Integrated microRNA and transcriptome profiling reveal key miRNA-mRNA interaction pairs associated with seed development in Tartary buckwheat (*Fagopyrum tataricum*)

Hongyou Li1*, Hengling Meng2, Xiaojian Sun1,3, Jiao Deng1, Taoxiong Shi1, Liwei Zhu1, Qiuyu Lv4 and Qingfu Chen1*

**Abstract**

**Background:** Tartary buckwheat seed development is an extremely complex process involving many gene regulatory pathways. MicroRNAs (miRNAs) have been identified as the important negative regulators of gene expression and performed crucial regulatory roles in various plant biological processes. However, whether miRNAs participate in Tartary buckwheat seed development remains unexplored.

**Results:** In this study, we first identified 26 miRNA biosynthesis genes in the Tartary buckwheat genome and described their phylogeny and expression profiling. Then we performed small RNA (sRNA) sequencing for Tartary buckwheat seeds at three developmental stages to identify the miRNAs associated with seed development. In total, 230 miRNAs, including 101 conserved and 129 novel miRNAs, were first identified in Tartary buckwheat, and 3268 target genes were successfully predicted. Among these miRNAs, 76 exhibited differential expression during seed development, and 1534 target genes which correspond to 74 differentially expressed miRNAs (DEMs) were identified. Based on integrated analysis of DEMs and their targets expression, 65 miRNA-mRNA interaction pairs (25 DEMs corresponding to 65 target genes) were identified that exhibited significantly opposite expression during Tartary buckwheat seed development, and 6 of the miRNA-mRNA pairs were further verified by quantitative real-time polymerase chain reaction (qRT-PCR) and ligase-mediated rapid amplification of 5′cDNA ends (5′-RLM-RACE). Functional annotation of the 65 target mRNAs showed that 56 miRNA-mRNA interaction pairs major involved in cell differentiation and proliferation, cell elongation, hormones response, organogenesis, embryo and endosperm development, seed size, mineral elements transport, and flavonoid biosynthesis, which indicated that they are the key miRNA-mRNA pairs for Tartary buckwheat seed development.

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Background
MicroRNAs (miRNAs), a class of endogenous non-coding small RNAs (sRNAs) that are 20–24 nt in length, play crucial regulatory functions in animals and plants by repressing their target genes expression at the transcription or post-transcription level [1–4]. In plants, the biosynthesis of miRNAs is a multistep pathway that is involved in many genes and enzymes [5]. Plant miRNA genes are initially transcribed into primary miRNAs (pri-miRNAs) by RNA polymerase II in the nucleus [5, 6]. Then, the pri-miRNAs are processed into miRNA precursors (pre-miRNAs) with stem-loop structures by the Dicer-like1 (DCL1), Hypostatic leaves1 (HYL1), and serrate (SE) protein complex [5–7]. Next, these pri-miRNAs are further cleaved into miRNA* duplexes under the action of DCL1, HYL1 and SE protein complex [5]. After methylation, the duplexes are methylated by the methyltransferase HUA enhancer1 (HEN1) [5]. Finally, the miRNA duplex is bound by Argonaute1 (AGO1) to form the RNA-induced silencing complex (RISC) to carry out its function by either cleaving target mRNAs or repressing the translation process [5, 9]. At present, miRNAs have been identified in numerous plants, and more and more evidence indicates that they play crucial roles in plant growth and development, secondary metabolism, biotic and abiotic stress tolerance, and signal transduction [4, 10–16].

Seed is the reproductive and the primary nutrient storage organ in many crop plants, and its developmental success or failure directly determines the final crop yield and seed quality, as well as whether genetic information can be successfully transmitted to the next generation [4]. Crop seed development is a complex biological process that involves many gene regulatory pathways [17, 18]. An increasing body of evidence shows that miRNAs participate in the regulation of seed development in crop plants. To date, thousands of miRNAs have been identified in the development of seed in multiple crop plants including rice [19, 20], maize [21, 22], wheat [23, 24], barley [25, 26], soybean [27, 28], peanut [18], Brassica napus [29, 30], narrow-leaved lupin [6], and common buckwheat [4] by using a high-throughput sequencing approach. These studies found that the expression of various miRNAs is extremely dynamic during seed development and some miRNAs are specifically expressed in seed, implying that miRNAs have very vital regulatory roles in seed development. In fact, a few miRNAs have been functionally demonstrated to play crucial regulatory roles in the seed development of model crop rice and a few other crops by negatively regulating their target genes expression. For example, miR159, miR160, miR397, miR398, and miR408 positively regulate rice grain size [31–35], while miR1432, miR156, miR167, miR396c, miR396e, and miR396f, and miR530 have opposite roles in regulating the grain size [35–41]. Notably, the conserved role of miR408 and miR160 in regulating seed size was also found in Arabidopsis and tobacco, and cotton, respectively [42, 43]. In addition to the role in seed size, miRNAs were also demonstrated to regulate nutrient accumulation in developing seed [4]. For instance, miR160 positively regulate starch accumulation in rice seed [4, 32]. EgmiR5179 and csa-miR167A regulate the oil and linolenic acid biosynthesis in oil palm and Camelina sativa seeds, respectively [44, 45].

Tartary buckwheat (Fagopyrum tataricum) is an annual medicinal and edible crop, belonging to the eudicot family Polygonaceae [46]. It is widely cultivated in Asia and Eastern Europe, especially in the mountainous areas of Southwest China [46]. Tartary buckwheat seed is a good source of nutrients including starch, protein, dietary fiber, fatty acid (linoleic acid), and various minerals [46]. Importantly, Tartary buckwheat seed also contains rich flavonoids especially rutin, which have been proved to be effective in preventing liver injury and especially inflammatory liver injury [43, 46]. Therefore, it is of great significance to understand the molecular mechanism of Tartary buckwheat seed development, which will be helpful in high-yield and quality breeding of Tartary buckwheat [46]. To date, several transcriptome analyses have been reported that examine the molecular mechanism of Tartary buckwheat seed development [4, 43, 46–48]. However, to our knowledge, no one has studied miRNAs in Tartary buckwheat, and whether and how to regulate the development of Tartary buckwheat seed is largely unclear. In this study, we first examined the conserved evolution of miRNA biosynthesis...
in Tartary buckwheat compared to other plants through homology identification and phylogenetic analysis of miRNA biosynthesis genes. Then, we identified the known and novel miRNAs in the developing seed of Tartary buckwheat and predicted their target genes. Finally, we performed integration analysis between miRNA and mRNA expression to insight into the miRNA-mediated molecular mechanisms of Tartary buckwheat seed development and identified key miRNA-mRNA interaction pairs for Tartary buckwheat seed development. Our results provide valuable information for enhancing the understanding of the miRNA-mediated regulatory mechanism of Tartary buckwheat seed development and aid in the Tartary buckwheat seed improvement.

Results
Identification, phylogeny, and expression profiles analysis of the miRNA biosynthesis genes in Tartary buckwheat

RDR, DCL, HYL, SE, HEN, HST and AGO genes have been demonstrated to play essential roles in plant miRNA biosynthesis [5]. As the first and most important step in studying the miRNA in Tartary buckwheat, we identified the orthologs of these genes in Tartary buckwheat. A total of 8 RDR, 4 DCL, 1 HYL, 2 SE, 1 HEN, 2 HST, and 8 AGO genes were identified in the Tartary buckwheat genome (Additional file 1: Table S1), respectively. Phylogenetic analysis revealed that plant RDR proteins can be divided into four clades, as previously defined by Qian et al. [49] (Fig. 1). Among the 8 FtRDR proteins, 3, 1, 3, and 1 belong to clades 1, 2, 3, and 4, respectively. In addition, the FtRDR proteins were closely homologous to A. thaliana RDR proteins. The DCL proteins can also be classed into four groups, and 4 FtDCL proteins showed a one-to-one counterpart with A. thaliana DCL proteins (Fig. 1). Similarly, the AGO proteins can be separated into four clades, but clade 4 only contained the grass AGO proteins as defined by Zhang et al. [50] (Fig. 1). For Tartary buckwheat AGO proteins, clade 1 was the biggest clade, which contained 6 FtAGO proteins. In contrast, both clades 1 and 2 only contained 1 FtAGO protein (Fig. 1).

RNA-seq data were further used to investigate the expression profiles of the 26 identified genes in roots, stems, leaves, flowers, and during seed development. As shown in Fig. 2, most RDR genes (6) had lower expression in all tissues. In contrast, 4 genes (FtAGO1, FtAGO2, FtAGO6, and FtSE1) had constitutive high expression in all tissues. The remaining 16 genes were expressed at a moderate level. Among them, 4 genes (FtHYL1, FtHST2, FtAGO4, and FtAGO8) exhibited specifically high expression in seeds. Furthermore, 6 genes (FtDCL1, FtDCL3, FtDCL4, FtAGO2, FtAGO3, and FtRDR4) showed significant differential expression (|log2(fold change)| > 1 and FDR value < 0.05) during seed development (Fig. 2). The 6 differentially expressed genes (DEGs) showed three expression patterns in the developing Tartary buckwheat seed (1) FtAGO2 and FtAGO3 were down-regulated only at the initial maturity stage (S3), (2) FtDCL1 and FtRDR4 showed a sustained decrease during seed development, and (3) FtDCL3 and FtDCL4 were up-regulated at the peak filling stage (S2) and down-regulated at the initial maturity stage (S3) (Fig. 2).

Sequencing of Tartary buckwheat seed sRNAs

To investigate the effects of miRNAs on seed development in Tartary buckwheat, we constructed and sequenced six sRNA libraries from seeds at three different developmental stages (Fig. 3). A total of 120.63 million raw reads were generated in six sRNA libraries (Additional file 1: Table S2). After filtration, 13.89 million, 17.09 million, 17.19 million, 14.54 million, 13.64 million, and 12.00 million clean reads were obtained for the six libraries, respectively (Additional file 1: Table S2). The length distribution displayed most the small RNA reads were 21–24 nt (Additional file 2: Figure S1). Among them, the 24 nt small RNA was the most abundant type and showed differential accumulation during Tartary buckwheat seed development (Additional file 2: Figure S1). To further obtain sRNA reads containing miRNAs, non-coding RNAs (including rRNAs, tRNAs, snRNAs, and snoRNAs) and repeat sequences were removed by comparing the clean reads to the Silv, GrRNAdb, Rfam, and Repbase databases. After discarding the non-coding RNAs and repeat sequences, a total of 10.45 million, 14.16 million, 14.60 million, 13.05 million, 6.80 million, and 5.63 million unannotated sRNA reads containing miRNAs were obtained for the six libraries, respectively (Additional file 1: Table S2). Among these unannotated clean reads, a total of 61.94, 66.76, 46.98, 39.20, 37.22, and 39.42% reads were mapped to the Tartary buckwheat reference genome, respectively (Additional file 1: Table S2).

Identification of known and novel miRNAs

To identify known miRNAs in the developing Tartary buckwheat seed, the mapped sRNA reads were subjected to Blastn search against miRBase v21.0. In total, 101 conserved miRNAs, belonging to 25 known miRNA families, were identified (Additional file 1: Table S3). Of these families, 17 families contained more than two members, three families (MiR390, MiR399, and MiR858) contained two members, and the remaining five families (MiR159, MiR394, MiR397, MiR828, and MiR845) only contained one member. The length of these known miRNAs ranged from 19 to 22 nt, and the numbers of miRNAs with length of 19, 20, 21, and 22 nt were 8, 29, 56, and 8, respectively (Additional file 1: Table S3). Among
these identified conserved miRNAs, 29 miRNAs were highly expressed, which have more than 1000 read counts at least in one library (Additional file 1: Table S3). The rest of miRNAs were expressed at a moderate level (36) or low level (36) which have < 10 read counts for each library, respectively (Additional file 1: Table S3). Notably, the highest expression miRNA was fta-miR159, and read counts ranged from 14,806 to 95,396 for each library.

To further identify novel miRNAs in the developing Tartary buckwheat seed, the remaining sRNA sequences that were not mapped to the Tartary buckwheat reference genome were analyzed using the miRdeep2 program. As a result, a total of 129 novel miRNAs were identified (Additional file 1: Table S4). The length of these novel miRNAs ranged from 18 nt to 24 nt, and the minimum free energy (AMFE) distribution ranged from $-116.2 \text{ kcal mol}^{-1}$ to $-26.2 \text{ kcal mol}^{-1}$ (Additional file
1: Table S4). Among these novel miRNAs, 66 miRNAs were assigned to 35 known miRNA families in the miRNA database, while the remaining 63 miRNAs had no similarity to any known family. Like the above identified conserved miRNAs, more than two-thirds of the novel miRNAs were expressed at a moderate or high level, and the highest expression was fta_novel_miR71 (Additional file 1: Table S4).

miRNAs target prediction and functional analysis
To understand the potential function of these identified miRNAs, the putative target genes of the 230 miRNAs were predicted using the TargetFinder software. A total of 3268 potential target genes were successfully predicted for among 213 miRNAs, including 2052 target genes from 101 known miRNAs and 1372 target genes from 112 novel miRNAs (Additional file 1: Table S5). These predicted target genes mainly encoded transcription factors (TFs, 285), protein kinases (254), phosphatase, E3 ubiquitin-protein ligase, proteins in hormone signal transduction and other cellular processes, and enzymes in various metabolisms (Additional file 1: Table S6). The main target TFs of these miRNAs are displayed in Additional file 1: Table S7, and the top three TFs were MYB (65), AP2/ERF (18), and NAC (18), respectively. Consistent with the results of previous studies, some conserved miRNAs targeted the known TFs. For instance,
miR156, miR160, miR164, miR171, miR172, miR319, miR396, and miR858 targeted SPL, ARF, NAC, GRAS, AP2-ERF, TCP, GRF, and MYB TFs, respectively (Additional file 1: Table S7). In addition, based on a homologous search of the putative target genes, the predicted target genes of 61 miRNAs, including 39 conserved and 22 novel miRNAs, were found to be the homologous genes of 47 known seed or organ size genes (Additional file 1: Table S8). Among these, the 39 conserved miRNAs were from 14 known miRNA families including MiR156/157 (10), MiR159 (1), MiR162 (1), MiR164 (1), MiR166 (2), MiR167 (1), MiR168 (4), MiR169 (1), MiR172 (5), MiR319 (2), MiR390 (2), MiR395 (2), MiR396 (4), and MiR530 (3). Notably, among these 61 miRNAs, most miRNAs were first found to target the known seed or organ size genes, except the members of the MiR156/157 and MiR396 families which are well known to target the seed size SPL and GRF TFs, respectively. Furthermore, based on a homologous search, 8 miRNAs (4 known and 4 novel) were identified to target the structural or regulatory genes of flavonoid biosynthesis (Additional file 1: Table S9). Among these, fta_novel_miR26 targeted phenylalanine ammonia-lyase (PAL), fta_novel_miR74 targeted 4-coumarate-CoA ligase (4CL), fta-miR394a-5p targeted flavonol synthase (FLS), fta_novel_miR1 targeted the homologous genes of AtMYB123 that regulate anthocyanindins biosynthesis, and fta-miR828-5p targeted the homologous genes of AtMYB75/90/113/114 that regulate anthocyanindins biosynthesis. In addition, fta-miR858a-5p and fta-miR858b-3p targeted 13 and 14 MYB TFs involved in the regulation of flavonol biosynthesis and anthocyanindins biosynthesis, respectively.

DEM in the development Tartary buckwheat seed and their targets

To identify Tartary buckwheat seed development-associated miRNAs and understand their potential regulatory mechanisms, the DEMs were identified by comparing the TPM expression value. A total of 76 miRNAs, including 39 conserved and 37 novel miRNAs, displayed significant differential expression (Fig. 4). Among these, 45, 43, and 22 miRNAs were found in the comparisons between S1 and S2, S1 and S3, and S2 and S3, respectively (Fig. 4a). When compared with S1, 1 miRNA (fta_novel_miR110) was differentially expressed in both S2 and S3 stages, and 22 and 17 miRNAs were specifically differentially expressed in the S2 and S3 stage, respectively (Fig. 4a). When compared with S2, 4 miRNAs were specifically differential expression in S3 stage (Fig. 4a). In addition, between stages S1 and S2, 19 and 26 miRNAs were up- or down-regulated, respectively (Fig. 4b). In the S1 vs. S3 comparison, 23 up-regulated and 20 down-regulated miRNAs were found. In the comparison of S2 vs. S3, 11 and 11 miRNAs were up- or down-regulated, respectively (Fig. 4b).

The expression heat map of all DEMs as shown in Fig. 4c. Among these DEMs, 26, 18, and 9 miRNAs displayed specifically high expression at the initial filling stage, peak filling stage, and initial maturity stage, respectively. Twelve miRNAs exhibited high expression at both the initial filling stage and peak filling stage. Notably, the expression of 9 novel miRNAs (fta_novel_miR17–25) was sustained growth during seed development (Fig. 4c). To verify the results of miRNA sequencing, quantitative stem-loop RT-PCR was performed to determine the expression level of 9 DEMs during Tartary buckwheat seed development. As shown in Fig. 5, the results were broadly consistent with those obtained in miRNA sequencing.

To better understand the functions of these DEMs, their predicted target genes were investigated and further subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. A total of 1534 target genes were identified for among 74 DEMs (Additional file 1: Table S5). Of these target genes, 850, 765, and 735 genes were targeted by 44, 41, and 22 DEMs in the S1 vs. S2, S1 vs. S3, and S2 vs. S3 comparisons, respectively. GO analysis showed that 514, 448, and 419 targets were assigned to biological processes, cellular
component, and molecular function categories for the S1 vs. S2, S1 vs. S3, and S2 vs. S3 comparisons, respectively (Additional file 2: Figure S2). There were 18, 14, and 14 GO terms in biological processes, cellular component, and molecular function categories for each comparison, respectively. The “metabolic process”, “cell part”, and “catalytic activity” were the greatest abundance terms for these three categories (Additional file 2: Figure S2). KEGG analysis displayed that a total of 332, 309, and 272 target genes were assigned to 61, 54, and 55 KEGG pathways for the S1 vs. S2, S1 vs. S3, and S2 vs. S3 comparisons (Additional file 1: Table S10), respectively. Most of these pathways were involved in the metabolism process, of which biosynthesis of “amino acids biosynthesis” (ko01230), “starch and sucrose metabolism” (ko00500) “phenylpropanoid biosynthesis” (ko00940), “purine metabolism” (ko00230), and “amino
sugar and nucleotide sugar metabolism" (ko00520) were the major pathways. In addition, a high number target genes were assigned to "plant hormone signal transduction" (ko04075). The top 50 pathways for each comparison are shown in Additional file 2: Figure S3.

Identification of the key miRNA-mRNA pairs related to Tartary buckwheat seed development
To identify potential miRNA-mRNA pairs related to Tartary buckwheat seed development, we performed expression correlation analyses between DEMs and target mRNAs that were differentially expressed during Tartary buckwheat seed development. Based on our previous transcriptome data [46], which have the same samples with small RNA sequencing, 439 out of 1534 target genes of 74 DEMs exhibited significant differential expression during Tartary buckwheat seed development. Correlation analyses between the 439 DEGs and their corresponding DEMs identified that 117 miRNA-mRNA pairs were negatively correlated (Additional file 1: Table S11). Among them, 65 miRNA-mRNA pairs, which consisted of 25 miRNAs and 65 target genes, showed significantly expression negative correlation ($R \geq 0.5, P < 0.05$) (Table 1 and Fig. 6). Based on a homologous annotation of these 65 target genes, 56 miRNA-mRNA pairs achieved homologous functional annotation (Table 1). These 56 miRNA-mRNA pairs were found to be significantly involved in cell differentiation and proliferation, cell elongation, hormone response and balance, organogenesis, embryo and endosperm development, transport of mineral elements (Pi, Fe and Mn), and fatty acid and flavonoid biosynthesis (Table 1). In addition, 2 calcium binding proteins, 1 receptor-like kinase, 1 CBL-interacting protein kinase, and 1 SUMO protease were identified in these 65 target genes, although their homologues in other plants were not reported to participate in seed development (Table 1). Notably, among these identified miRNA-mRNA pairs, 5 miRNA-mRNA pairs, including fta-miR156c-5p-FtPinG0000496000.01, fta-miR167c-5p-FtPinG0002560000.01, fta-miR396a-5p-FtPinG0002594600.01, and fta_novel_miR17-FtPinG0007576600.01, might be involved in seed size regulation of Tartary buckwheat because the target mRNAs were homologous to the known genes related to rice seed size (Table 1). Furthermore, fta-miR858a-5p exhibited conspicuous negative correlation with several MYB TFs which are the well-known regulators of plant flavonoid biosynthesis (Table 1).

qRT-PCR and 5’-RLM-RACE validation of the key miRNA-mRNA pairs related to Tartary buckwheat seed development
To further confirm these identified key miRNA-mRNA pairs related to Tartary buckwheat seed development.
| miRNA | Target gene ID | Annotation of targets | Function of targets | Orthologs | Pearson | P-value |
|-------|----------------|-----------------------|---------------------|-----------|---------|---------|
| fta-miR156c-5p | FtPinG000490000001 | Squamosa promoter binding-like protein 16 | Grain size and quality | OsSPL16/ OsByg031600 | −0.70892 | 0.00340 |
| fta-miR156h-5p | FtPinG000140000001 | Nodulin MtN21-like transporter family protein | NA | ATIG21890.1 | −0.86151 | 0.00021 |
| Fta-miR157-5p | FtPinG000240000001 | NAC domain-containing protein 1 | Involved in shoot apical meristem formation and auxin-mediated lateral root formation, auxin response, plant growth and abiotic stress resistance | AT1G56100.1 | −0.89303 | 0.00275 |
| Fta-miR164a-5p | FtPinG000024000001 | NAC domain-containing protein 1 | Involved in shoot apical meristem formation and auxin-mediated lateral root formation, auxin response, plant growth and abiotic stress resistance | AT1G56100.1 | −0.81870 | 0.00281 |
| Fta-miR164c-5p | FtPinG000661000001 | Homeodomain-leucine zipper protein 8 | Involved in cell differentiation and proliferation, auxin response | AT1G32880.1 | −0.77425 | 0.00131 |
| Fta-miR390a- | FtPinG000821300001 | Polygalacturonase inhibiting protein | Involved in defense response | AT5G06800.1 | −0.72038 | 0.00261 |

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| miRNA     | Target gene ID                          | Annotation of targets                                                                 | Function of targets                                                                 | Orthologs | Pearson | P-value |
|-----------|----------------------------------------|---------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|-----------|---------|---------|
| fta-miR396a-5p | FtPinG000068070001.01 | Sucrose synthase                                                                      | Starch and cellulose biosynthesis, grain size and grain weight                      | SUS3/  |
|           |                                        |                                                                                       |                                                                                    | OX07g0616800 | -0.72690 | 0.00059 |
|          | FtPinG00031720001.01 | Neutral ceramidase                                                                    | Sphingolipid homeostasis, oxidative stress responses, ceramide catabolic process, long-chain fatty acid biosynthetic | ATG07380.1. | -0.78815 | 0.00060 |
|          | FtPinG00055177001.01 | Phosphoglucomutase 2                                                                 | Plant growth; seed, root, male and female gametophyte development, and carbohydrate partitioning | ATG07330.1. | -0.69606 | 0.00076 |
|          | FtPinG00050402001.01 | Sulfur dioxygenase                                                                    | Embryo and endosperm development, abiotic stress resistance,                        | TIG53580.1 | -0.778492 | 0.00799 |
|          | FtPinG00070606001.01 | Kinase interacting (KIP1-like) family protein                                         |                                                                                     | AT2G22560.1 | -0.65160 | 0.01957 |
| fta-miR396c-3p | FtPinG00050402001.01 | Sulfur dioxygenase                                                                    |                                                                                     | AT1G07380.1 | -0.69606 | 0.00076 |
|          | FtPinG00055177001.01 | Phosphoglucomutase 2                                                                 | Plant growth; seed, root, male and female gametophyte development, and carbohydrate partitioning | ATG07330.1. | -0.69606 | 0.00076 |
|          | FtPinG00050402001.01 | Sulfur dioxygenase                                                                    | Embryo and endosperm development, abiotic stress resistance,                        | TIG53580.1 | -0.778492 | 0.00799 |
| fta-miR396d-3p | FtPinG00070606001.01 | Kinase interacting (KIP1-like) family protein                                         |                                                                                     | AT2G22560.1 | -0.65160 | 0.01957 |
|          | FtPinG00050402001.01 | Sulfur dioxygenase                                                                    |                                                                                     | AT1G07380.1 | -0.69606 | 0.00076 |
|          | FtPinG00055177001.01 | Phosphoglucomutase 2                                                                 | Plant growth; seed, root, male and female gametophyte development, and carbohydrate partitioning | ATG07330.1. | -0.69606 | 0.00076 |
|          | FtPinG00050402001.01 | Sulfur dioxygenase                                                                    | Embryo and endosperm development, abiotic stress resistance,                        | TIG53580.1 | -0.778492 | 0.00799 |
| miRNA      | Target gene ID     | Annotation of targets                                      | Function of targets                                                                                     | Orthologs       | Pearson        | P-value   |
|------------|--------------------|-------------------------------------------------------------|--------------------------------------------------------------------------------------------------------|-----------------|----------------|-----------|
| FtPinG0007576600.01 | Argonaute protein | Regulate the development of embryo and shoot meristem development, embryo development, seed size | AG0177/AG240838                                                                                       |                | -0.54760       | 0.00002   |
| fta_novel_miR37   | FtPinG00040723000.01 | CBL-interacting protein kinase                              | Response to salt and osmotic stress, protein phosphorylation                                           | AT3G57630.1     | -0.56995       | 0.00126   |
| fta_novel_miR41   | FtPinG0006514000.01 | Intrinsic thylakoid membrane protein                        | NA                                                                                                     | AT2G17750.1     | -0.87363       | 0.00773   |
| fta_novel_      | FtPinG00024713000.01 | Carboxyesterase 13                                           | NA                                                                                                     | AT3G48700.1     | -0.55115       | 0.00159   |
| fta_novel_      | FtPinG0007248800.01 | Receptor-like protein kinase                                | Defense response to bacterium, protein phosphorylation                                               | AT4G23180.1     | -0.79183       | 0.00150   |
| fta_novel_      | FtPinG0005410000.01 | MYB transcription factor 73                                 | Auxin and abiotic stress responses a                                                                   | MYB73/AT4G37260.1 | -0.63527       | 0.01789   |
| fta_novel_      | FtPinG00036174000.01 | GRAS transcription factor 15                                | Cell differentiation, repressing the seed maturation programme,                                        | AT4G36710.1     | -0.88771       | 0.01772   |
| fta_novel_      | FtPinG0002993000.01 | C2H2 zinc finger protein                                    | Transfer cell differentiation                                                                         | AT4G27240.1     | -0.65506       | 0.00569   |
| fta_novel_      | FtPinG0006513600.01 | Galactosyltransferase family protein                        | Embryo development                                                                                  | AT4G32930.1     | -0.64824       | 0.00570   |
development, qRT-PCR and 5′-RLM-RACE were performed to analyze fta-miR156c-5p-FtPinG000496000.01, fta-miR164a-5p-FtPinG000240100.01, fta-miR166c-3p-FtPinG000845600.01, fta-miR167c-5p-FtPinG000256000.01, fta-miR396a-5p-FtPinG000068000.01, and fta novel_mir118-FtPinG0003617400.01 pairs, which were involved in seed size, cell differentiation and proliferation, and auxin response, respectively. qRT-PCR results indicated that the expression of these miRNAs was obviously negatively correlated with their corresponding target genes expression (Fig. 7). 5′-RLM-RACE results showed that these miRNAs could cleave their corresponding target genes (Fig. 8).
Discussion

MiRNAs, a class of negative regulators of gene expression, play key roles in numerous plant developmental processes including seed development [10, 51]. However, it is not clear how and whether to regulate the development of Tartary buckwheat seed remains unexplored. Here, to better understand the miRNAs-mediated molecular mechanisms of Tartary buckwheat seed development, we systematically identified the miRNA biosynthesis genes in the Tartary buckwheat genome and miRNA during Tartary buckwheat seed development. More importantly, we performed integrated analysis of miRNA and target mRNA expression profiles during Tartary buckwheat seed development, and identified the key miRNA-mRNA pairs for Tartary buckwheat seed development.

miRNA biosynthesis orthologs within the Tartary buckwheat genome

Numerous studies have shown that several different genes, including RDR, DCL, HYL, SE, HEN, HST, and AGO, play crucial roles in plant miRNA biosynthesis [5]. In this study, we identified 8 RDR, 4 DCL, 1 HYL, 2 SE, 1 HEN, 2 HST, and 8 AGO genes in the Tartary buckwheat genome. The phylogenetic analyses showed that 8 RDR, 4 DCL, and 8 AGO could be divided into 4, 4, and 3 subfamilies, respectively. The results were consistent with the previous studies in other plants [6, 49, 50, 52, 53], and indicated that the evolution of these gene families was conserved in these plants. Expression analysis revealed that 1 AGO-like FtAGO1 had the highest expression in seeds in these identified genes, 1 AGO-like FtAGO4 exhibited specific high expression in seeds, and 2 AGO-like FtAGO2 and FtAGO3 exhibited significant differential expression during seed development. Notably, OsAGO17, which belongs to the same subfamily as these four FtAGO genes, had been functionally demonstrated to positively regulate the grain size and grain weight in rice [54, 55]. This suggested that these four FtAGO genes might also play a similar role with OsAGO17 in Tartary buckwheat seed development. In addition, we also found that FtHYL1, FtHST2, and FtAGO8 displayed especially high expression in seeds, and FtDCL1, FtDCL3, FtDCL4, and FtRDR4 were obviously differentially expressed during seed development. These indicated that these miRNA biosynthesis genes might also have important regulatory roles in Tartary buckwheat seed development.

Characteristics of sRNAs during Tartary buckwheat seed development

By sequencing six sRNA libraries from three differently developmental stages seeds, we obtained abundant sRNAs with a length of 18–30 nt. Among them, the 24 nt sRNAs were the most abundant in developmental Tartary buckwheat seed, which was similar to previous observations in the developmental seed of many plants [6, 56]. Furthermore, the 24 nt sRNAs exhibited differential accumulation during Tartary buckwheat seed development. These suggested that the 24 nt sRNAs might play crucial roles in Tartary buckwheat and other plant seed development. It has been reported that many of the 24 nt sRNAs were heterochromatic siRNAs (hetsiRNAs), which mediate transcriptional gene silencing through DNA methylation (RdDM) [56]. Generally, the 24 nt sRNAs need to be imported into the nucleus before they were methylated, and the process was mediated by AGO4 [57]. Notably, our study observed that FtAGO8, which was the AGO4 ortholog, exhibited special high expression in the development Tartary buckwheat seed. These observations implied that FtAGO8 might has a role similar to AGO4 in 24-nt sRNAs methylation, and these 24-nt sRNAs might mediate transcriptional gene silencing through the RdDM pathway during Tartary buckwheat seed development.
Known and novel miRNAs and their target genes in the developmental Tartary buckwheat seed

In the Tartary buckwheat genome, 278 miRNAs have been predicted through a genome-wide bioinformatics analysis [58]. In our study, a total of 230 miRNAs, including 101 known and 129 novel miRNAs, were identified during Tartary buckwheat seed development. Among the miRNAs, more than two-thirds were expressed at high and moderate levels. These indicated that most miRNAs in the Tartary buckwheat genome were involved in the seed development, and further suggested that Tartary buckwheat seed development was an extremely complex biological process. Notably, the fta-miR159 exhibited the highest expression in the developmental Tartary buckwheat seed. In strawberry, Fa-miR159b and Fa-miR159b were more highly expressed in developing fruit and played a key regulatory role in fruit development [4, 59]. In rice, osa-miR159 was also more highly expressed in developing seed and positively regulated grain length and width [4, 20, 33, 35]. Therefore, our results suggested that fta-miR159 might also possess a crucial regulation role in the Tartary buckwheat seed development.

Using TargetFinder software, we identified 3268 potential target genes from 213 miRNAs. Among these target genes, the largest number were encoded TFs. Notably, among these miRNAs with target gene encoded TFs, some miRNAs such as miR156, miR160, miR396, and miR858 targeted the SBP, ARF, GRF, and MYB TFs, respectively. Interestingly, these miRNAs have been experimentally verified to target these corresponding TFs in other plants [32, 36, 37, 60]. This suggested that these miRNA targets were highly conserved in different plants and further confirmed the high reliability of target gene prediction.

![Fig. 8 S' RLM-RACE verification of six identified key miRNA-mRNA interaction pairs for Tartary buckwheat seed development. The numbers above sequences indicate the detected cleavage site of independent clones](image-url)
miR858, were found to be specifically differential expressed miRNAs, including miR530, miR828, and miR858. In addition, 37 novel miRNAs and several candidate miRNAs for Tartary buckwheat seed development, miR396, miR397, miR389, and miR408 have been functionally demonstrated to regulate flavonoid biosynthesis in many seed plants and miR408 were also found to be differentially expressed during seed development in many seed plants [4, 18–30]. These suggested that these known miRNAs might perform their regulatory roles in a specific stage during Tartary buckwheat seed development, suggesting that these DEMs might perform their regulatory roles in a specific stage during Tartary buckwheat seed development and miRNAs with the same expression pattern might have a similar regulatory role in the developing Tartary buckwheat seed. Notably, some DEMs such as miR156, miR160, miR166, miR167, miR168, miR395, miR396, miR397, miR398, miR399, and miR408 were also found to be differentially expressed during seed development in many seed plants [4, 18, 19, 21–30]. In rice, miR156, miR160, miR167, miR396, miR397, miR398, and miR408 have been functionally verified to regulate the rice seed development [31–38, 40]. These suggested that these known miRNAs were the key and conserved miRNAs in different plant seed development regulation and could also be used as candidate miRNAs for Tartary buckwheat seed development. In addition, 37 novel miRNAs and several conserved miRNAs, including miR530, miR828, and miR858, were found to be specifically differential expression in developing Tartary buckwheat seed when compared with other seed plants. This indicated that these miRNAs might have a specific regulatory function in Tartary buckwheat seed development. Furthermore, all these information indicated that conserved and diverse miRNA-mediated regulatory mechanisms in seed development might exist in different seed plants.

**DEM analysis during Tartary buckwheat seed development**

In many plants, a larger number of DEMs have been identified in the developing seed [4, 6, 18–30]. In this study, we identified 76 DEMs during Tartary buckwheat seed development. Expression profiles analysis revealed that these DEMs possessed stage-specific highly expressed patterns during Tartary buckwheat seed development, suggesting that these DEMs might perform their regulatory roles in a specific stage during Tartary buckwheat seed development and miRNAs with the same expression pattern might have a similar regulatory role in the developing Tartary buckwheat seed. Notably, some DEMs such as miR156, miR160, miR166, miR167, miR168, miR395, miR396, miR397, miR398, miR399, and miR408 were also found to be differentially expressed during seed development in many seed plants [4, 18, 19, 21–30]. In rice, miR156, miR160, miR167, miR396, miR397, miR398, and miR408 have been functionally verified to regulate the rice seed development [31–38, 40]. These suggested that these known miRNAs were the key and conserved miRNAs in different plant seed development regulation and could also be used as candidate miRNAs for Tartary buckwheat seed development. In addition, 37 novel miRNAs and several conserved miRNAs, including miR530, miR828, and miR858, were found to be specifically differential expression in developing Tartary buckwheat seed when compared with other seed plants. This indicated that these miRNAs might have a specific regulatory function in Tartary buckwheat seed development. Furthermore, all these information indicated that conserved and diverse miRNA-mediated regulatory mechanisms in seed development might exist in different seed plants.

**Integration analysis of miRNA and target mRNA**

Integrated miRNA and its target mRNA expression analysis could be helpful in understanding the function of miRNAs and identifying the functional miRNA-mRNA pairs related to seed development [67]. To our knowledge, there was no integration analysis of miRNA and target mRNA performed in previous studies of plant seed development. In this study, we identified 65 significantly negatively correlated miRNA-mRNA pairs (R ≥ 0.5, P < 0.05), which consisted of 25 miRNAs and 65 corresponding target genes. Based on homologous queries of these miRNAs corresponding target genes, 56 miRNA-mRNA pairs obtained functional annotations. These miRNA-mRNA pairs are involved in different aspects, most significantly cell differentiation and proliferation, cell elongation, hormones response and balance, organogenesis, embryo and endosperm development, mineral elements transport and accumulation, and flavonoid biosynthesis. These suggested that these identified miRNA-mRNA pairs were the key miRNA-mRNA pairs related to Tartary buckwheat seed development.

**In rice**, OsSPL16 [68, 69], OsNAC024 [70], OsSUS [71], OsGIF1 [38, 72], and OsAGO17 [54] genes have been functionally demonstrated to regulate the seed size. In this study, we found the target mRNAs from 5 miRNA-mRNA pairs (fta_novel_miR26, fta_novel_miR74, and fta-miR394a-5p) were identified to target the flavonoid biosynthesis structural genes PAL, 4CL, and FLS, respectively. Furthermore, 5 miRNAs (fta_novel_miR1, fta_novel_miR58, fta-miR858a-5p, fta-miR858a-5p, and fta-miR858b-3p) were found to target the orthologs of known flavonoid biosynthesis regulatory genes in Arabidopsis thaliana [63–66]. These implied that these miRNAs could regulate flavonoid biosynthesis in Tartary buckwheat by regulating the expression of the structural or regulatory genes of flavonoid biosynthesis.

**Integrated miRNA and its target mRNA expression analysis could be helpful in understanding the function of miRNAs and identifying the functional miRNA-mRNA pairs related to seed development [67].**

In this study, we identified 65 significantly negatively correlated miRNA-mRNA pairs (R ≥ 0.5, P < 0.05), which consisted of 25 miRNAs and 65 corresponding target genes. Based on homologous queries of these miRNAs corresponding target genes, 56 miRNA-mRNA pairs obtained functional annotations. These miRNA-mRNA pairs are involved in different aspects, most significantly cell differentiation and proliferation, cell elongation, hormones response and balance, organogenesis, embryo and endosperm development, mineral elements transport and accumulation, and flavonoid biosynthesis. These suggested that these identified miRNA-mRNA pairs were the key miRNA-mRNA pairs related to Tartary buckwheat seed development.

In rice, OsSPL16 [68, 69], OsNAC024 [70], OsSUS [71], OsGIF1 [38, 72], and OsAGO17 [54] genes have been functionally demonstrated to regulate the seed size. In this study, we found the target mRNAs from 5 miRNA-mRNA pairs (fta-miR156c-5p-FtPinG0000496000.01, fta-miR167c-5p-FtPinG0002560000.01, fta-miR396a-5p-FtPinG0002560000.01, fta-miR396b-3p-FtPinG0000680700.01, fta-miR530c-5p-FtPinG0002594600.01) were homologous with these 5 rice seed size genes, respectively, which indicated that these genes might have a conserved regulatory role in the seed size of Tartary buckwheat seed and the corresponding miRNAs were the key miRNAs for controlling Tartary buckwheat seed size. Notably, to date, these seed size genes had not been identified as the target genes of any miRNAs in previous studies. Therefore, our results provided the first-hand information on the post-transcription regulation of these seed size genes. It is well known that the Ca ²⁺ signal transduction pathways play important roles in many developmental processes including seed development in plants [73, 74]. In this study, we found that three of the 56 identified miRNA-mRNA pairs were involved in Ca ²⁺ signal transduction because the target mRNAs were homologous to the calcium binding protein and CBL-interacting protein kinases, respectively. This suggested that these miRNAs could regulate Tartary buckwheat seed development.
development through Ca²⁺ signal transduction pathways. In a previous study, we found that the total seed flavonoid content was dynamically accumulated during Tartary buckwheat seed development, and identified one SG7 subgroup R2R3-MYB TF gene (FtPinG0009153900.01) was the key regulatory gene of flavonoid biosynthesis in the developing Tartary buckwheat seed [46]. Interestingly, in this study, we found fta-miR858a-5p-FtPinG0009153900.01 pair displayed significantly expression negative correlation. In addition, fta-miR858a-5p also exhibited conspicuous negative correlation with the orthologs of Arabidopsis thaliana MYB123, which was the well-known regulator of proanthocyanidin accumulation in developing seed [64]. These indicated that the flavonoid biosynthesis of Tartary buckwheat seed is involved in miRNA-mediated post-transcription regulation and fta-miR858a-5p is the key regulator.

To further verify the reliability of these identified miRNA-mRNA pairs related to Tartary buckwheat seed development, we carried out qRT-PCR and 5′-RLM-RACE analyses for among 6 miRNA-mRNA pairs that are involved in seed size, cell differentiation and proliferation, and auxin response. These results showed that the expression of these miRNAs and their corresponding target genes were obviously negatively related and could cleave their corresponding target genes. The above results indicated that these identified miRNA-mRNA pairs could be considered as the candidate miRNA-mRNA pairs for regulating the development of Tartary buckwheat seed, and also suggested that combining small RNA and transcriptome analysis is an effective method for identifying key miRNAs that are involved in plant seed development.

Conclusion

The present work is the first attempt to integrate miRNA and mRNA expression data to identify key regulatory miRNA-mRNA pairs in developing Tartary buckwheat seed. A total of 230 miRNAs, including 101 known and 129 novel miRNAs, were first identified in Tartary buckwheat. Among these miRNAs, 76 showed significant differential expression during Tartary buckwheat seed development, and 1543 of their target genes were identified. Additionally, 25 miRNAs corresponding to 56 target genes were identified as key candidate miRNA-mRNA pairs for Tartary buckwheat seed development. The integrated analyses of miRNAs and target mRNAs in this study not only provide the first new insights into miRNA-mediated regulation in Tartary buckwheat seed development, but also provide a basis for further research into the functions of these candidate miRNAs and their targets in Tartary buckwheat seed development and for Tartary buckwheat seed improvement.

Methods

Plant materials and sample preparation

Tartary buckwheat cultivar Jinqiao No. 2, which was obtained from the Research Center of Buckwheat Industry Technology of Guizhou Normal University (Guiyang, Guizhou, China), was used in this study. The seed samples used for sRNA sequencing were same as our previous transcriptome analysis [46]. In brief, seeds were collected at 12 (S1: initial filling stage), 15 (S2: peak filling stage), and 21 (S3: initial maturity stage) days after pollination (DAP) [46].

Identification, phylogeny, and expression profiles analysis of the miRNA biosynthesis genes in the Tartary buckwheat genome

The Tartary buckwheat genome information was downloaded from http://www.mbkbase.org/Pinku1 [58]. All protein sequences from annotated genes were used to build a local protein blast database in BioEdit software (Version 7.0.9.0). The protein sequences of RDR1–6, DCL1–4, AGO1–10, HEN1, HYL1, SE, and HST genes from Arabidopsis thaliana were used as queries to carry out local blastp with e-values <e⁻⁵ to identify orthologs in the Tartary buckwheat genome. The conserved domain of all obtained non-redundant sequences were further examined in the Pfam (http://pfam.sanger.ac.uk) [75], and the final orthologs were ensured. The RDR, DCL, and AGO protein sequences from Tartary buckwheat, Arabidopsis thaliana, rice, and maize were used to construct the phylogenetic trees using the Maximum Likelihood method in MEGA 7.0.21 software. Expression analysis of these identified miRNA biosynthesis genes in Tartary buckwheat was performed using RNA-seq data from 4 organs (root, stem, leaf, and flower) [58] and three developmental-stage seeds [46]. The expression heatmaps of these genes were generated by using TBtools software [76].

RNA extraction, sRNA library construction, and sequencing

Total RNA was extracted from three samples as previously described [46] and used for sRNA and qRT-PCR assay. RNA quality and concentration were determined via 1.2% agarose gel electrophoresis and NanoDrop 2000c spectrophotometer (NanoDrop, Wilmington, DE, USA), respectively.

For construction and sequencing of the sRNA libraries, two replicates were performed for each sample. Firstly, sRNAs were isolated from total RNA by polyacrylamide gel electrophoresis (PAGE). Next, the isolated sRNAs were added to a 5′ RNA adaptor and a 3′ RNA adaptor by using T4 RNA ligase (TaKaRa, Dalian, China). Then, sRNAs with added 5′ and 3′ RNA adaptors were reverse transcribed into single-stranded cDNA sequences.
using RT-PCR. Follows, the single-stranded cDNA was further synthesized into double-stranded cDNA by PCR amplification using adapter primers. Finally, the PCR product was purified and subjected to high-throughput sequencing by using the Illumina SE50 system at Biorunner Technologies Co., Ltd. (Beijing, China).

**Bioinformatics analysis of sequencing data**

Raw reads were processed to generate clean reads by removing the low-quality reads, reads containing poly-N or poly-A sequences, reads lacking the 3′ adaptor sequence, and reads with length < 18 nt or > 30 nt, and adapter sequences. The clean reads were then mapped against the Tartary buckwheat genome (http://www.mbkbase.org/Pinku1/) [58]. The Silva database, GtRNAdb database, Rfam database and Repbase database were used to filter the rRNA, tRNA, snRNA, and snoRNA to produce the unannotated reads containing miRNA [4]. Unannotated reads were blasted against miRBase to search for known miRNAs [77]. The potential novel miRNAs were predicted by using the miRDeep2 program [78]. The miFam.dat (http://www.mirbase.org/ftp.shtml) was used to investigate the miRNA family class of these identified miRNAs. The miRNA expression abundances were calculated and normalized by transcript per million (TPM), and differential expression analysis was carried out by the DESeq R package with FDR (false discovery rate) value < 0.05 and |log2(fold change)| > 1 as the threshold for significant difference. The potential target genes of miRNA were predicted by using TargetFinder software [79]. The predicted target genes were subjected to GO (http://geneontology.org/) [80] and KEGG (www.kegg.jp/kegg/kegg1. html) [81] pathway analysis to predict and classify possible functions.

**Identification of the key miRNA-mRNA pairs for Tartary buckwheat seed development**

Expression profiles of all the DEMs target genes were analyzed by using transcriptomic data from our previous report [46]. Then, the significantly different expression target genes were identified and functional analysis was performed by GO, KEGG and homologous annotation. Finally, expression correlation analysis between DEMs and differentially expressed target genes was performed. The miRNAs and target mRNA pairs with $R \geq 0.5$ and $P$ value < 0.05 were identified, and the key miRNA-mRNA pairs for Tartary buckwheat seed development were obtained by homologous annotation of target miRNAs.

**qRT-PCR validation of DEMs and its differently expressed target genes**

Total RNA was isolated from three different developmental stages of Tartary buckwheat seeds by using the EASYspin Plus Plant RNA Kit (Aidlab, Beijing, China) and digested by DNase I (TaKaRa, Dalian, China) to remove the genomic DNA. Then, 2 μg total RNA was reverse transcribed into single-stranded cDNA using the miRcute miRNA First-Strand cDNA Synthesis Kit (Tiangen, Beijing, China) following the manufacturer’s instructions. The qRT-PCR was performed on a CFX96 Real-time System (BIO-RAD, USA) using the miRcute Plus miRNA qPCR Kit (SYBR Green) (Tiangen, Beijing, China). Three independent biological replicates were used, and the $FtU6$ was used as the reference gene. The relative expression level of each miRNA was calculated by using the $2^{-ΔΔCt}$ method. All primers used in this experiment are listed in Additional file 1: Table S12.

For differently expressed target genes, reverse transcription and qRT-PCR reactions were carried out by using PrimeScript™ RT Master Mix (Perfect Real Time) (TaKaRa, Beijing, China) and TB Green* Premix Ex Taq™ (Tli RNaseH Plus) (TaKaRa, Beijing, China), respectively. $FtActin$ was used as the reference gene. All samples were performed three independent biological replicates. The relative expression level of each gene was calculated by using the $2^{-ΔΔCt}$ method. The primers used in this experiment are listed in Additional file 1: Table S12.

**Validation of the miRNA-directed cleavage of their predicted targets**

The 5′-RLM-RACE method was used to investigate the miRNA-directed cleavage of their predicted target mRNA in vitro. Total RNA was ligated to an RNA adaptor and was reverse transcribed using the First-Choice® RLM-RACE Kit (Ambion, USA) according to the manufacturer’s instructions. The 5′-RLM-RACE was performed as described previously by DeBoer et al. [6]. The primers used in this experiment are listed in Additional file 1: Table S13.

**Abbreviations**

DEMs: Differentially expressed miRNAs; qRT-PCR: Quantitative real-time polymerase chain reaction; 5′-RLM-RACE: Ligase-mediated rapid amplification of 5′ cDNA ends; miRNAs: MicroRNAs; sRNAs: Small RNAs; DCL1: Dicer-Like 1; HYL1: Hyponastic Leaves 1; SE: Serrate; HEN1: Hua enhancer 1; AGO1: Argonaute 1; RISC: RNA-induced silencing complex; DEGs: Differentially expressed genes; TFs: Transcription factors; hetriRNAs: Heterochromatic siRNAs; AME: Minimum free energy; PAL: Phenylalanine ammonia-lyase; 4CL: 4-coumarate-CoA ligase; FLS: Flavonol synthase; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; DAP: Days after pollination.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s12870-021-02914-w.

Additional file 1: Table S1. Characteristic features of all the identified DCL, AGO, HYL1, SE, HST, and RDRs in tartary buckwheat genome. Table
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Not applicable.

Authors’ contributions
HRL and QFC designed the research. HYL carried out the experiments and wrote the manuscript. HYL, HLM, XQS, JD, and LW performed data analysis. QYL, TXS and QFC reviewed and revised the manuscript. All authors read and approved the final manuscript.

Availability of data and materials
The datasets supporting the conclusions of this article are included within the article and its additional files. The dataset and materials presented in the investigation is available by request from the corresponding author.

Declarations
Ethics approval, guidelines and consent to participate
The plant material (Jinqiao No. 2) was a Tartary buckwheat cultivar in China, which was collected by our lab (Research Center of Buckwheat Industry Technology of Guizhou Normal University, Guiyang, Guizhou, China). All authors stated that this study comply with the Chinese legislation and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
1Research Center of Buckwheat Industry Technology, Guizhou Normal University, Guiyang 550001, China. 2Key Laboratory of High-Quality Crops Cultivation and Safety Control of Yunnan Province, Honghe University, Honghe 661100, China. 3School of Life Sciences, Guizhou Normal University, Guiyang 550025, China. 4School of Big Data and Computer Science, Guizhou Normal University, Guiyang 550025, China.

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