-miR042-3p	UGAUUGAGCCGUGCCAAUAUC	21	–	11.20	–	–	WD and FYVE containing protein 3 (TC418522); Papain-like cysteine proteinase (TC448847).
Ta-miR107-2-3p	AAAAUACUUGUCGGAGAAUG	21	–	12.66	–	–	Uncharacterized protein (CA664743; DR734972; CK215832)
Ta-miR106-5p	CGGUGGAGCUGGUUGAUGGAC	21	–	141.21	–	–	MYB39 (BE497135); HD-ZIP ROC8 (TC459241); SRG1 (CD454006); alpha-glucosidase (TC393877).
Cluster IV							
Ta-miR154-5p	GGCGAGGGACAUCACUGUACA	22	–	–	1772.21	7485.33	Nucleoredoxin (CA605146)
Ta-miR051-3p	AAUAAGUGUGUAGUUGCUACU	21	1.18	–	10.19	15.73	Serine/threonine-protein phosphatase PP2A-4 (TC437472); BONZAI 3 (TC398798).
Cluster V							
Ta-miR068-5p	CUCUCUCGGGAGGGCUGAUC	20	14.64	8.58	1.50	1.36	
Ta-miR057-1-3p	UGGCCGUUGGUAGAGUAGGAGA	22	67.80	13.28	2.74	2.63	hypothetical protein (TC415409); ribosomal protein S14 (TC421110).
Ta-miR007-5p	CUUAAUUUUGUAAUCUUCUGG	21	109.64	149.93	–	–	NBS-LRR protein (TC426546); superkiller viralicidic activity 2-like 2 (TC441282).
Ta-miR007-3p	AGAAGAUUAGAAGAUUAAGCA	21	666.30	1234.41	–	–	V-type proton ATPase (TC454891); 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (CV759216).
Cluster VI							
Ta-miR158-3p	AAGACAACUAAUUUGGGACGG	21	–	–	–	12.72	pre-mRNA-splicing factor (TC435842); receptor protein kinase (TC390384).
Ta-miR159-3p	UGUAGAAUAGGCACCCGUGC	21	–	–	–	14.83	ATP sulfurylase (TC415167); DCN1 protein (TC382978); acetylglucosaminyltransferase (TC385422).
Ta-miR033-3p	UCAAAGGAUGAGCAAUACU	20	1.77	3.94	–	10.09	Adenosylhomocysteinase (TC437024); met-10+ protein (TC423089); chaperonin (TC384436).

Table 1 Novel miRNAs highly expressed in developing wheat grains (Continued)

Ta-miR053-3p	AGGUGGUUAGGAUACUCGGCU	21	1.85	4.15	1.83	10.62	Acetyl-CoA carboxylase (CK153030)
Ta-miR039-5p	CAGAACCAGAAUGAGUAGCUC	21	18.93	–	21.23	–	NAC (TC389150); Elongation factor 1 (TC380217); Ubiquitin-protein ligase (GH729553).
Ta-miR034-5p	UGAGAUGAGAUUACCCCAUAC	21	85.17	75.79	56.76	163.67	F-box domain containing protein (TC411563); hypothetical protein (CJ660567).

grains, and gene specific qRT-PCR was performed. The expression analysis demonstrated that all detected target genes were strongly down-regulated during grain-filling (Figure 9), which were negatively correlated to the expression profiles of their corresponding miRNAs (Additional file 4). These results suggested that miRNAs play a crucial regulatory role during the developmental processes of grain-filling in wheat.

Discussion

For most eukaryotic cells, miRNAs are a class of regulatory sRNAs involved in gene regulation. In plants, some miRNAs play crucial roles in various developmental processes, such as the control of root and shoot architecture, transitions from the vegetative to reproductive phase, and leaf and flower morphogenesis [22,28]. In wheat, 43 miRNAs have been detected in pooled tissue samples (leaves, roots, and spikes) as well as stressed leaves [15,36]. Here, we identified 540 conserved miRNA families and 182 novel miRNA families, with many being differentially expressed during grain-filling in wheat. Despite the growing knowledge about miRNA functions in plants, only those of highly conserved miRNAs, such as miR156 and

miR172, have been investigated in crop species. For example, miR156 in rice regulates SPL (Squamosa Promoter-binding protein-Like), which promotes panicle branching and higher grain productivity [37]. Likewise, miR172 regulates AP2-like genes that are involved in controlling floral organ identity in rice, maize, and barley [38-40]. Both of these miRNAs were significantly up-regulated and expressed at high levels in our wheat grains during the filling process. We also predicted several new potential targets for miR156 and miR172, such as MYB-related protein (TC#: TC370322) and the starch negative regulator RSR1 (GB#: CA486144).

The processes by which cereal grains mature strongly influence their yield and flour quality. Formation of the starchy endosperm, which is the greatest contributor to human nutrition, occurs in several stages [40]. In wheat, a major transition point occurs at about 14 d after pollination, essentially marking the end of endosperm cell division and the start of grain-filling (i.e., deposition of starch and gluten proteins) in those cells [31,40]. After approximately 28 d, that deposition of storage reserves decreases and the grains begin to desiccate. To investigate the regulatory mechanisms mediated by miRNAs in wheat,

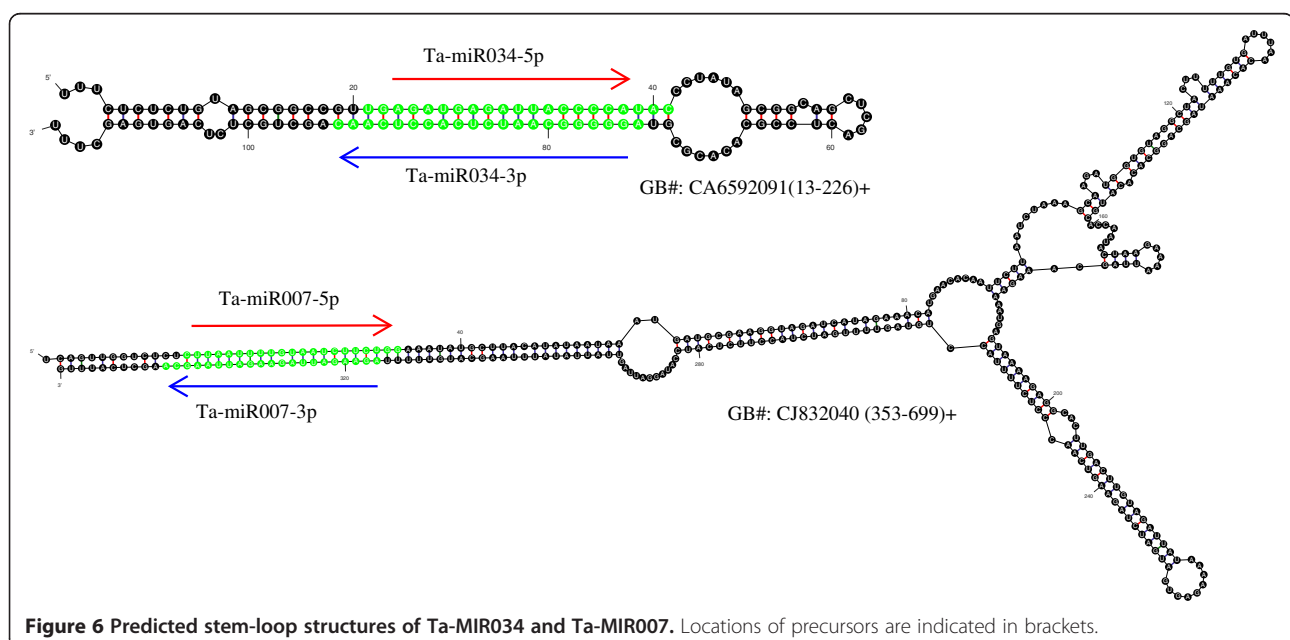


Figure 6 Predicted stem-loop structures of Ta-MIR034 and Ta-MIR007. Locations of precursors are indicated in brackets.

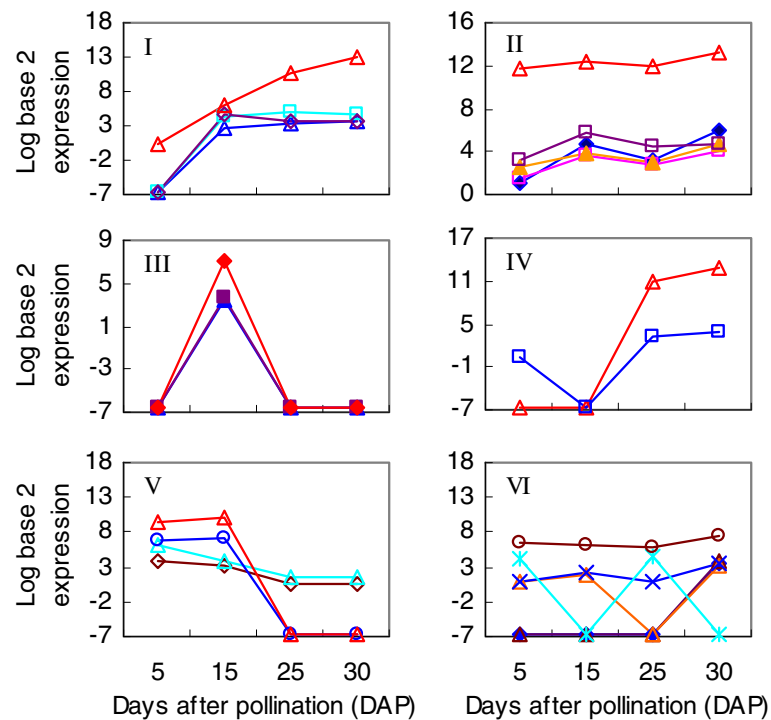


Figure 7 Expression patterns of novel wheat miRNAs in Clusters I through VI during grain development. Abundance of expression was normalized to log base-2 values. I-VI, clusters indicated in Table 1.

especially those involved in grain-filling, we used high-throughout sequencing for profiling at 5, 15, 25, and 30 DAP, and identified a number of grain filling-associated miRNAs (Additional file 5 and Table 1), which were significantly up- or down- regulated during grain formation. Several grain filling –associated miRNAs identified in this study have also been previously detected in other cereal seeds. For example, miR156a, miR167d, miR168a, and miR172a are significantly up-regulated in barley grains [28]. Additionally, these miRNAs (except miR172a) were also highly expressed in developing seeds of maize [41] and rice [7,11]. Therefore, all of these data suggest that miRNAs play crucial regulatory roles during grain development in cereal crops.

Target prediction indicated that grain filling-associated miRNAs identified in this study potentially target to a set of wheat genes, which are involved in various biological processes including carbohydrate metabolism, protein metabolism, developmental processes, transcription, transport, cell organization and biogenesis, response to stress, signal transduction and phytohormone signaling (Additional file 4 and Table 1). Of those predicted targets, some may be involved in regulating starch accumulation or grain formation. For example, the triose phosphate translocator (TPT), a potential target of miR1855, is an integral membrane protein found in the inner membrane of *Arabidopsis* chloroplasts [42]. There, it is responsible for exporting all of the

carbohydrates produced during photosynthesis. Repression of the TPT leads to an increase in starch synthesis in that species. Furthermore, phosphoglycerate kinase (a potential target of miR1037) might participate in regulating carbon-partitioning between starch and sucrose through a series of reactions [43]. In addition, miR172 potentially targets AP2 domain transcription factors CA486144 and CA626451, with the former being a negative regulator of starch production, which is repressed during the middle stages of grain-filling [44]. The expansin gene TC418521, a potential target of miR1137, was highly abundant during early grain expansion but later repressed, implying that levels of expansin are a possible factor in determining grain size [45]. Notably, miR1042 potentially targets a PLAC8 family protein (TC383018), which functions in regulating cell numbers and affects organ size in maize [46]. SNF1-related protein kinase regulatory subunit beta-1 (TC371426), a potential target of miR1211, has vital roles in a signal transduction cascade that controls gene expression and carbohydrate metabolism in higher plants [47]. Its counterpart in barley is thought to modulate the accumulation of storage products during grain-filling [48]. DRG1 (TC386119), a potential target of Ta-miR128-5p, may be crucial to the regulation of either vesicle transport or the activity of enzymes for processing storage proteins [49]. Finally, Ta-miR004-1-5p potentially represses sucrose-phosphate synthase, which is negatively correlated with the accumulation

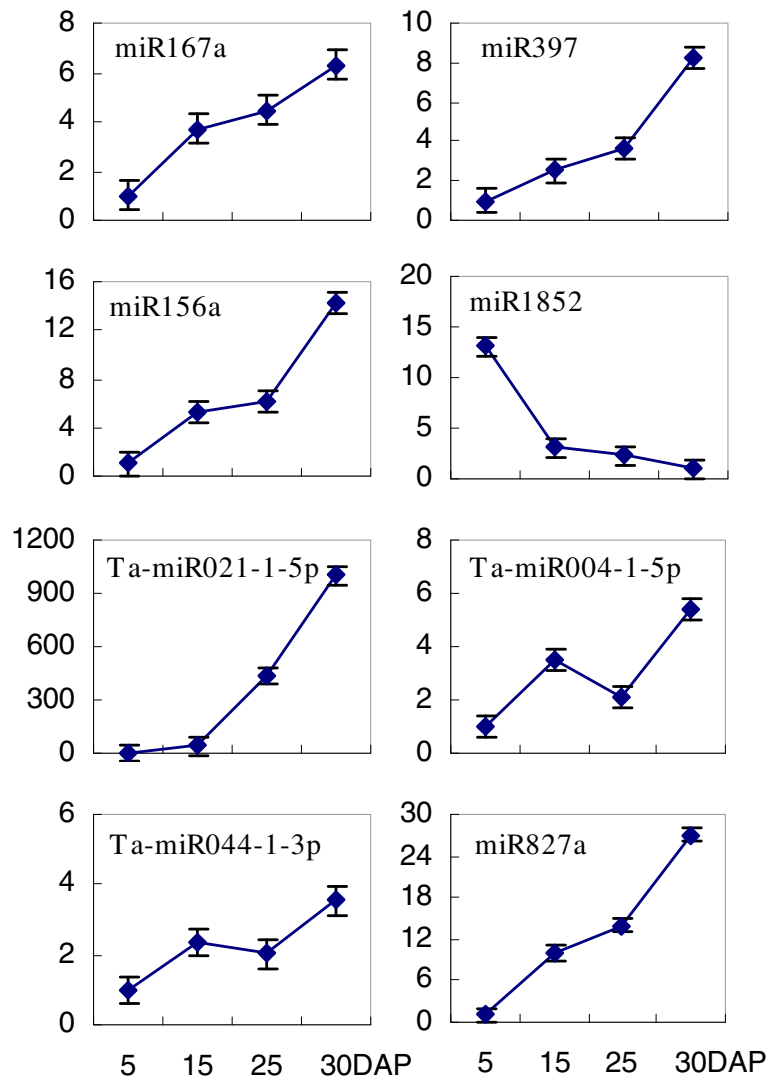


Figure 8 Verification of expression patterns for 8 miRNAs in developing grains. DAP, days after pollination. Data for miRNA expression were normalized to endogenous *actin* [GB#: AB181991]. Values are presented as fold-changes (mean \pm SD) when compared with levels at 5 DAP. Table 1 Novel miRNAs highly expressed in developing wheat grains.

of starch [50]. Overall, our results demonstrate that miRNA-mediated mechanisms operate in the regulation of wheat grain development.

Conclusions

We identified 104 miRNAs (86 conserved and 18 novel ones) associated with grain-filling from wheat and their potential targets are involved in various biological processes, e.g., carbohydrate and protein metabolism, general plant development, transcription, cellular transport, cell organization and biogenesis, stress responses, signal transduction, and phytohormone signaling. These miRNA-mediated networks play crucial roles during the maturation of wheat grains, and our miRNA

data are a valuable resource for future molecular studies that focus on the control of grain development in cereal crops.

Methods

Plant materials

Seeds of wheat (*Triticum aestivum* L. cv. Yumai 18) imbibed water for 24 h and were then transferred to a cold chamber (4°C in the dark). After 4 weeks of vernalization, they were planted in pots and cultured in a naturally lit phytotron glasshouse (25°C/20°C day/night). Developing grains were harvested from the middle four rows of a head at 3 to 33 DAP. Each experiment comprised 100 grains, with three replicates.

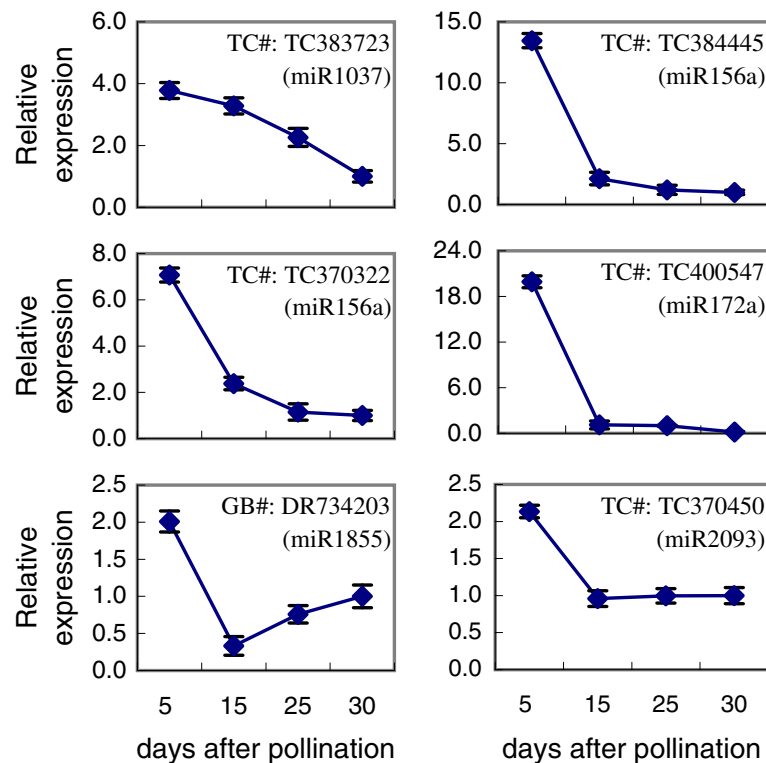


Figure 9 Expression patterns of selected miRNA targets. Accession numbers obtained from either DFCI Wheat Gene Index (<http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=wheat>) (TC#) or GenBank (GB#) are shown. Corresponding miRNAs are indicated in brackets. Data were normalized to endogenous *actin* [GB#: AB181991].

Samples were immediately frozen in liquid nitrogen and stored at -80°C .

Total RNA isolation

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Prior to nucleic precipitation, two extra chloroform washes were performed. A 1% agarose gel, stained by ethidium bromide, was run to determine the preliminary integrity of the RNA. All RNA samples were quantified and examined with an ND 1000 spectrophotometer (Nanodrop) for contamination with either protein (A260 nm/A280 nm ratios) or reagent (A260 nm/A230 nm ratios). The RNA integrity number (RIN) was >8 for all samples, as determined with a 2100 Bioanalyzer (Agilent Technologies).

Construction of sRNA libraries and deep-sequencing

Total RNAs, extracted from developing wheat grains at 5, 15, 25, and 30 DAP, were purified by electrophoretic separations on a 15% TBE-Urea denaturing PAGE gel. The sRNA regions corresponding to 18- to 30-nucleotide bands were excised and recovered. Those sRNAs were then 5' and 3' RNA adapter-ligated by T4 RNA ligase. At each step, their lengths were verified before they were purified.

The adapter-ligated sRNAs were transcribed into cDNA by Super-Script II Reverse Transcriptase (Invitrogen). We performed PCR-amplifications with primers that annealed to the ends of the adapters. Amplified cDNA constructs were also purified and recovered. The final quality of the cDNA library was ensured by examining its size, purity, and concentration with the 2100 Bioanalyzer. Four libraries, one for each sampling time point, were used for sequencing with the Illumina Genome Analyzer at the Beijing Genomics Institute, Shenzhen, China.

Analysis of sequencing data

Automated base calling of the raw sequence and vector removal were performed with PHRED and CROSS MATCH programs [51,52]. Any low-quality reads as well as reads with adaptor contamination that were not ligated to any other sequences were discarded. The remaining, high-quality, sequences were trimmed of their adapter sequences; only sequences between 18 nt and 30 nt were analyzed further. The clean reads were mapped to all of the wheat EST sequences obtained from NCBI by SOAP2 (<http://soap.genomics.org.cn/>). They were aligned to known miRNA precursors (<http://www.mirbase.org/>) to determine how many identified miRNAs were present. The remaining sequences were used in a BLASTN search of the

Rfam database (<http://rfam.sanger.ac.uk/>) to remove most of the non-siRNA and non-miRNA sequences. Putative origins for the remaining sequences were identified by a BLASTN search against the wheat EST database from NCBI. The protein-coding EST sequences were removed and the remaining non-coding candidate ESTs, with perfect matches to sRNA sequences, were used for fold-back via Mireap (<http://sourceforge.net/projects/mireap>) based on critical criteria described by Meyers et al. [33]. The secondary structure of precursors were predicted by the Mfold web server (<http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form>) [53].

Expression analysis of miRNAs based on deep-sequencing data

The frequency of miRNAs was normalized according to the expression of transcripts per million (TPM). For each sample, $TPM = (\text{Actual miRNA count} / \text{Total count of clean reads}) \times 10^6$. The fold-change in miRNA expression at 15, 25, and 30 DAP versus 5 DAP was calculated as $\text{Fold-change} = \log_2(\text{treatment} / \text{control})$, where 5 DAP served as the control. All p -values were determined according to the following formula:

$$p(x|y) = \binom{N_2}{N_1}^y \frac{(x+y)!}{x!y! \left(1 + \frac{N_2}{N_1}\right)^{(x+y+1)}} \quad \begin{aligned} C(y \leq y_{\min}|x) &= \sum_{y=0}^{y \leq y_{\min}} p(y|x) \\ D(y \geq y_{\max}|x) &= \sum_{y \geq y_{\max}} p(y|x) \end{aligned}$$

Prediction and annotation of potential miRNA targets

Predictions of putative targets were performed by using the most abundant miRNA variants as queries against the TAGI database at psRNATarget (<http://plantgrn.noble.org/psRNATarget/>) [35]. The following default parameters were applied: maximum expectation value, 3; target accessibility – allowed maximum energy to unpair the target site (UPE), 25; flanking length around the target site for target accessibility analysis, 17 bp upstream and 13 bp downstream; and the range of central mismatch leading to translational inhibition, 9 to 11 nt. To acquire a better annotation of the potential targets, we performed BLAST searches for their sequences against the NCBI database.

Expressing validation of miRNAs and their targets

We verified the patterns of expression by five conserved wheat miRNAs -- miR167a, miR397, miR156a, miR1852, and miR827a – plus three novel miRNAs -- Ta-miR021-2-5p, Ta-miR004-1-5p, and Ta-miR044-1-3p. A One Step PrimeScript® miRNA cDNA Synthesis Kit (Perfect Real Time; TaKaRa) was used for the RT reactions. The temperature program was adjusted to run for 60 min at 37°C, 5 s at 85°C, and then 4°C. For each miRNA, three biological replicates were performed. qRT-PCR was

conducted on a Bio-Rad IQ5 Real-Time PCR Detection System. Each reaction included 2 µL of product from the diluted RT reactions, 1.0 µL of each primer (forward and reverse), 12.5 µL of SYBR® Premix Ex Taq™ (Perfect Real Time; TaKaRa), and 8.5 µL of nuclease-free water. The reactions were incubated in a 96-well plate at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 30 s. All reactions were run in three replicates for each sample. The *actin* gene (GB#: AB181991) served as the endogenous control. We also selected six target genes -- TC383723, TC384445, TC370322, TC400547, TC370450, and DR734203 -- for grain filling-induced miR1037, miR156a, miR172a, miR1855, and miR2093 in order to validate their expression profiles in developing grains via qRT-PCR. All primers are listed in additional file 9.

Additional files

Additional file 1: Annotation and distribution of sRNAs. The classifications are listed for all sRNAs detected in the four libraries from developing wheat grains at 5, 15, 25, and 30 DAP.

Additional file 2: Conserved miRNAs identified in developing wheat grains. Listed sequences and abundances for all conserved miRNAs detected.

Additional file 3: Conserved miRNAs differentially expressed during grain development. Listed sequences, abundances, as well as ratios of change in expression for differentially expressed miRNAs.

Additional file 4: Conserved miRNAs potentially associated with grain-filling. Listed sequences, abundances and target descriptions for 86 conserved miRNAs potentially involved in the control of grain-filling.

Additional file 5: Novel miRNAs identified in developing grains. Listed sequences, abundances and precursors for novel miRNAs identified.

Additional file 6: Secondary structures of 24 highly expressed novel miRNAs. Secondary structures of 24 highly expressed novel miRNAs. Demonstrated stem-loop structures for novel miRNAs presented in Table 1.

Additional file 7: Predicted targets and annotation for grain filling-regulated conserved miRNAs. Listed the targets and annotations for conserved miRNAs from Clusters I through VIII, as shown in Figure 5.

Additional file 8: Predicted targets for novel miRNAs. Listed the targets and annotations for 24 miRNAs highly expressed in developing grains and presented in Table 1.

Additional file 9: Primers used in this study.

Abbreviations

DAP: Days after pollination; miRNAs: microRNAs; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction; siRNAs: Short-interfering RNAs; TPM: Transcripts per million.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

FM performed the bioinformatics analysis and miRNA validation. HL and KW were involved in preparing the plant materials, deep-sequencing, and characterization of the miRNA targets. LL and SW participated in the expression profiling of miRNA targets. YZ take part in the statistic analysis. FM, JY, and YL designed and prepared the manuscript. All authors read and approved the final manuscript.

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