Identification of microRNAs in developing wheat grain that are potentially involved in regulating grain characteristics and the response to nitrogen levels

Gege Hou¹, Chenyang Du¹, Honghuan Gao¹, Sujun Liu¹, Wan Sun¹, Hongfang Lu¹, Juan Kang¹, Yingxin Xie¹, Dongyun Ma¹,²* and Chenyang Wang¹,²*

Abstract

Background: MicroRNAs (miRNAs) play crucial roles in the regulation of plant development and growth, but little information is available concerning their roles during grain development under different nitrogen (N) application levels. Our objective was to identify miRNAs related to the regulation of grain characteristics and the response to different N fertilizer conditions.

Results: A total of 79 miRNAs (46 known and 33 novel miRNAs) were identified that showed significant differential expression during grain development under both high nitrogen (HN) and low nitrogen (LN) treatments. The miRNAs that were significantly upregulated early in grain development target genes involved mainly in cell differentiation, auxin-activated signaling, and transcription, which may be associated with grain size; miRNAs abundant in the middle and later stages target genes mainly involved in carbohydrate and nitrogen metabolism, transport, and kinase activity and may be associated with grain filling. Additionally, we identified 50 miRNAs (22 known and 28 novel miRNAs), of which 11, 9, and 39 were differentially expressed between the HN and LN libraries at 7, 17, and 27 days after anthesis (DAA). The miRNAs that were differentially expressed in response to nitrogen conditions target genes involved mainly in carbohydrate and nitrogen metabolism, the defense response, and transport as well as genes that encode ubiquitin ligase. Only one novel miRNA (PC-5p-2614_215) was significantly upregulated in response to LN treatment at all three stages, and 21 miRNAs showed significant differential expression between HN and LN conditions only at 27 DAA. We therefore propose a model for target gene regulation by miRNAs during grain development with N-responsive patterns.

Conclusions: The potential targets of the identified miRNAs are related to various biological processes, such as carbohydrate/nitrogen metabolism, transcription, cellular differentiation, transport, and defense. Our results indicate that miRNA-mediated networks, via posttranscriptional regulation, play crucial roles in grain development and the N response, which determine wheat grain weight and quality. Our study provides useful information for future research of regulatory mechanisms that focus on improving grain yield and quality.

Keywords: Nitrogen application level, Differentially expressed miRNAs, Grain development, Wheat
Background

As one of the largest and most widely planted food crop species in the world, wheat provides approximately 20% of the required human calorie intake [1]. With the rapid increase in the global population, increasing wheat yields is a major goal to meet future food demands [2]. Additionally, with increasing economic development, improving wheat quality, including increasing the protein content, is another important objective for wheat production. Conventional breeding approaches, which depend on the manipulation of genetic variation, have been successful at producing wheat cultivars with improved grain yield and quality; however, understanding the molecular mechanisms that regulate wheat development and grain quality would benefit further wheat improvement programs via modern genetic engineering technology [3].

Small RNAs (sRNAs) are ubiquitous components of plant transcriptomes; they have been found to regulate cellular metabolism, differentiation and growth and are involved in defense against viruses and mobile genetic elements in plants [4]. MicroRNAs (miRNAs) are single-stranded, noncoding sRNAs produced from endogenous transcripts with an imperfect self-complementary stem–loop structure [5, 6]. miRNAs play crucial regulatory roles in organisms by targeting specific mRNAs for cleavage or translational repression [7–9]. It has been reported that miRNAs affect various biological processes, such as the development of leaves, roots, and flowers, and affect grain filling [7, 10–12]. Grain filling is a critical phase in crop grain development. In rice, osa-miR167 regulates the auxin-miR167–ARF8-OsGH3.2 pathway, which functions during grain filling [13]. High-throughput sequencing revealed that the differentially expressed miRNAs between superior and inferior wheat grain are likely related to cell division, carbohydrate metabolism and hormone biosynthesis [14]. Han et al. [15] demonstrated that miR164, miR160, and miR169 show different expression profiles during grain filling, suggesting that their functions are coordinated during the different stages of wheat seed development. Similarly, Meng et al. [12] identified 873 miRNAs from wheat grain and suggested that many of them potentially regulate grain filling. Using a genome-wide investigation of miRNAs in different wheat tissues, Sun et al. [16] characterized 64 miRNAs that are expressed in developing wheat grain and that might play important functions in regulating grain development. Furthermore, miRNAs that respond to nitrogen (N) deficiency in higher plants have been identified in previous studies [17–19]. In maize, miR169, miR399, miR518 and miR408 respond to N in the roots and leaves, but miR160, miR167, miR168 and miR395 respond to N deficiency only in the roots [18]. Several miRNAs, including miR167, miR171, miR398, miR827, miR408, and miR857, have been identified as being responsive to low-N conditions [20], and the wheat miRNA TamiR444a, which responds to nitrogen deficiency, is involved in plant adaptation to nitrogen starvation stress [21]. Zuluaga et al. [22] identified 84 novel and 161 conserved miRNAs in durum wheat in response to N starvation and found that ttu-miR169c and ttu-novel-61 were strongly downregulated under N-starvation conditions. Some miRNAs, such as miR159a, miR159b and miR399, showed significantly different expression profiles under low N treatment compared with high N treatment [23]. However, little information is available concerning the regulatory role of miRNAs in wheat grain in response to N deficiency, especially at different developmental stages.

Nitrogen is extremely important for plant growth and for agricultural productivity of many crop species [24]. Together with other N metabolites, nitrate can also act as a signal that regulates global gene expression [25]. In China, applying N fertilizer is an important agronomic practice to increase yield and economic productivity [26]. In addition to its effect on yield, N fertilizer is one of the key agronomic means of increasing grain protein content and improving wheat grain quality [27, 28]. However, excessive applications of N fertilizer have resulted in environmental pollution [29] and soil acidification [30]. Many efforts have been made to improve N fertilizer-use efficiency to reduce the waste of resources and to solve eco-environmental problems. Understanding the molecular mechanism of plant responses to N fertilizer applications might enable the development of crop varieties with increased nutrient-use efficiency and high grain yield and quality. To gain further insight into the role of wheat miRNAs that respond to different N fertilizer levels wheat grain development, the wheat cultivar Zhengmai 119 (ZM119), which has a high protein content, was grown under high nitrogen (HN) and low nitrogen (LN) conditions, and miRNA expression patterns were evaluated during grain development. The findings in this study will provide new insights into the regulatory function of wheat miRNAs that not only are associated with grain yield and quality but also respond to N supply levels.

Results

Grain characteristics

As shown in Table 1, the wheat cultivar ‘ZM119’ grown under HN conditions had a high grain yield, thousand-kernel weight, total protein content and protein fraction content (albumin, globulin, gliadin, and glutenin), whereas wheat grain from plants grown under LN conditions had low values. Correspondingly, the dough (rheological) characteristics of the wheat flour from the HN treatment had higher values than did the dough from the LN treatment. These results confirm that, compared with no N treatment,
Table 1 Grain characteristics of the wheat cultivar ‘ZM119’ grown under HN and LN conditions

| Items                        | HN               | LN               |
|------------------------------|------------------|------------------|
| Thousand kernel weight (g)   | 50.23 ± 1.10     | 48.52 ± 1.60     |
| Grain yield (kg ha⁻¹)        | 7184.6 ± 378.0   | 4870.2 ± 28.1___|
| Protein content (mg g⁻¹)     | 153.38 ± 1.94    | 112.36 ± 0.52    |
| Albumin content (mg g⁻¹)     | 31.69 ± 0.25     | 24.94 ± 0.41     |
| Globulin content (mg g⁻¹)    | 10.32 ± 0.86     | 7.33 ± 0.20      |
| Gliadin content (mg g⁻¹)     | 34.44 ± 0.10     | 18.07 ± 0.75     |
| Glutenin content (mg g⁻¹)    | 61.88 ± 4.60     | 50.45 ± 4.89     |
| Water absorption (%)         | 63.55 ± 0.40     | 60.35 ± 0.49     |
| Dough development time (min) | 6.95 ± 0.35      | 1.90 ± 0.14      |
| Dough stability (min)        | 9.00 ± 1.41      | 4.70 ± 0.98      |

Data represent the mean ± standard deviation. Within the same row, mean values followed by different lowercase letters indicate significant differences at \( P < 0.05 \) (least significant difference).

N fertilizer applications can increase grain yield and improve wheat grain quality.

Deep sequencing of sRNAs in developing wheat grain from plants grown under two N fertilizer levels

To investigate the role of miRNAs in the developing wheat grain under different N fertilizer application levels, we sampled wheat grain at 7, 17, and 27 days after anthesis (DAA) under LN and HN conditions (three biological replicates each) and constructed a total of 18 sRNA libraries (Additional file 1: Table S1). The mean data of the three biological replicates are listed in Table 2.

After sequencing, 10,475,211 to 11,752,733 redundant raw reads were produced per library (Table 2). After removing the low-quality reads, 3,955,306 to 6,430,010 clean reads were obtained from each library, representing 36.16–60.05% of the total redundant raw reads. The number of unique raw reads ranged from 1,561,169 to 2,014,214, and the number of unique clean reads ranged from 891,960 to 1,412,072, representing 52.37–70.00% of the total unique raw reads. The majority of the miRNAs in the constructed libraries were between 21 and 24 nt in length (Fig. 1), with the 21-nt class (59.83–74.34%) being the most frequent of the total reads, followed by the 24-nt miRNAs (7.08–14.64%). The 21-nt miRNAs were more frequent in the HN-7 and LN-7 libraries, whereas the 24-nt miRNAs were well represented in the LN-17 and HN-27 libraries.

Identification of known miRNAs and predicted novel miRNAs

To identify the conserved miRNAs in the constructed libraries in this study, the unique reads from each library were subjected to homology searches with known mature miRNAs from plants within miRBase (Additional file 2: Table S2). Additionally, novel miRNAs were predicted from unannotated sequences without similarity to known miRNAs (Additional file 2: Table S2, Additional file 3: Table S3). Since many miRNAs have not been deposited into miRBase, the novel miRNAs identified in this study were also compared with previously published wheat miRNAs [12, 14–16, 31–35]. In total, the sequences of 39 miRNAs from among the newly identified miRNAs were the same as those of previously found novel miRNAs (Table S2; the miRNAs are labeled in yellow). Most of the novel miRNAs had a relatively low expression level. The most abundant novel miRNAs were tae-miR9655-p5 and tae-miR2916-p3_2ss2TC17CA, which accounted for 24, 656 reads and 27,735 reads in the LN-17 and LN-27 libraries, respectively. A total of 205, 327, and 322 unique miRNAs (of which 92, 190, and 191 were novel, respectively) were found in the HN-7, HN-17, and HN-27 libraries, respectively (Fig. 2). Additionally, a total of 143 miRNAs (55 novel ones) were found in the three HN-7, HN-17, and HN-27 libraries. The HN-7 and HN-17 libraries shared the same 19 miRNAs, of which 11 were novel. There were 245, 332, and 233 miRNAs (of which

Table 2 Sequencing data of the small RNA libraries constructed from developing wheat grain produced under two nitrogen fertilizer levels

| Type                  | LN-7              | LN-17             | LN-27              | HN-7              | HN-17             | HN-27              |
|-----------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
|                      | No.               | %                 | No.               | %                 | No.               | %                 | No.               | %                 |
| Redundant             | 10,621,127        | 100               | 11,752,733        | 100               | 10,989,065        | 100               | 10,652,837        | 100               | 10,948,017        | 100               | 10,475,211        | 100               |
| 3 AD and length filter| 3,809,346         | 36.38             | 6,655,958         | 56.05             | 5,115,582         | 46.94             | 4,985,140         | 46.62             | 6,192,961         | 56.53             | 3,646,829         | 34.73             |
| Other filters         | 381,771           | 3.57              | 898,931           | 7.72              | 1,320,520         | 12.09             | 332,596           | 3.13              | 799,749           | 7.31              | 780,664           | 7.46              |
| Clean reads           | 6,430,010         | 60.05             | 4,196,843         | 36.23             | 4,461,963         | 40.96             | 5,335,101         | 50.25             | 3,955,306         | 36.16             | 6,047,718         | 57.81             |
| Unique                | 1,698,982         | 100               | 1,624,844         | 100               | 1,849,117         | 100               | 1,561,169         | 100               | 1,604,716         | 100               | 2,014,214         | 100               |
| 3 AD and length filter| 510,620           | 30.66             | 715,762           | 44.70             | 860,978           | 46.58             | 506,277           | 32.44             | 656,487           | 40.75             | 580,730           | 28.94             |
| Other filters         | 16,242            | 0.95              | 17,121            | 1.05              | 19,361            | 1.05              | 14,521            | 0.93              | 17,403            | 1.09              | 21,412            | 1.06              |
| Clean reads           | 1,172,120         | 68.39             | 891,960           | 54.25             | 968,778           | 52.37             | 1,040,371         | 66.63             | 930,826           | 58.16             | 1,412,072         | 70.00             |

No., number of reads; %, percentage of reads. The data are the means of three biological replicates.
121, 199, and 118 were novel, respectively) expressed in the LN-7, LN-17, and LN-27 libraries, respectively. We found that 146 miRNAs (including 53 novel ones) were expressed in all three LN libraries. When the effects of the two N fertilizer application levels were compared, LN-7 and HN-7 were found to share 189 expressed miRNAs, including 83 novel miRNAs. LN-17 and HN-17 shared 283 expressed miRNAs, of which 163 were novel. It is clear that the 17 DAA libraries (HN-17 and LN-17) contained more expressed miRNAs than did the other libraries.

**Differentially expressed miRNAs in the developing wheat grain**

To identify the differentially expressed miRNAs in developing wheat grain, we analyzed the relative abundance of miRNAs in the HN-7, HN-17, and HN-27 libraries, and $P \leq 0.05$ was used as a threshold to determine significant changes in expression during grain development. Similarly, the abundance of miRNAs in LN-7, LN-17, and LN-27 was also compared. A total of 179 miRNAs (91 known and 88 novel miRNAs) were identified in the comparisons of the three HN treatment libraries (Additional file 4: Tables S4–2 and S4–4). Additionally, 80 known and 76 novel miRNAs were identified among the three LN treatment libraries (Table S4–1, S4–3). All of these miRNAs belong to 51 miRNA families. As shown in Additional file 4 (Table S4–1), the known miRNAs that were differentially expressed between the three LN treatment libraries were divided into four groups. In group A-I, the expression of the identified miRNA showed an increasing trend with grain development; there were 17 miRNAs in this group, including two miR156s and three miR167s, of which tae-miR9674b-5p (Table S4–1) and tae-miR9622a-3p were the most abundant (Table S4–1). The tae-miR9666b-3p miRNA showed an increasing degree of expression in developing grain, with 3, 213, and 674 normalized reads in the LN-7, LN-17, and LN-27 libraries, respectively. This expression profile might indicate that these miRNAs have more important regulatory roles at later stages of grain development. Group A-II miRNAs show a decreasing expression profile.
profile with grain development, and there were 30 miRNAs in this group, including two miR159s, three miR171s, and four miR169s. In this group, ata-miR5168-3p was the most abundant, and ata-miR169d-3p_L-2R + 2 showed large fold-changes of −3.81, −1.18, and −4.99 for the LN-17 vs. LN-7, LN-27 vs. LN-7, and LN-27 vs. LN-7 comparisons, respectively. The high levels of expression at the early stages of wheat grain development might be related to grain endosperm differentiation and cell division. The members of group A-III showed a single expression peak, with the highest abundance at 17 DAA. In this group, there were 18 miRNAs, of which tae-miR9655-3p was the most abundant. In addition, there were 15 miRNAs in groups A-IV, including three miR159s, four miR166s, and two miR319s (Table S4–1).

Similarly, there were four miRNA groups (for the known miRNAs) for the LN treatment libraries (Table S4–2). Group B-I contained 15 miRNAs, including two miR156s, one miR408, and three miR9666s. There were 30 miRNAs in group B-II belonging to 17 miRNA families. The 24 miRNAs in group B-III included three miR160s, three miR167s, and two miR531s, of which tae-miR160 (with an increase from 4129 reads to 6157 reads) was the most abundant. There were 22 miRNAs in groups B-IV, including four miR159s and four miR166s. The fold-changes for the LN-17 vs. HN-7 and LN-27 vs. HN-27 comparisons were −2.23 and −2.19, respectively, for tae-miR159a (Table S4–2). We noticed that some miRNAs exhibited the same expression profiles during grain development in both the HN and LN treatments. For example, tae-miR408_L-1, osa-miR827, tae-miR9666b-3p, tae-miR9666a-3p, and tae-miR9664–3p_L-1 were in group I (A-I, and B-I) in both the HN and LN treatments. Moreover, miR168, miR396, and miR530 were in group II (A-II, and B-II), and miR-528, miR-1127, and miR9655 were all in group III (A-III, and B-III) (Table S4–1, S4–2). These miRNAs exhibiting the same expression patterns between the two N application levels might be closely related to grain development. Of course, some miRNAs showed different expression profiles with respect to N application levels, which indicates that the regulatory role of these miRNAs might be related to N supply status. For example, ppt-miR894_L-1R + 1 was differentially expressed only in the HN treatment, whereas tae-miR9652-5p showed differential expression only in the LN treatment.

In addition, the expression patterns of novel miRNAs during grain development for the LN treatment and the HN treatment are shown in Table S4–3 and Table S4–4, respectively. Similar to the known miRNAs, the novel miRNAs identified among the LN treatments were also divided into four groups (Table S4–3). Group C-I contains 24 novel miRNAs, of which tae-miR2916-p5_2s2AG17TG was the most abundant, and 15 miRNAs were not expressed in the LN-7 library. There were 18 and 31 novel miRNAs in groups C-II and C-III, respectively. Only three novel miRNAs were in group C-IV, of which tae-miR9674a-p5 was the most abundant (Table S4–3). Similarly, the differentially expressed novel miRNAs in the HN treatment libraries were also classified into four groups (Table S4–4). In group D-I, there were 30 novel miRNAs, of which 21 showed no expression in the HN-7 library but were highly expressed in the HN-27 library. There were 12 miRNAs in group D-II, of which tae-miR171a-p5 exhibited a large expression fold change (−4.87) from 7 DAA to 17 DAA. There were 40 and 6 novel miRNAs found in groups D-III and D-IV, respectively (Table S4–4).

Target gene functions of miRNAs differentially expressed during grain development

Based on Gene Ontology (GO) assignments, we identified 3011 potential target genes for 354 differentially expressed miRNAs. The target functions of the miRNAs were analyzed according to the “biological process” (A), “cellular component” (B), and “molecular function” (C) GO categories. In the “biological process” category, the target genes were associated largely with 10 GO terms (Fig. 3), with the three most represented being “growth and development” (24.61%), “regulation of transcription” (22.55%), and “cellular process” (14.11%). Twelve molecular function GO terms were identified, with the three most frequent being “protein binding” (28.28%), “ATP binding” (18.32%), and “DNA/RNA binding” (11.68%). For “cellular component”, the most abundant

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**Fig. 3** The major GO categories of “cellular component”, “biological process”, and “molecular function” for the predicted genes of all the differentially expressed miRNAs during grain development.
GO term was “nucleus” (59.77%), followed by “mitochondrion”, with 19.01%.

To explore the roles of these differentially expressed miRNAs in grain development, we listed the known miRNAs and the novel miRNAs and their target functions in Tables 3 and 4, respectively (target gene IDs in Additional file 5: Table S5). It is clear that the target genes have diverse functions, and the encoded gene products include transcription factors, involve Skp-cullin-F-box (SCF) ubiquitin ligase activity, encode Cytokinin Response Factor 1 (CRF), encode protein kinases and encode heat-shock proteins (HSPs). tae-miR9674b-5p, tae-miR160, ata-miR166a-3p, and tae-miR159a are relatively abundant during all grain development stages (RAGS), and their target genes have multiple functions and encode proteins such as SCF proteins, protein kinase 20-like, serine carboxypeptidase, kinesin-2-like protein pollen semisterility 1 (PSS1) and MYB transcription factors (Table 3), indicating that these miRNAs might have multiple and important roles during all stages of grain development. The miRNAs osa-miR171a, ata-miR172c-3p, three mir396s (ata-mir396a-5p, ata-mir396e-5p, and ata-mir396c-5p), ata-mir3933-5p_L-1R + 1 and ata-mir5168-3p were relatively more abundant during early stages of development and were predicted to target genes encoding UDP-glycosyltransferase, metalloendopeptidase, neural precursor cell-expressed developmentally downregulated 8 (NEDD8)-specific protease, and glucosidase.

Cell differentiation plays an important role in determining wheat grain size. To clarify the potential regulatory mechanisms underlying cell differentiation, we constructed a miRNA target gene GO network by selecting miRNAs and their target genes based on their GO annotation (Fig. 4a).

There are 27 miRNAs and 20 genes in this network, with five, five, and three miRNAs belonging to the miR171, miR166, and miR319 families, respectively. It is clear that miR166 members target mainly transcription factor-encoding and nucleic acid-related genes, and the miR171 family members target genes mainly involved in the hormone response. Additionally, we constructed a network with miRNAs that had GO annotations related to seed development (Fig. 4b). Fifteen miRNAs and 16 genes are included in this network, including ata-miR167b-3p, which is predicted to target glycine cleavage system H protein 2. The tae-miR815c-p5_2ss6CT21CT miRNA might regulate grain development by targeting cytochrome P450s, which play roles in carbohydrate metabolism. Additionally, a network including eight miRNAs and eight target genes was constructed with GO annotations related to the heat stress response (Fig. 4c). This network shows that the miRNA targets include genes that encode chaperones, transcription factors, HSPs, DnaJ, and components of the abscisic acid (ABA) pathway, indicating that the miRNAs might participate in the heat stress response by regulating various pathways. Additionally, miR172 members might play an important role in the response to heat stress by regulating chaperones.

miRNAs that are differentially expressed between the two N levels and their potential target gene functions

A comparison between HN and LN revealed 40 (25 known and 15 novel), 44 (26 known and 18 novel), and
| Grain filling stages | miRNA               | Abundance (RPM) | P-value | Abundance (RPM) | P-value | Target function                                                                 |
|----------------------|---------------------|-----------------|---------|-----------------|---------|---------------------------------------------------------------------------------|
|                      |                     | LN-7            | LN-17   | LN-27           | HN-7    | HN-17                                                                          | HN-27    |                                                   |
| Relative abundance   | tae-miR9674b-5p     | 13,242          | 14,580  | 19,780          | 2.10E-05| SCF ubiquitin ligase complex; calcium-dependent protein kinase 20-like        |                                                   |
| during grain filling  | tae-miR160          | 3696            | 6919    | 5125            | 3.34E-07| serine carboxypeptidase; transcription factor; Auxin reponse factor            |                                                   |
| stages (RAGS)        | ata-miR166a-3p      | 9081            | 2818    | 4010            | 1.05E-08| kinesin-1-like protein PSS1; transport                                          |                                                   |
|                      | tae-miR159a         | 5372            | 1381    | 2706            | 1.28E-06| MYB; 40D repeat                                                                 |                                                   |
|                      | osa-miR171a         | 1235            | 113     | 58              | 1.10E-09| DELLA proteins RGL2; malate dehydrogenase                                      |                                                   |
| Relative abundance   | ata-miR172c-3p      | 162             | 104     | 0               | 1.65E-05| cytokinin response factor; Dna1                                                |                                                   |
| at early stage       | ata-miR396a-5p      | 1869            | 967     | 964             | 4.70E-07| phosphatidylinositol 4-phosphate 5-kinase 1; glutamate dehydrogenase; response to GA |                                                   |
| (RAES)               | ata-miR396c-5p      | 144             | 16      | 7               | 4.11E-06| GFPase activity; HSP protein; RPM1                                              |                                                   |
|                      | ata-miR396e-5p      | 2264            | 772     | 709             | 7.40E-08| transcription factor TFID; ribonuclease P activity                              |                                                   |
|                      | ata-miR393-5p,L-1R+1| 325             | 45      | 37              | 6.88E-07| auxin-activated signaling; protein TRANSPORT INHIBITOR RESPONSE 1; LRR kinase  |                                                   |
|                      | ata-miR167f-3p,L-1R+1| 61              | 26      | 19              | 5.86E-04| ubiquitin ligase complex; lysosomal alpha-mannosidase                           |                                                   |
|                      | ata-miR169e-5p      | 38              | 5       | 2               | 1.23E-06| serine-type endopeptidase; peptidase                                           |                                                   |
|                      | osa-miR168a-5p      | 108             | 84      | 72              | 4.97E-03| WRKY; Cu4-RING E3 ubiquitin ligase;                                             |                                                   |
|                      | osa-miR168a-3p,L-3  | 115             | 50      | 23              | 1.79E-04| ubiquitin domain-containing protein DSK2b                                       |                                                   |
|                      | ata-miR169g-3p,L+1R-1| 250             | 7       | 0               | 4.17E-07| ubiquitin protein ligase; RPM protein                                          |                                                   |
|                      | osa-miR171e-5p-ss2GA21AG | 344 | 12        | 0              | 1.51E-06| protein binding                                                                |                                                   |
|                      | osa-miR530-5p,L+1   | 126             | 61      | 0               | 3.00E-04| SCF ubiquitin ligase complex; fructose-bisphosphate aldolase                   |                                                   |
|                      | ata-miR5168-3p      | 5692            | 638     | 454             | 2.00E-10| kinesin-1-like protein PSS1; Transcription factor; fructose-bisphosphate aldolase |                                                   |
|                      | osa-miR530-5p,R+1  | 52              | 36      | 0               | 3.00E-04| fructose-bisphosphate aldolase; HSP70.                                         |                                                   |
|                      | osa-miR159a1.L+1   | 86              | 15      | 41              | 9.85E-03| MYB; W540 repeat protein                                                       |                                                   |
|                      | tae-miR9776.L-1R+1  | 190             | 167     | 145             | 3.54E-03| cell division; O-methyltransferase                                            |                                                   |
|                      | osa-miR166g-3p,L+1  | 118             | 14      | 16              | 1.10E-05| transcription factor; transport                                                |                                                   |
|                      | tae-miR171a        | 696             | 125     | 126             | 1.00E-10| DELLA protein RGL2-like; malate dehydrogenase                                  |                                                   |
|                      | ata-miR394-5p       | 286             | 42      | 98              | 3.46E-06| SCF ubiquitin ligase complex; glutathione transferase activity                 |                                                   |
|                      | ata-miR9772a-5p,L-2R+2 | 47        | 21      | 16              | 1.17E-04| SCF ubiquitin ligase complex                                                   |                                                   |
100 (48 known and 52 novel) differentially expressed miRNAs at 7 DAA, 17 DAA, and 27 DAA, respectively (Additional file 6: Table S6–1, S6–2, and S6–3). To further explore the role of these miRNAs in grain development and the response to N supply levels, the criteria of \( \log_2(\text{fold change}) \geq 1.0 \) and reads per million of total miRNA reads (RPM) \( \geq 20 \) were further used to select the differentially expressed miRNAs in the constructed libraries. For the LN-7 vs. HN-7, LN-17 vs. HN-17, and LN-27 vs. HN-27 comparisons, the ratios of upregulated miRNAs were 2/6, 0/6, and 5/10 for the known miRNAs, respectively, and were 5/5, 2/3, and 8/24 for the novel miRNAs, respectively (Table 5).

The differentially expressed miRNAs and their target gene functions are listed in Table 6. For the known miRNAs, the abundance of sbi-miR399a was significantly higher in the HN treatments (HN-7 and HN-17) than in the LN treatments (LN-7 and LN-17), and tae-miR9664–3p_L-1 showed significantly higher expression in the HN treatment than in the LN treatment at 17 and

### Table 3 Conserved miRNAs differentially expressed in developing wheat grain and their predicted target gene functions (Continued)

| Grain filling stages | miRNA | Abundance (RPM) | \( P \)- value | Abundance (RPM) | \( P \)- value | Target function |
|----------------------|-------|----------------|--------------|----------------|--------------|----------------|
|                      |       | LN-7  | LN-17 | LN-27 | HN-7 | HN-17 | HN-27 | |
| Relative abundance at middle stage (RAMS) | ato-miR528-5p | 39 | 46 | 3 | 4.89E-04 | 28 | 38 | 23 | 1.99E-02 | lectin-rich repeat receptor kinase |
|                      | bdi-miR531_L-4_R+1_1ss5SCT | 17 | 33 | 18 | 7.10E-03 | 12 | 38 | 23 | 1.27E-02 | NAD(P)H dehydrogenase; ABC transporter |
|                      | ato-miR172b-3p | 22 | 66 | 34 | 1.65E-05 | 26 | 36 | 20 | 1.35E-02 | Cytokinin response factor; chaperone binding; |
|                      | tae-miR1127b-3p_1ss12TC | 7 | 18 | 4 | 3.57E-02 | 2 | 26 | 13 | 2.84E-04 | UDP-glucuronate decarboxylase; SCF ubiquitin ligase complex |
|                      | tae-miR9652-5p | 3 | 28 | 0 | 3.46E-02 | 0 | 18 | 10 | 2.63E-02 | retrograde transport |
|                      | tae-miR167c-5p | 681 | 793 | 595 | 4.30E-02 | 638 | 790 | 336 | 2.95E-05 | SCF ubiquitin ligase complex |
|                      | tae-miR9657a-3p | 37 | 124 | 91 | 6.02E-05 | 23 | 87 | 68 | 5.70E-05 | serine-type endopeptidase inhibitor activity; amino acid transport; WRKY |
|                      | tae-miR9655-3p | 21 | 1031 | 312 | 4.10E-07 | 0 | 1099 | 333 | 2.78E-07 | L-fucosidase; SCF ubiquitin ligase complex |
|                      | tae-miR9658-3p | 24 | 37 | 20 | 1.10E-02 | 19 | 53 | 15 | 4.54E-04 | SCF ubiquitin ligase complex; RPM1 |
| Relative abundance at later stage (RALS) | bdi-miR156h-3p_L+1 | 32 | 110 | 207 | 5.24E-04 | 23 | 103 | 219 | 9.19E-06 | WRKY transcription factor |
|                      | zma-miR156d-3p_L +1_1ss9TC | 5 | 50 | 114 | 5.71E-05 | 9 | 28 | 64 | 3.78E-06 | catalytic activity |
|                      | ato-miR167b-5p | 111 | 576 | 889 | 1.38E-06 | 124 | 634 | 648 | 4.41E-07 | SCF ubiquitin ligase complex |
|                      | tae-miR319 | 179 | 227 | 682 | 3.93E-06 | 130 | 199 | 1018 | 1.94E-06 | MYB transcription factor; DnaJ protein |
|                      | osa-miR827 | 34 | 174 | 1002 | 7.10E-09 | 41 | 566 | 682 | 2.09E-07 | SPX-domain-containing genes |
|                      | tae-miR408_L-1 | 28 | 207 | 213 | 6.71E-06 | 25 | 127 | 204 | 6.88E-05 | L-ascorbate oxidase; LPEAT1-like |
|                      | tae-miR9666a-3p | 0 | 439 | 4812 | 0.00E+00 | 0 | 492 | 911 | 3.68E-07 | Alternative splicing regulator; regulatory protein NPSR |
|                      | tae-miR9666b-3p | 3 | 213 | 674 | 4.59E-07 | 0 | 255 | 318 | 1.46E-07 | DNA-directed RNA polymerase II subunit 1 |
|                      | tae-miR9664–3p_L-1 | 15 | 29 | 55 | 4.78E-03 | 0 | 62 | 151 | 1.67E-07 | RPM1 protein; zinc finger CCCH domain-containing; glucosidase |
|                      | hvu-miR399 | 0 | 24 | 66 | 2.40E-05 | 0 | 54 | 62 | 2.18E-05 | NB-ARC domain; glucosyltransferase |
|                      | lus-miR159b_R+1_1ss21CT | 101 | 42 | 150 | 9.28E-04 | 78 | 22 | 179 | 2.10E-04 | MYB |
|                      | osa-miR319a-3p-2-3p_R+1 | 387 | 210 | 608 | 1.23E-05 | 507 | 193 | 537 | 3.02E-05 | SCF ubiquitin ligase complex; MYB |

*Abundance is indicated by normalized reads (reads per million of total miRNA reads (RPM))
*RPM \( \geq 20 \) in these libraries
*The values are the means of three biological replicates
*\( P \)-value < 0.05 (5.00E-02) means a significant difference among different developmental stages
### Table 4: Differentially expressed novel miRNAs associated with grain development and their target gene functions

| Grain filling stages | miRNA | Abundance (RPM) | P-value | Abundance (RPM) | P-value | Target function |
|----------------------|-------|----------------|---------|----------------|---------|-----------------|
|                      |       | LN-7 | LN-17 | LN-27 |                      |       |                      |
|                      |       | HN-7 | HN-17 | HN-27 |                      |       |                      |
| Relative abundance during grain filling stages (RAGS) | tae-miR2916-p5_2ss2AG17TG | 1070 | 3667 | 9557 | 2.49E-06 | 1610 | 2067 | 2868 | 1.52E-05 | protein serine/threonine kinase; glutathione S-transferase (GSTF1); DELLA |
|                      |       | tae-miR2916-p3_2ss2TC17CA | 2518 | 7110 | 27,736 | 5.92E-08 | 3507 | 4610 | 7049 | 1.66E-06 | Peroxidase; fructose-bisphosphate aldolase; GTPase |
|                      |       | tae-miR9655-p5 | 2936 | 24,657 | 11,329 | 2.48E-05 | 668 | 20,120 | 11,554 | 1.30E-09 | transcription factor; oxidation-reduction |
| Relative abundance at early stages (RAES) | tae-miR166d-p3 | 107 | 46 | 70 | 5.58E-03 | 83 | 56 | 80 | 1.97E-02 | cell differentiation; kinesin-1-like protein PSS1 |
|                      |       | PC-Sp-30718_164 | 39 | 7 | 0 | 6.33E-04 | 49 | 2 | 15 | 4.94E-05 | BRASSINOSTEROID INSENSITIVE 1; 1,3-beta-D-glucan synthase |
|                      |       | tae-miR815c-p5_2ss6CT21CT | 107 | 92 | 21 | 3.62E-04 | 121 | 61 | 51 | 4.76E-04 | UDP-arabinose 4-epimerase 1-like; transcription factor TGA2-like; DnaJ |
|                      |       | tae-miR9662b-p5 | 128 | 15 | 15 | 5.04E-07 | 153 | 31 | 18 | 4.56E-05 | beta-fructofuranosidase |
|                      |       | tae-miR9662a-p5 | 95 | 62 | 46 | 5.53E-04 | 112 | 77 | 67 | 3.96E-02 | beta-fructofuranosidase; |
|                      |       | tae-miR9672b-p5 | 125 | 33 | 0 | 1.57E-04 | 94 | 30 | 0 | 1.65E-03 | LRR receptor-like serine/threonine-protein kinase FLS2-like |
|                      |       | tae-miR9779-p3 | 244 | 40 | 11 | 1.15E-03 | 361 | 43 | 93 | 9.91E-04 | hexose transmembrane transport |
| Relative abundance at middle stages (RAMS) | tae-miR5384-p5 | 577 | 627 | 132 | 7.30E-06 | 579 | 686 | 489 | 1.07E-03 | UDP-glycosyltransferase 83A1-like; ethylene-responsive transcription factor 1B |
|                      |       | PC-Sp-3645_2157 | 33 | 175 | 148 | 1.88E-05 | 41 | 154 | 105 | 1.81E-05 | Metalloendopeptidase; SCF ubiquitin ligase |
|                      |       | PC-Sp-4780_1750 | 4 | 231 | 101 | 3.90E-05 | 0 | 135 | 104 | 1.09E-06 | aspartic endopeptidase; transcription factor |
|                      |       | PC-Sp-24289_247 | 2 | 23 | 3 | 1.20E-03 | 0 | 26 | 16 | 6.58E-04 | NEDD8-specific protease; protein EKERIFERUM 1-like |
|                      |       | PC-Sp-18073_397 | 0 | 66 | 26 | 3.34E-03 | 0 | 69 | 26 | 3.51E-06 | beta-glucosidase; BRASSINOSTEROID INSENSITIVE 1-like |
|                      |       | PC-Sp-21502_303 | 23 | 34 | 0 | 1.30E-03 | 38 | 30 | 25 | 1.03E-02 | Metalloendopeptidase; aspartic-type endopeptidase; MYB |
|                      |       | tae-miR164-p3 | 68 | 159 | 143 | 1.24E-03 | 57 | 165 | 131 | 3.76E-03 | auxin response factor 3-like; transcription factor IIB |
|                      |       | tae-miR164b-p3 | 16 | 62 | 18 | 1.71E-03 | 16 | 40 | 21 | 3.79E-03 | auxin response factor 2-like; high-affinity nitrate transporter 2.1-like |
|                      |       | tae-miR1137a-p5_2ss9GA20GC | 0 | 28 | 2 | 3.31E-02 | 0 | 13 | 2 | 1.77E-03 | DNA mismatch repair protein MSH6 |
|                      |       | tae-miR2275-p5 | 0 | 26 | 13 | 1.82E-04 | 0 | 28 | 2 | 1.66E-04 | LRR receptor-like serine/threonine-protein kinase FLS2; BRASSINOSTEROID INSENSITIVE 1 |
|                      |       | PC-Sp-21100_312 | 0 | 35 | 17 | 2.14E-03 | 0 | 27 | 9 | 2.75E-03 | glycerol-3-phosphate dehydrogenase; protein gar2 |
|                      |       | PC-Sp-15070_514 | 0 | 61 | 25 | 7.94E-04 | 0 | 44 | 41 | 2.64E-06 | transcription factor |
|                      |       | tae-miR9653b-p3 | 6 | 36 | 5 | 8.36E-03 | 20 | 21 | 0 | 5.81E-04 | protein binding |
|                      |       | PC-Sp-11233_746 | 38 | 65 | 9 | 6.74E-05 | 22 | 58 | 33 | 1.38E-02 | Metalloendopeptidase |
|                      |       | PC-Sp-16735_445 | 10 | 43 | 13 | 3.99E-06 | 0 | 38 | 23 | 1.72E-05 | O-acetyltransferase WSD1-like |
27 DAA. Additionally, ata-miR172b-5p had a higher expression level in the LN-27 treatment than in the HN-27 treatment. For the novel miRNAs, we found more differentially expressed miRNAs at the late developmental stages (LN-27 and HN-27). PC-5p-26413_215 had significantly higher expression in the LN treatment than in the HN treatment at the three grain developmental stages and potentially regulates BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 (BAK1) and LRR receptor-like serine/threonine-protein kinase. We found that 16 novel miRNAs were significantly upregulated in the HN-27 library compared to the LN-27 library, and these include two tae-miR2916 family members (tae-miR2916-p3_2ss12TC19AT and tae-miR9655-p5) that show fold-changes of 1.74–1.98 between LN-27 and HN-27. Both PC-3p-13925_573 and PC-3p-13909_573 were more abundant in LN-27 than in HN-27 and potentially function by targeting defense (peroxidase activity)- and transcription factor-encoding genes. PC-5p-36040_123 also showed higher expression in LN-27 compared to HN-27, and its predicted target gene product is disulfide isomerase, which indicates that it might be related to the regulation of grain storage and protein quality.

A network of the miRNAs that are differentially expressed between the HN and LN treatments was constructed on the Cytoscape platform v.3.6.0 (https://cytoscape.org) [36] (Additional file 7: Figure S1). The differentially expressed miRNAs have multiple potential functions, such as targeting carbohydrate metabolism, nitrogen metabolism, signal transduction, hormone responses, and defense. However, it is clear that eight miRNAs, tae-miR2916-p5_2ss2AG17TG, PC-3p-4066_99, tae-miR5386-p5, PC-5p-26413_215, ata-miR164a-5p, tae-miR9655-p5, PC-3p-21502_303, and sbi-miR399a, have the same target—genes encoding transcription factors. Apart from carbohydrate metabolism and signal transduction, five miRNAs, tae-miR2916-p3_2ss2TC17CA, PC-3p-15988_474, tae-miR530-5p_L + 1, tae-miR2592bj-p3_2ss12TC19AT, and ata-miR528-5p, were predicted to target genes that function in transport. We also noticed that tae-miR9664–3p_L-1 not only has multiple potential functions but also is linked to the transcription factor group and the transport group via the RPP13 and RPM1 proteins. The results of these analyses indicate that the differentially expressed miRNAs might respond to different nitrogen fertilizer conditions by regulating multiple metabolic pathways. Additionally, transcription factors and transport proteins may play important roles in the response to N supply status. We also constructed a model to show how the regulation of differentially expressed miRNAs is related to grain development and the response to N supply (Fig. 5).

**Table 4** Differentially expressed novel miRNAs associated with grain development and their target gene functions (Continued)

| Grain filling stages | miRNA | Abundance (RPM) | P-value | Abundance (RPM) | P-value | Target function |
|----------------------|-------|-----------------|---------|-----------------|---------|----------------|
|                      |       | LN-7 | LN-17 | LN-27 | HN-7 | HN-17 | HN-27 |
| PC-5p-12716_642      |       | 0    | 101   | 75    | 1.14E-04 | 0    | 111   | 79     | 6.00E-04 | MAPK cascade; BRASSINOSTEROID INSENSITIVE 1 |
| tae-miR6300-p5       |       | 23   | 312   | 268   | 4.86E-05 | 25   | 258   | 253   | 1.42E-05 | protein RPM1; response to nitrate |
| Relative abundance at later stages (RALS) | tae-miR2592bj-p3_2ss12TC19AT | 82   | 193   | 380   | 6.17E-04 | 114  | 217   | 135   | 4.57E-04 | protein serine/threonine phosphatase; transporter; protein RPM1-like |
| PC-5p-31845_154      |       | 0    | 8     | 21    | 4.35E-03 | 0    | 8     | 11    | 5.36E-03 | receptor-like serine/threonine-protein kinase SD1–6 |
| PC-5p-1231_4743      |       | 52   | 260   | 372   | 1.48E-06 | 40   | 339   | 453   | 1.35E-07 | serine-type endopeptidase |
| PC-5p-13909_573      |       | 0    | 47    | 66    | 6.93E-05 | 0    | 42    | 56    | 7.87E-05 | GTPase |
| PC-3p-13925_573      |       | 17   | 11    | 55    | 1.16E-06 | 5    | 9     | 87    | 6.72E-06 | serine-type carboxypeptidase; valine-RNA ligase |
| tae-miR408-p5_1ss20GA |       | –    | 16    | 17    | 5.81E-04 | –    | 16    | 22    | 9.12E-03 | BRASSINOSTEROID INSENSITIVE 1 |

*Abundance is represented by normalized reads (reads per million of total miRNA reads (RPM))

*RPM ≥ 20 in these libraries

*The values are the means of three biological replicates

*P-value < 0.05 (5.00E-02) means a significant difference among different developmental stages

Validation of identified miRNAs and their target genes

To confirm the miRNA expression in this study and to validate the deep sequencing results, we randomly selected four and five miRNAs (including one novel) from the LN and HN libraries, respectively, for quantitative real-time polymerase chain reaction (qRT-PCR) assays. The expression patterns of these miRNAs were similar to those obtained from deep sequencing, suggesting that...
the sRNA sequencing results obtained in this study are reliable (Fig. 6).

To verify the expression patterns of the potential miRNA targets, four target genes (the gene IDs and the primer sequences are given in Additional file 8: Table S7) were selected for gene-specific qRT-PCR under the HN and LN treatments (Fig. 7). The expression levels of PC-5p-3645_2157, tae-miR9655-p5, tae-miR9655-3p, and tae-miR319 were found to be upregulated with grain development, and their predicted target genes showed downregulated expression patterns. These results indicate that a negative relationship exists between target gene expression and their corresponding miRNA expression profiles.

**Discussion**

**Grain characteristics under different N treatments**

Nitrogen plays an important role in plant growth and agricultural production [24, 26], and N fertilization is an effective agronomic practice to increase wheat grain yield and improve grain processing quality [27, 28]. Here, HN treatment resulted in higher grain yields, protein content and dough development time, which is consistent with the results of previous reports [27, 28, 37].

| Library       | Known Up | Known Down | Known Total | Novel Up | Novel Down | Novel Total | Total Up | Total Down | Total Total |
|---------------|----------|------------|-------------|----------|------------|-------------|----------|------------|-------------|
| LN-7 vs. HN-7 | 2        | 4          | 6           | 5        | 0          | 5           | 7        | 4          | 11          |
| LN-17 vs. HN-17 | 0       | 6          | 6           | 2        | 1          | 3           | 2        | 7          | 9           |
| LN-27 vs. HN-27 | 5       | 10         | 15          | 8        | 16         | 24          | 13       | 26         | 39          |

**Table 5** Number of miRNAs that show differential expression between the HN and LN treatments
| Cluster | miRNA ID | Abundance (RPM) | P-value | Abundance (RPM) | P-value | Abundance (RPM) | P-value | Target function |
|---------|----------|----------------|---------|----------------|---------|----------------|---------|-----------------|
|         |          | LN7 | HN7 | LN17 | HN17 | LN27 | HN27 | LN7 | HN7 | LN17 | HN17 | LN27 | HN27 |
| Known  | bdi-miR156b-5p | 19 | 81 | 4.27E-02 | – | – | – | – | – | – | – | – | – |
|         | tae-miR159a-5p | 18 | 38 | 1.75E-02 | – | – | – | – | – | – | – | – | – |
|         | osa-miR159a.1_L + 1 | – | – | – | – | – | 41 | 85 | 1.39E-02 | MYB; WD40 repeat-like protein |
|         | at-a-miR164a-5p | – | – | – | – | – | 19 | 54 | 4.90E-02 | glutathione S-transferase; MAP kinase |
|         | at-miR169d-5p | – | – | – | – | – | 7 | 23 | 2.13E-02 | protein serine/threonine kinase; ABC transporter C family member 13 |
|         | at-miR169d-3p_L-2R + 2 | – | – | – | – | – | 27 | 92 | 8.22E-05 | galactinol--sucrose galactosyltransferase 2; E3 ubiquitin-protein ligase |
|         | at-miR172b-5p | – | – | – | – | – | 43 | 2 | 1.38E-03 | – |
|         | sbi-miR399a | 27 | 113 | 5.40E-03 | 9 | 109 | 1.05E-03 | – | – | Oxidoreductase; transcription factor |
|         | hvu-miR399 | – | – | 24 | 54 | 4.73E-03 | – | – | UDP-glucosyltransferase; transcription factor |
|         | at-miR528-5p | – | – | – | – | – | 3 | 23 | 2.20E-02 | lectin-rich repeat receptor kinase |
|         | osa-miR827 | – | – | 174 | 566 | 2.36E-05 | – | – | – |
|         | tae-miR9653a-3p | – | – | – | – | – | 181 | 0 | 9.46E-04 | – |
|         | tae-miR9659-3p | 55 | 0 | 6.03E-03 | – | – | – | – | – | – | – | – | – |
|         | tae-miR9661-5p_1ss14GA | – | – | – | – | – | 22 | 0 | 5.49E-03 | – |
|         | tae-miR9664--3p_L-1 | 15 | 0 | 2.00E-02 | 29 | 62 | 4.34E-03 | 55 | 151 | 2.29E-03 | RPM1; Gibberellin receptor; peptidyl-prolyl cis-trans isomerase; |
|         | tae-miR9666a-3p | – | – | – | – | – | 4812 | 911 | 1.38E-07 | Alternative splicing regulator; regulatory protein NPR5-like |
|         | tae-miR9666b-3p | – | – | – | – | – | 674 | 318 | 2.57E-04 | stress response |
|         | tae-miR9672a-3p_L + 2R-2 | – | – | – | – | – | 15 | 41 | 4.01E-02 | replication factor C subunit 1 |
|         | tae-miR9774_L + 2 | – | – | – | – | – | 107 | 42 | 1.12E-03 | – |
|         | tae-miR9777_L + 2 | – | – | – | – | – | 14 | 46 | 2.94E-02 | geraniol 8-hydroxylase-like; SCF ubiquitin ligase complex |
| Novel   | PC-3p-40666_99 | 32 | 0 | 1.06E-02 | – | – | – | – | – | – | transcription factor activity |
|         | PC-5p-11080_759 | 72 | 21 | 1.73E-02 | – | – | – | – | 0 | 30 | 3.60E-03 | – |
|         | PC-5p-26413_215 | 23 | 4 | 3.03E-02 | 23 | 9 | 4.43E-02 | 11 | 0 | 5.99E-03 | BRI 1-associated receptor kinase 1-like; LRR receptor-like serine/threonine-protein kinase |
|         | PC-3p-43479_87 | 24 | 5 | 4.85E-02 | – | – | – | – | – | – | oxidation-reduction process; transcription factor |
|         | PC-5p-16963_436 | – | – | 32 | 91 | 8.60E-04 | 0 | 74 | 1.78E-02 | – |
|         | tae-miR1137a-p3_1ss9CT | – | – | 19 | 25 | 1.92E-02 | – | – | – | peroxidase activity |
|         | PC-3p-15988_474 | – | – | – | – | – | 206 | 51 | 9.29E-04 | regulation of transcription |
|         | PC-5p-6533_1330 | – | – | – | – | – | 269 | 47 | 3.16E-03 | – |
|         | PC-3p-33976_137 | – | – | – | – | – | 25 | 0 | 3.29E-03 | RPM1 |
We also observed that the gluten (gliadin and glutenin) content (27.80 mg g\(^{-1}\)) increased more than did the albumin and globulin content (9.74 mg g\(^{-1}\)) under HN treatment, indicating different responses of the grain protein component to N fertilizer. According to the national standards of high-quality wheat [38], the grain under HN conditions had a high protein content, and its dough rheological characteristics (water absorption, dough development time and stability) met the standard for strong-gluten wheat, which confirms that N fertilizer applications can improve grain processing quality.

**miRNAs involved in wheat grain development**

A better understanding of the regulatory roles of miRNAs in grain development and the response to nitrogen application levels will provide new perspectives for improving grain yield and quality. Previous studies have identified several conserved miRNAs in wheat grain, such as miR159, miR160, miR164, and miR156 [12, 15]. In this study, we also identified several highly conserved miRNA families, such as miR156, miR396, miR160, miR172, and miR164, indicating that these miRNAs are abundant in wheat grain and potentially play important regulatory roles in grain development. The reproductive stage of wheat from flowering to maturity includes the grain formation and filling stages; the number of endosperm cells in wheat is directly related to grain yield and quality [39]. A rapid increase in the number of endosperm cells occurs at 6–9 DAA, and the final number is determined by 14–18 DAA [39]. Members of the osa-miR171a, ata-miR172c-3p, miR396 (ata-miR396e-5p, ata-miR396a-5p, and ata-miR396c-5p) and ata-miR5168-3p families were the most abundant miRNAs at 7 DAA, suggesting that these miRNAs might be involved in the early stages of grain development. Higher abundances of miR171a and miR172 members were also found in the early developing grain by Meng et al. [12]. Additionally, by analyzing the miRNA target gene GO terms, we identified 27 miRNAs with target gene products related to cell differentiation (Fig. 4), including osa-miR171a and.

![Table 6](image-url)
ata-miR172c-3p. It has been reported that miR172 members control floral organ identity in rice, maize, and barley by regulating the expression of APETALA2 (AP2)-like genes [40–42]. The floral and seed development of transgenic rice overexpressing miR172b were defective; the plants also presented relatively low spikelet fertility and reduced seed weight [42]. Here, the predicted target of ata-miR172c-3p is the CRF gene. CRFs compose a small subset of APETALA2/ethylene response factor (AP2/ERF) transcription factors that regulate plant development as part of the cytokinin signal transduction pathway [43], suggesting that ata-miR172c-3p might be involved in wheat seed development via cytokinin regulation. A positive correlation between cytokinin level during grain development and final grain weight has been reported in maize and wheat [44, 45]. Apart from cell differentiation, we also found that the target gene products of ata-miR172c-3p (DnaJ) and ata-miR172b-3p (chaperone binding) are involved in seed development and the response to heat stress (Fig. 4), which might indicate that they play important regulatory roles in determining wheat grain size and weight. Ma et al. [46] found that the miR171-SCL module plays a key role in mediating gibberellin (GA)-DELLA signaling in the coordinated regulation of leaf growth and chlorophyll biosynthesis in the light. We also predict that potential targets for osa-miR171a are genes that encode proteins such as the DELLA protein RG2-like, which is a regulator in the GA signaling pathway. It has been shown that miR396 members affect rice yield by regulating grain size [47], and the base substitution from TT to AA of the target genes can counteract the suppression by miR396, resulting in the activation of the hormone response and an increase in grain size [48, 49]. Schommer et al. [50] also found that the transcription factor TCP4, which is a direct regulator of miR396b, can repress cell proliferation by activating various pathways. Here, three miR396s (ata-miR396e-5p, ata-miR396a-5p, ata-miR396c-5p) were relatively abundant at 7 DAA, and the encoded proteins of the target genes function in response to GA, function as transcription factors, are GTPases, and act as protein kinases, suggesting that the regulation of these
Fig. 6 Verification of the expression patterns of nine miRNAs present in developing wheat grain. The different lowercase letters above the columns indicate significant differences ($P < 0.05$).
miRNAs might be related to grain size. All these results suggest that these miRNAs may play crucial roles in regulating grain endosperm cell development, which is directly related to grain size. However, additional research is needed to explore the target genes and their functions.

Grain filling is an important process in kernel development and ultimately determines kernel weight and flour quality. It has been suggested that 14–18 DAA is a major transition point for the formation of the wheat starchy endosperm and essentially marks the end of endosperm cell division and the deposition of starch and gluten protein [39]. Meng et al. [12] identified some miRNAs that might be involved in the control of grain filling; these miRNAs are involved in various biological processes by targeting many different wheat genes, such as those involved in carbohydrate metabolism, protein metabolism, transport, and transcription. In our study, we identified several conserved miRNAs and novel miRNAs that not only were highly abundant in wheat grain but also were significantly up- or downregulated during grain development; examples include tae-miR160 and ata-miR166a-3p. The gene products of the potential gene targets of these miRNAs include transcription factors, auxin response factors (ARFs), serine carboxypeptidases, kinesin-1-like protein PSS1, and proteins involved in transport. It is well known that miR160 directs the cleavage of several ARF mRNAs to regulate plant growth and seed germination [51, 52]. Expression of a miR160-resistant version of ARF17 in plants causes dramatic developmental defects, and overexpression of miR160 results in fewer starch granules and disorganized root tips [52, 53]. Analysis of the conservation and diversification of the miR166 family in plants showed that miR166 members play a wide and important regulatory role in seed development [54]. Therefore, these data suggest that these miRNAs have multiple regulatory functions in grain filling. In this study, we also identified some significantly upregulated novel miRNAs (such as ae-miR5384-p5, PC-5p-3645_2157, PC-3p-4780_1750, PC-5p-24289_247, PC-5p-18073_397, and PC-3p-21502_303) in the middle stage of grain development (17 DAA). The predicted proteins encoded by the targeted genes include UDP-glycosyltransferase, NEDD8-specific protease, metalloendopeptidase, and glucosidase, which...
indicate that these upregulated miRNAs might be related to carbohydrate and nitrogen metabolism, which would affect starch deposition and protein accumulation.

Some miRNAs, such as tae-miR319, hvu-miR399, and miR9666, showed little or no expression during the early stage (7 DAA) but then became upregulated as grain development progressed, and these miRNAs were most abundant at 27 DAA. The potential encoded proteins of the genes targeted by these miRNAs include MYBs, DnaJ, NB-ARC domain-containing proteins, and alternative splicing regulators. miR319 is a conserved microRNA that regulates TCP transcription factors involved in various developmental pathways, such as hormone biosynthesis, signaling, and leaf development and senescence [55, 56]. We also predicted that one of the potential targets of miR319 in the response to heat stress is the gene encoding the DnaJ protein. High-temperature stress at grain filling is a major problem in the production of high-quality wheat, and further exploration of the internal mechanism of these miRNAs in response to heat stress would be a potential way to reduce the effects of high-temperature stress.

Grain quality is a complex trait and includes not only protein content but also wheat gluten polymer structure [57]. Chen et al. [58] reported that cysteine and methionine metabolism pathways have an important role in storage protein biosynthesis via the synthesis of sulfur-containing amino acids. Here, KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis showed that the target genes of bdi-miR531_L-4R + 1_1ssSCT and ata-miR396e-5p were annotated as involved in cysteine and methionine metabolism. The formation of disulfide (SS) bonds regulated by protein disulfide isomerase is a key factor that determines gluten protein polymerization [57, 59]. We identified one novel miRNA, PC-5p-36040_123, which is predicted to target protein disulfide isomerase, that was significantly downregulated at HN-27. The downregulated expression under high nitrogen treatment is predicted to cause an increase in disulfide isomerase activity and to promote grain protein polymer formation. In addition to SS bonds, hydrogen or electrostatic bonds or other noncovalent bonds also participate in the formation of gluten protein polymers [57]. Additional information concerning the regulatory function of the identified miRNAs and their relationship to the protein polymer structures in grain and flour is needed.

**Differentially expressed grain miRNAs in response to nitrogen application levels**

Many miRNAs have been identified that respond to nutrients and abiotic stress, such as salt and drought stress [60]. In this study, we identified 50 miRNAs (including 28 novel ones) that showed significant differential expression between HN and LN treatments. The expression of miR399 is upregulated by phosphorus (P) deficiency [61] and is related to P homeostasis by regulating the expression level of the target PHO2 gene, which encodes a ubiquitin-conjugating E2 enzyme, UBC24 [62]. Baek et al. [63] also found that AtMYB2, a transcription factor, can regulate miR399 expression levels in response to P starvation. Similarly, Zhao et al. [23] reported that Tamir399 was downregulated under conditions of N deficiency. In our study, we found that the expression of two miR399s (sbi-miR399a and hvu-miR399) was significantly downregulated in the LN treatment. The two miRNAs are involved in regulating target genes encoding transcription factors, oxidoreductase (defense response), and UDP-glucosyltransferase, suggesting that these miRNAs possibly mediate signal transduction in response to low N by repressing translation.

Arabidopsis miR169 has been shown to be involved in adaptation to LN stress [64]. Zhao et al. [19] also reported that two novel miR169 species (miRC10 and miRC68) critically regulate the adaptation to LN in maize seedlings via their interaction with the mRNA that targets a nuclear transcription factor subunit. The strong downregulation of miR169 in Arabidopsis under LN affects the expression of its targets, NFXA (Nuclear Factor X, subunit A) family members involved in the ABA-dependent pathway [65]. We found that two miR169s (ata-miR169d-3p_L-2R + 2 and ata-miR169d-5p) had significantly lower expression in LN-27 than in HN-27, and the predicted target gene functions included kinase activity and E3 ubiquitin-protein ligase activity. It was previously reported that Arabidopsis MIEL1 E3 ligase negatively regulates ABA signaling sensitivity by promoting MYB96 turnover [66]. Thus, the regulation of the grain response to N supply levels by miR169 might involve the ABA signaling pathway.

Previous studies have shown that miR827 responds strongly to P starvation by targeting genes encoding SPX-MFS proteins predicted to be implicated in P sensing or transport [67]. There is crosstalk between nitrogen and P sensing in Arabidopsis [68], and overexpression of miR827, which targets nitrogen limitation adaption (NLA) mRNA, in Arabidopsis also resulted in similar physiological responses in both NLA and PHO2 (PHOSPHATE2-ubiquitin conjugase) mutants grown under either low nitrate or high P [68]. Fertilizer experiments also confirmed the interaction between N and P fertilizers, and the combination of N and P improved wheat grain yield and quality [69]. Here, we identified one osa-miR827, which is predicted to target a gene encoding an SPX-domain-containing protein that showed significant downregulation in LN-17. Liu et al. [70] found that NLA responded to the remobilization of nitrate between sources and sinks in Arabidopsis by regulating the degradation of NRT1.7, which depended on miR827. This finding suggests that miR827...
might influence N accumulation and remobilization by regulating NLA. Additionally, the mode of action occurs via a posttranslational regulatory pathway, not at the level of transcription [71]. For all the identified differentially expressed miRNAs in our study, only one, PC-5p-26413_215, was significantly upregulated in the LN treatment at all three stages. The target gene of PC-5p-26413_215 is predicted to encode BAK1. It is well known that brassinosteroids (BRs) control various aspects of plant development and growth and that BR perception and signal transduction require active BRASSINOSTEROID INSENSITIVE 1 (BRI) and BAK1 [71]. However, additional work is needed to confirm the function of the target genes and the regulatory mechanism of this miRNA.

Nitrogen is an essential inorganic nutrient for wheat growth and plays many important roles in grain development. In this study, we found that 21 novel miRNAs were significantly up- or downregulated only in the LN-27 treatment (examples are PC-5p-6533_1330, PC-3p-15988_474, tae-mir9779-p3, PC-3p-11233_746, and tae-miR5384-p3) in comparison with HN-27, and they are predicted to target multiple genes involved in defense (peroxidase), encoding transcription factors (MYB), involved in nitrogen metabolism (metalloendopeptidase), involved in carbohydrate metabolism (UDP-glycosyltransferase), and involved in transport (hexose transport). Different expression patterns between the LN and HN treatments have also been reported by Zhao et al. [23]. Thus, the differentially expressed miRNAs and their target genes suggest that there are multiple N-responsive modules in wheat grain and that miRNAs may mediate the plant responses and adaptation to N deprivation via posttranscriptional or posttranslational regulation.

Conclusions
We identified 79 miRNAs (46 known and 33 novel miRNAs) that showed significant differential expression during wheat grain development under both HN and LN treatments. We also identified 50 miRNAs (22 known and 28 novel miRNAs) that responded to the level of applied nitrogen. The potential targets of these miRNAs are involved in multiple biological processes, including cell differentiation, carbohydrate metabolism, transcription, signal transduction, transport, and defense. Our results indicate that posttranscriptional regulation by miRNA-mediated networks can play a crucial role in grain development and the N response, which determine wheat grain weight and quality. Our work provides useful information for further exploration of the mechanisms by which miRNAs function to improve grain yield and quality.

Methods

Experimental design and sample preparation
Seeds of the winter wheat cultivar ‘ZM119’, provided by Henan Academy of Agricultural Sciences, were grown during the 2016–2017 growing season at the Scientific and Educational Park of Henan Agricultural University, Yuanyang, China (35°06’ N, 113°56’ E). The cultivar ‘ZM119’ was bred by Henan Academy of Agricultural Sciences, and approved by Henan Province Crop Variety Approval Committee in 2014. The organic matter content in the topsoil was 10.1 g kg$^{-1}$, and the available phosphorus and available potassium contents were 35.0 mg kg$^{-1}$ and 110.6 mg kg$^{-1}$, respectively. Two N fertilizer treatments with three replicates each were applied: LN (0 kg ha$^{-1}$) and HN (210 kg ha$^{-1}$). The area of each plot was 21 m$^2$ (3 m × 7 m), and each plot received 315 g of P (P$_2$O$_5$) and 315 g of K (K$_2$O) before sowing. Fifty percent of the total N (in the form of urea) was applied before sowing, and 50% was topdressed at the elongation stage. Seeds were planted on 14 October 2016 at a density of 200 seeds m$^{-2}$. At flowering, wheat spikes having a similar size and flowering on the same day were marked. The wheat spikes were sampled at 7, 17, and 27 DAA. The sampled grain was immediately frozen in liquid nitrogen and then stored in an ultra-low-temperature refrigerator until use. Afterward, mature grain was ground in a Cyclotec Sample Mill (Foss Tecator AB, Höganas, Sweden) after cleaning and removing the foreign grain.

Small RNA library construction and RNA sequencing
To construct eighteen sRNA libraries, six independent samples, HN-7, HN-17, and HN-27 as well as LN-7, LN-17, and LN-27 (grain sampled at 7, 17, and 27 DAA under HN and LN conditions, respectively) with three biological replicates were separately extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The RNA quantity and quality were assessed using a Bioanalyzer 2100 and an RNA 6000 Nano LabChip Kit (Agilent Technologies, Santa Clara, CA, USA). RNA preparations with a RIN (RNA integrity number) > 7.0 were used for sRNA library construction. Small RNAs were first ligated to 5’-RNA/DNA chimeric oligonucleotide adaptors (Illumina) and then 3’-adaptor to generate ligation products. cDNAs were then produced by reverse transcribing the ligation products. The cDNAs were sequenced using an Illumina HiSeq 2500 instrument (Illumina, San Diego, CA, USA) according to the manufacturer’s recommended protocol.

Bioinformatic analysis and miRNA identification
The raw sequencing reads in the miRNA libraries were subjected to an Illumina pipeline filter (Solexa 0.3), and then an in-house program, ACGT101-miR (LC Sciences, Houston, TX, United States), was used to process the dataset for removing adaptor dimers, common RNA family members, repeats and low-complexity reads. Unique sRNA sequences between 18 and 25 nucleotides...
in length were subsequently mapped to specific species precursors in miRBase 21.0 (http://www.mirbase.org) using BLAST searches to identify conserved miRNAs and novel 5p- and 3p-derived miRNAs [72]. Internal mismatches in the sequence and length variations at the 3’ and 5’ ends were allowed in the alignment process. The unique sequences that mapped to specific mature miRNA species in the hairpin arms were characterized as known miRNAs. A potential novel 5p- or 3p-derived miRNA was identified when unique sequences were mapped to the other arm of known specific species of precursor hairpins, opposite to the annotated mature miRNA arm. The remaining sequences were further matched to other selected species precursors in miRBase 21.0 by BLAST searches, and the mapped pre-miRNAs were further used as BLAST queries to identify their genomic locations by searching the genomes of specific species. The results of the above two searches were classified as known miRNAs. Finally, unmapped sequences were queried via BLAST against the specific genomes, and hairpin RNA structures containing the sequences from the flanking 120-nt sequences were predicted using RNAfold software (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). The key criteria for miRNA prediction are reported in the literature [73]. The secondary structures of precursors were also predicted using RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). The raw data have been submitted to the Sequence Read Archive (SRA) at the NCBI (https://www.ncbi.nlm.nih.gov) under accession number PRJNA563099.

Identification of differentially expressed miRNAs
The expression levels were normalized as RPM values. A t-test was applied to evaluate statistical significance. If the RPM ratios between different treatments were greater than 2 (fold change ≥ 2) and if the P-value was ≤ 0.05, then the miRNAs were defined as differentially expressed miRNAs.

miRNA target prediction and annotation
The genes targeted by miRNAs were predicted by identifying miRNA-binding sites using computational target prediction algorithms (Target Finder). Target searching was performed using the Triticum aestivum L. IWGSC library (http://plants.ensembl.org/index.html). Potential miRNA targets were functionally annotated by querying the target sequence via BLAST against the NCBI library (https://www.ncbi.nlm.nih.gov/). The most abundant miRNA targets were also annotated with GO terms (http://www.geneontology.org/) and KEGG pathways (http://www.genome.jp/kegg/).

miRNA and target mRNA quantification using qRT-PCR
Reverse transcription reactions were carried out using miRNA First-Strand cDNA Synthesis SuperMix (TransScript) according to the manufacturer’s instructions. A SYBR PrimeScript miRNA RT-PCR Kit was used to perform qRT-PCR in a fluorescence detection system (TianGen Biotech, Beijing, China) following the manufacturer’s instructions. All reactions were performed in triplicate for each sample. The 2^ΔΔCt method was then used to calculate the relative expression levels of the miRNAs [74]. Target genes of the miRNAs that were differentially expressed during grain development in the HN and LN treatments were selected to validate their expression patterns in developing wheat grain via qRT-PCR. Fisher’s least significant difference test (LSD) was subsequently used to distinguish differences in relative expression levels between different development stages using SPSS (Statistical Program for Social Science) software; P < 0.05 was considered statistically significant. All primer sequences used and the target gene identification results are given in Table S7 (Additional file 8).

Determination of grain total protein content and the individual fraction contents
Four grain protein fractions, albumins, globulins, gliadins, and glutenins, were extracted using the sequential extraction methods of Liu et al. [75]. The total protein content and the individual fraction contents in each sample were analyzed by an automatic Kjeldahl analyzer (Kjeltec 2300, Foss Tecator, Sweden) following the method of ICC (1994) Standard Method 105/2 (International Association for Cereal Chemistry, Vienna, Austria). Differences between HN and LN were evaluated with t-test via SPSS; A value of P < 0.05 was considered to be statistically significant.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12870-020-2296-7.

Additional file 1: Table S1. Illumina DNA sequencing data for the small RNA libraries.
Additional file 2: Table S2. miRNAs identified in this study.
Additional file 3: Table S3. Novel miRNAs identified in developing wheat grains at two different N levels.
Additional file 4: Table S4. Conserved and novel miRNAs that are differentially expressed during wheat grain development.
Additional file 5: Table S5. Target function of differentially expressed miRNAs during grain filling under HN and LN treatments.
Additional file 6: Table S6. Wheat grain miRNAs that are differentially expressed between the HN and LN treatments and their predicted targets as determined by Cytoescape.
Additional file 7: Figure S1. Relationships between differentially-expressed miRNAs identified between the HN and LN treatments and their predicted targets.
Additional file 8: Table S7. Oligonucleotide primers used for qRT-PCR assays in this study.
Abbreviations

ABA: Abscisic acid; ARF: Auxin response factor; BAK1: BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1; BRI: BRASSINOSTEROID INSENSITIVE 1; CBF: Cytokinin response factor; DIA: Days after anthesis; GA: Gibberellin; GO: Gene ontology; GSTF: Glutathione S-transferase; HN: High nitrogen treatment; HSP: Heat-shock protein; LN: Low nitrogen treatment; LRR: Leucine-rich repeat; miRNA: microRNA; N: Nitrogen; NLA: Nitrogen limitation adaption; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction; SCF: Skp-cullin-F-box; SPXSYK1/PHO81/XPRI; sRNA: Small RNA

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Authors’ contributions

CYW and YXX helped design the experiments; DYM designed the experiments and revised the manuscript. GGH carried out the experiments and wrote the draft. CYD and HHK performed the qPCR and prepared some of the figures. HFL and JK carried out the field trials and sampling. WS and SJA performed the grain quality analyses. All authors have contributed to the corrections and approval of the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files. The sequencing raw reads were submitted to the NCBI database with the accession number PRJNA563099 (https://www.ncbi.nlm.nih.gov).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

1. College of Agronomy/National Engineering Research Center for Wheat, Henan Agricultural University, Zhengzhou 450002, China. 2. The National Key Laboratory of Wheat and Maize Crop Science, Henan Agricultural University, Zhengzhou 450002, China.

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