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Research article

MicroRNA and regulation of auxin and cytokinin signalling during post-mowing regeneration of winter wheat (*Triticum aestivum* L.)

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ABSTRACT

Winter wheat not only provides adequate fresh forage grass in winter, but also ensures ample grain production in summer. The mechanisms underlying the regeneration of winter wheat after mowing or grazing are not well understood. In this study, the miRNA expression profile of winter wheat was determined using RNA sequencing and the endogenous auxin and *cis*-zeatin concentrations, as well as the expression of related miRNA-targeted genes, were measured. During the post-mowing regeneration of winter wheat, the concentrations of endogenous indole-3-acetic acid (IAA), methyl indole-3-acetate (ME-IAA), and indole-3-carboxaldehyde (ICA) decreased, while those of *cis*-zeatin (cZ) increased. Moreover, 15 novel miRNAs and three known miRNAs were found to be involved in the synthesis and signalling transduction of auxins and cytokinins (CKs). Among these miRNAs, miR1153-y, miR5059-x, miR2916-x, novel-miR1532–3p, novel-miR1060–3p, and novel-miR0890–3p, were found to be negatively correlated with the expression of their target genes including *auxin response GH3.7*, *auxin response factor (ARF)*, *type-A two-component response regulator (A-ARR)*, *aldehyde dehydrogenase (ALDH)*, and *O-glucosyltransferase (CISZOG)*. Furthermore, miR1153-y was identified as mediating the cleavage of *GH3.7* by RACE assay. In turn, these genes inhibited the biosynthesis and signalling of IAA and activated CK signal transduction, resulting in the rapid regeneration of mowed winter wheat. This study revealed that some miRNAs exert a positive regulatory effect on the post-mowing regeneration of winter wheat by controlling the synthesis and signal transduction of IAA and CK, and our founding will aid developments in biotechnology aimed at improving the post-mowing regeneration ability of winter wheat.

1. Introduction

Dual-purpose winter wheat (*Triticum aestivum* L.) is utilized globally (Arzadun et al., 2006) to supply an adequate quantity and quality of forage during winter, as well as high grain yields in summer (Kim and Anderson, 2015). Although wheat has been widely planted for grain production in northern China, it is rarely used for forage production or grazing (Tian et al., 2012). Dual-purpose wheat can improve soil utilization efficiency as it provides fresh, green forage during winter in northern China, when warm-season forages, such as alfalfa, do not grow (Kim and Anderson, 2015; Tian et al., 2012). Compared with other small grain species that can be planted for winter forage, the main advantages of wheat are its excellent tolerance to abiotic stresses (cold and drought stress) and its relatively high nutritive value, including high protein, energy, and mineral content, alongside a low fibre concentration (Butchee and Edwards, 2013; Juskiw et al., 2000; Kim and Anderson, 2015). Moreover, the use of winter wheat allows for more consistent and productive forage for the entire winter and early spring compared with that of other grain species (Juskiw et al., 2000). However, the regeneration that occurs after the mowing of wheat seedlings seriously affects grain production during the harvest season (Giunta et al., 2015; Tian et al., 2012). Thus, it is of practical significance to study the mechanisms

Abbreviations: CK, cytokinin; cZ, *cis*-zeatin; DE miRNA, differentially expressed microRNA; IAA, indole-3-acetic acid; miRNA, microRNA.

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underlying the regeneration of mowed wheat.

Most of the true leaves of wheat seedlings on the ground are removed during mowing or grazing, and morphological changes in the regenerated parts are largely attributed to the larger size of young leaves. The conversion of a young leaf into a mature leaf is the result of cell proliferation and expansion (Breuninger and Lenhard, 2010). During early leaf growth, cell proliferation occurs throughout the entire leaf primordium, which generates new cells, while the size of the leaf remains relatively small and constant. In the later growth phase, cell proliferation in the developing leaves ceases, and a large increase in leaf size is mainly achieved through cell expansion (Gonzalez et al., 2012). Cell expansion may play a more important role during the growth of young wheat leaves after mowing. It is known that phytohormones, especially auxins and cytokinins (CKs), play key regulatory roles throughout leaf development (Gonzalez et al., 2012; Schruff et al., 2006).

Auxin and CKs are important plant-growth regulators that also play important regulatory roles during vegetative growth (George et al., 2008; Moskovitz et al., 2008). CKs are involved in various developmental processes in plants, including the development of both leaf shape and size (Breuninger and Lenhard, 2010). Changes in the concentrations of CKs in the shoot apical meristem are the main factors that induce leaf development and differentiation (Kyozuka, 2007). Moreover, cell expansion is the main factor responsible for variation in leaf size (Breuninger and Lenhard, 2010). CKs stimulate cell expansion during leaf growth, and an increase in CK concentration leads to a higher cell expansion rate (Skalák et al., 2019). CKs reportedly stimulate the regrowth of barley after mowing (Christiansen et al., 1995). Moreover, several important signal transducers in the CK signalling pathway, such as type-A and type-B *two-component response regulators (ARRs)* also play important roles during leaf development (Argueso et al., 2010; Kieber and Schaller, 2018). Auxins also play important regulatory roles in leaf development and growth (Keller et al., 2004). Exogenously applied auxins significantly decrease the length, weight, and area of treated leaves compared with that in untreated leaves (Keller et al., 2004). Moreover, shade has been found to inhibit cell proliferation by increasing auxin concentrations while reducing cytokinin concentrations, thereby suppressing the growth of soybean leaves (Wu et al., 2017). In addition, *auxin response factor2 (ARF2)* encodes a member of a family of transcription factors that regulate gene expression in response to auxin; this factor represses the growth of *Arabidopsis* by affecting cell division and expansion (Okushima et al., 2010; Schruff et al., 2006).

The compensatory regeneration of winter wheat after mowing or grazing is important for maintaining nutrient accumulation in wheat seedlings, as this affects both spring elongation and summer grain harvest. Regeneration is also a key factor in determining whether wheat can be used for dual purposes (Giunta et al., 2015). Many previous studies focused on wheat germplasm screening, cultivation, grazing practices, and evaluation of the economic benefits of using dual-purpose wheat; studies that examine the regeneration mechanism of winter wheat after mowing or grazing have rarely been conducted. We hypothesized that miRNAs and miRNA-mediated regulation of gene expression play an important role in the rapid regeneration of wheat seedlings after mowing or grazing. To verify this hypothesis, the miRNA expression profile of wheat seedlings after mowing was observed by RNA-sequencing and the expression of key targeted genes during the rapid regeneration was examined.

2. Materials and methods

2.1. Plant materials and growth rate determination

Wheat (*Triticum aestivum* L. 'Xinong9106') was bred by the Key Laboratory of Wheat Biology and Genetic Breeding, Ministry of Agriculture and Rural Affairs, P.R. China. This variety was selected because of its high biomass and regeneration ability after mowing at the seedling stage (Liu et al., 2018). Seeds were planted in the experimental fields of Northwestern A&F University, Yangling, Shaanxi, China (34°16′56.24″N, 108°4′27.95″E). After five tillers were observed on the seedlings, 2 cm-long pseudostem from the base of the stem was harvested, for use as the control (T0). The leaves located approximately 3 cm from the base of the stem were mowed and 2 cm-long pseudostems from the base of the stem were harvested at 2 (T1), 24 (T2), and 72 h (T3) after mowing; these were the three treatment groups (Fig. 1). The growth rate and height of seedlings were determined at 0, 24, 48, and 72 h after mowing; 15 plants were harvested for each evaluation.

2.2. RNA isolation and small RNA library construction

Three biological replicates for each treatment (T0, T1, T2, T3) were sequenced and each biological replicate contained 15 plants, for a total of 12 RNA sequencing (RNA-Seq) samples. After total RNA extraction using TRIzol reagent (Invitrogen, Carlsbad, NM, USA), the quantity, purity, and integrity of the resulting RNA were confirmed using NanoDrop One (Thermo Fisher Scientific, Waltham, MA, USA) and Agilent 2100 (Agilent Technologies, Santa Clara, CA, USA). The RNA samples used for small RNA library construction had a RNA integrity number (RIN) $\geq$8 (Additional File 1). Small RNA library preparation was performed using the TruSeq Small RNA Sample Prep Kit (Illumina, San Diego, CA, USA). Adapters at the 3′ and 5′ ends were added to 1 μg RNA, and the adapter-ligated RNAs were reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen). The 140–160 bp-size PCR products were enriched to generate a cDNA library, which was then sequenced using Illumina HiSeq™ 2500 at the Gene Denovo Biotechnology Company (Guangzhou, China).

2.3. Small RNA analysis and miRNA identification

Raw reads generated from the Illumina HiSeq™ 2500 sequencing were filtered to obtain high-quality reads; this was done by removing the following: low quality reads [containing more than one low quality (Q-value $\leq$ 20) base or unknown nucleotides], adaptor sequences, $\leq$18 bp reads (not including adapters), and poly-A sequences. All the clean tags were aligned with small RNAs in the GeneBank database (Release 209.0), Rfam database (11.0, http://xfam.org/), and the reference genome (IWGSC RefSeq v1.0 assembly, https://wheat-urgi.versailles.inra.fr/) to identify and remove rRNA, scRNA, snoRNA, snRNA, tRNA, and other RNA sequences that mapped to the exons, introns, and repeat sequences. The remaining clean reads were used as search terms in the miRbase database (Release 21.0, http://www.mirbase.org/) to identify known miRNAs and other known other miRNAs (miRNAs from wheat and other plants). All the unannotated tags were aligned with the reference genome to predict novel miRNAs using MIREAP v0.2 software (https://sourceforge.net/projects/mireap/).

2.4. Differentially expressed (DE) miRNA analysis

The miRNA expression levels were calculated and normalized to transcripts per million (TPM). The formula used was TPM = (actual miRNA counts/total counts of clean tags) $\times$ 10^6. To identify differentially expressed (DE) miRNAs across groups, miRNAs with a fold change $\geq$2 and P value $\leq$ 0.05 were identified as significant DE miRNAs. For trend cluster analysis of miRNAs, OmicShare Tools (http://www.omicshare.com/tools/Home/Soft/getsoft?l=en-us) were used, and the significance of the miRNA enrichment was determined using a permutation test ($P < 0.05$).

2.5. Target gene prediction and functional annotation

Based on all the identified miRNAs, candidate genes were predicted using Mireap and psRNATarget (http://plantgrn.noble.org/psRNATarget). The default criteria were as follows: no more than four mismatches between miRNA and target (G-U bases count as 0.5

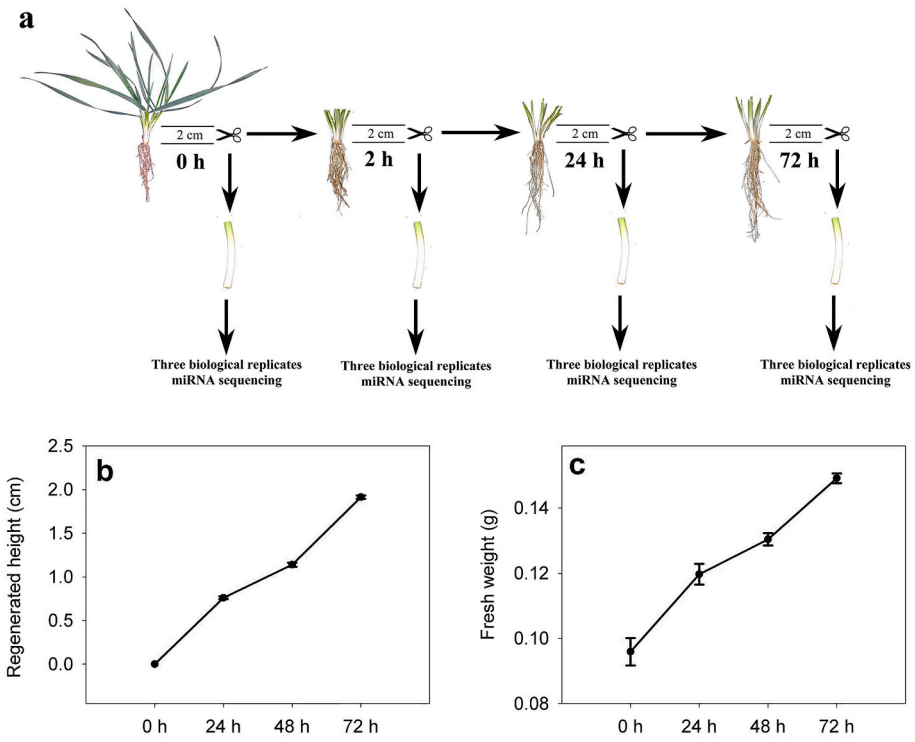

Fig. 1. Experimental procedure and sampling method for this study. The wheat leaves above 3 cm from the base of stem was cut and 2 cm-long pseudostem from the base of stem was harvested at 0, 2, 24, and 72 h after mowing. (**a**) Regeneration and sampling of forage wheat after mowing. (**b**) The height of regenerated seedling leaves. (**c**) The fresh weight of regenerated seedling leaves.

mismatches); no more than two adjacent mismatches in the miRNA/target duplex; no adjacent mismatches at positions 2–12 of the miRNA/target duplex (the 5′ end of the miRNA); no mismatches at positions 10–11 of the miRNA/target duplex; no more than 2.5 mismatches at positions 1–12 of the miRNA/target duplex (the 5′ end of the miRNA); minimum free energy (MFE) of the miRNA/target duplex ≥75% of the MFE of the miRNA bound to its perfect complement. Functional annotation of the target genes was conducted using Gene Ontology (GO) (http://www.geneontoloty.org/) enrichment analysis and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (http://www.genome.jp/kegg/). For GO and KEGG enrichment, the calculated *P* values were subjected to FDR Correction, using FDR ≤0.05 as a threshold. GO terms or KEGG pathways meeting this condition were defined as significantly enriched GO terms or KEGG pathways for DE miRNAs.

2.6. Measurement of auxins and cis-zeatin

HPLC-grade acetonitrile (ACN) and methanol (MeOH) were purchased from Merck (Darmstadt, Germany). Standards were purchased from Olchemim Ltd. (Olomouc, Czech Republic) and Sigma (St. Louis, MO, USA). Acetic acid was sourced from Sinopharm Chemical Reagents (Shanghai, China). Three biological replicates were used for quantification, and 15 seedlings were harvested for each biological replicate. Fresh plant materials were harvested, weighed, snap-frozen in liquid nitrogen, and stored at −80 °C. Endogenous phytohormone determination was performed in accordance with the protocol proposed by Pan et al. (2008). Plant materials (500 mg fresh weight) were ground into a powder and extracted using methanol (80%, v/v) at 4 °C. The extract was centrifuged at 12,000×*g* at 4 °C for 15 min. The supernatant was collected and dried under a stream of nitrogen gas, and was subsequently re-dissolved in methanol (30% v/v). The solution was centrifuged and the supernatant was collected for LC-MS analysis.

Sample extracts were analysed using an LC-ESI-MS/MS system (HPLC, Shim-pack UFLC SHIMADZU CBM30A system, Shimadzu, Japan; MS, Applied Biosystems 6500 Triple Quadrupole, Thermo Fisher Scientific). HPLC conditions were as follows: column, waters ACQUITY UPLC HSS T3 C18 (1.8 μm, 2.1 × 100 mm); solvent system, water (0.04% acetic acid), acetonitrile (0.04% acetic acid); gradient program, 95:5 (v/v) at 0 min, 5:95 at 11.0 min, 5:95 at 12.0 min, 95:5 at 12.1 min, 95:5 at 15.0 min; flow rate, 0.35 mL/min; temperature, 40 °C; injection volume, 5 μL. The effluent was connected to an ESI-triple quadrupole-linear ion trap (Q TRAP)-MS.

The effluent was analysed using an API 6500 Q TRAP LC/MS/MS system, equipped with an ESI Turbo Ion-Spray interface, operating in the positive ion mode and controlled by Analyst 1.6 software (AB Sciex, Framingham, MA, USA). ESI source operation parameters were as follows: ion source, turbo spray; source temperature, 500 °C; ion spray voltage (IS), 5500 V; curtain gas (CUR) set at 35.0 psi; collision gas (CAD) set to medium. DP and CE for individual MRM transitions were established with further DP and CE optimization. A specific set of MRM transitions was monitored for each period based on the plant hormones eluted within this period. The quantification of endogenous hormones was performed using a standard curve (Additional File 2). Duncan's multiple range test ($P < 0.05$) was used to analyse the differences among treatments.

2.7. Quantitative real-time PCR for targeted genes

Five biological replicates were used to detect the expression of target genes and 15 plants were used for each biological replicate. For RNA reverse transcription, 2 μg of total RNA was reverse-transcribed using a PrimeScript™ RT reagent kit (TaKaRa BIO, Japan). Gene-specific primers were designed using Primer Premier 6.0; these are shown in Additional File 3. Quantitative real-time PCR was performed in a 20 μL volume following the manufacturer's instructions (SYBR® Advantage® qPCR Premix, TaKaRa BIO). A three-step PCR was used set to the following conditions: pre-denaturation at 95 °C (3 min), 40 cycles of 95

°C (10 s), 60 °C (30 s), and 72 °C (60 s). For gene expression normalization, *Actin (gene ID: AB181991)* was used as an internal reference.

2.8. MiRNA-directed cleavage of target mRNAs

To verify miRNA-directed cleavage of target mRNAs, all mRNAs in Fig. 6 were selected to analysis the cleavage site targeted by miRNAs. The miRNA-directed cleavage was verified using a 5′ RACE assay in accordance with a previously published protocol (De Paola et al., 2012). The RACE assay was conducted using a FirstChoice® RLM-RACE Kit (Thermo Fisher Scientific). The specific forward primers used for targeted mRNAs were shown in Additional File 4, and the reverse primers comes from the kit. Total RNA was treated with tobacco acid pyrophosphatase (TAP) and 5′ RACE adapter was then ligated to the 5′ of the cleaved mRNA. After mRNAs were reverse transcribed, a nested PCR was used to amplify 200–500bp DNA fragments near the miRNA-directed cleavage site. PCR products were collected and sequenced for each mRNA, to confirm miRNA-directed cleavage site. For each target mRNA, 30 clones were sequenced to confirm cleavage site.

2.9. Data analysis

Microsoft Excel 2016 (Microsoft Corp., Redmond, WA, USA) and SigmaPlot 12.5 (Systat Software Inc., San Jose, CA, USA) were used for data arrangement and visualization. IBM SPSS Statistics 19.0 (IBM Corp., Armonk, NY, USA) was used for ANOVA analysis. The interaction diagram for miRNAs and mRNAs was created with Cytoscape 3.4.1 (https://cytoscape.org/).

3. Results

3.1. Regeneration of winter wheat, and measurement of endogenous phytohormones

The regenerative performance of wheat was observed (Fig. 1 and Additional File 5). The fresh weight of the aboveground parts of wheat increased by more than 50% 72 h after mowing (Fig. 1**c**). At the same time, the castrated young leaves expanded by approximately 2 cm (Fig. 1**b**). Continuous photography in the incubator (set to a temperature of 15/20 °C and a 10/14 h dark/light cycle), revealed that winter wheat changed significantly 1 h after mowing, and that rapid growth occurred over the following 72 h (Additional File 5). Endogenous CK and auxin concentrations were determined during the regeneration of mowed winter wheat. The *cis*-zeatin (cZ) content in the mowed winter wheat significantly increased 2 h after mowing, this value then increased to more than three-fold, and then stabilized at 24 h (Fig. 2**a**). The concentration of indole-3-acetic acid (IAA) in the pseudostem significantly decreased 2 h after mowing and kept on decreasing until it reached a stable value at 24 h (Fig. 2**b**). Unlike IAA, the methyl indole-3-acetate (ME-IAA) content in the mowed wheat increased significantly after 2 h of mowing, then decreased significantly at 24 h and 72 h (Fig. 2**c**). The indole-3-carboxaldehyde (ICA) content in the wheat did not change significantly at 2 h and 24 h after mowing, but showed a downward trend between 2 h and 24 h, and then decreased significantly at 72 h (Fig. 2**d**). Overall, the auxin content of wheat seedlings decreased after mowing. These results indicate that cZ and the three auxins play an important role in the rapid regeneration of.

3.2. Overview of small RNA profiles in all samples

High-throughput sequencing of miRNA libraries generated from wheat samples T0-1, T0-2, T0-3, T1-1, T1-2, T1-3, T2-1, T2-2, T2-3, T3-1, T3-2, and T3-3 produced 15,412,064, 13,409,848, 19,372,964, 14,515,377, 16,008,404, 17,603,520, 17,031,248, 16,966,593, 16,588,116, 16,885,703, 18,772,019, and 20,409,399 unfiltered sequence reads, respectively (Table 1). After filtering out low-quality reads, more than 90% of the clean reads of all samples were selected.

and searched in Genbank, Rfam, and the reference genome databases (Table 1).

The most abundant classes of clean tags were observed to have lengths ranging from 21 to 24 nucleotides (nt), particularly 21 nt and 24 nt (Fig. 3). After the rRNA, snRNA, snoRNA, tRNA, and fragments produced during mRNA degradation were filtered out, known miRNA and novel miRNA sequences were identified. In all samples, more than 75% of the clean tags were identified as being previously known types of RNA, while approximately 10–25% of the tags were identified as miRNAs. The number of identified unique and total tags of miRNA are shown in Table 2. Most of the identified miRNA tags were determined to be known tags in the database (11–23%), while novel miRNA tags accounted for 0.2–0.4% of all samples. Unique tags represented approximately 25–35% of all clean tags (Table 1); approximately 0.70–1.00% of the unique tags in all samples were identified as unique

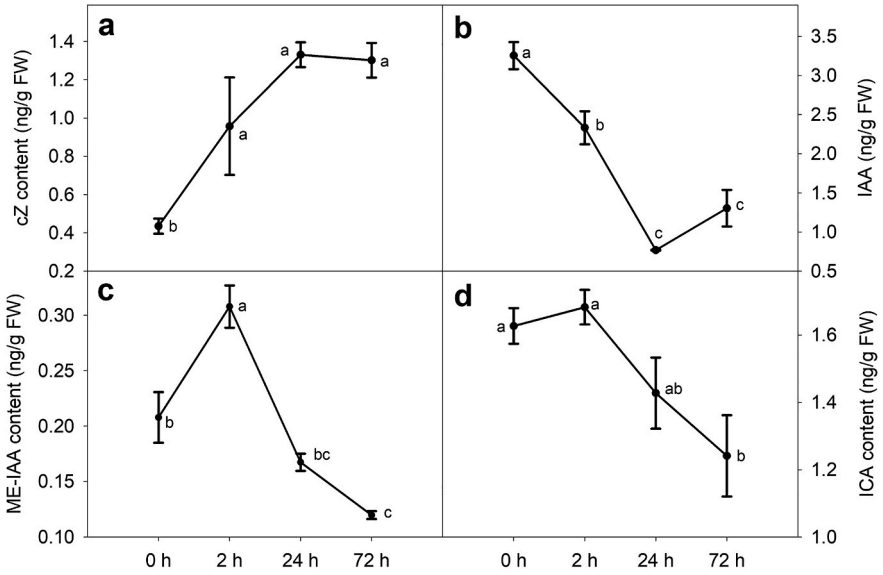

Fig. 2. The concentration of endogenous auxins and *cis*-zeatin in wheat seedlings after mowing. The wheat seedlings were harvested at 0, 2, 24, and 72 h after mowing. cZ, *cis*-zeatin; IAA, indole-3-acetic acid; ME-IAA, methyl indole-3-acetateand; ICA, indole-3-carboxaldehyde.

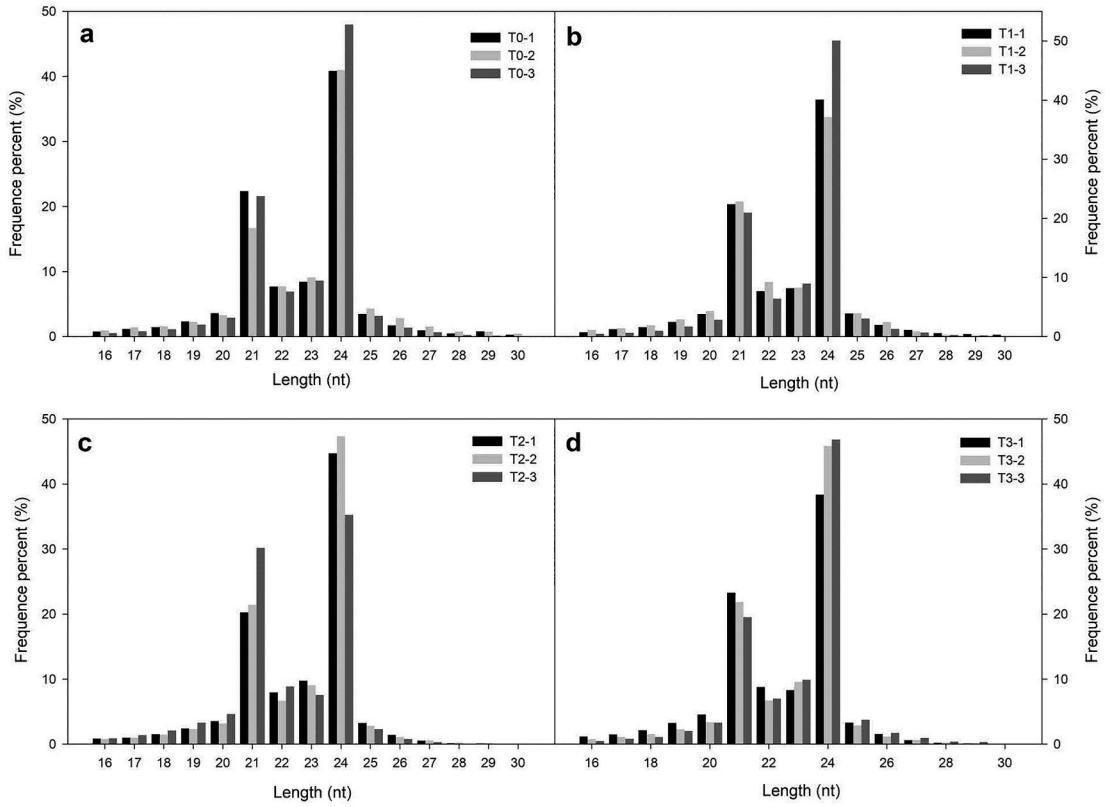

Fig. 3. The frequency percent of identified miRNAs in all samples. T0, T1, T2, and T3 represent samples harvested at 0, 2, 24 and 72 h after mowing, respectively.

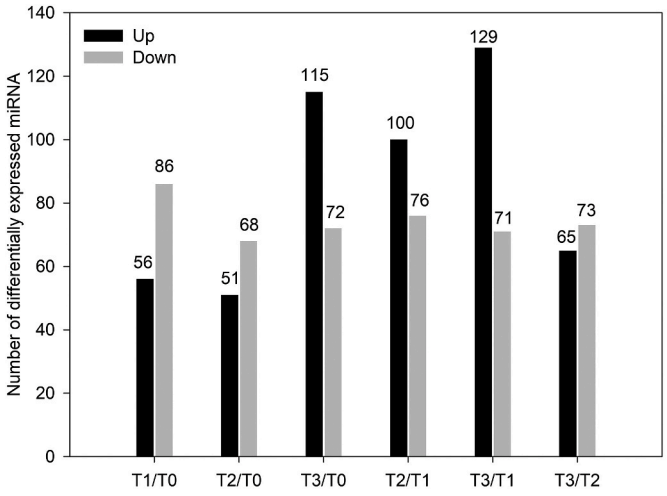

Fig. 4. The number of differentially expressed miRNA in all samples. T0, T1, T2, and T3 represent samples harvested at 0, 2, 24 and 72 h after mowing, respectively.

miRNAs (Table 2). Of all the unique miRNAs, more than 90% were known unique miRNAs and approximately 0.06–0.08% were novel unique miRNAs (Table 2). In total, more than 4000 novel miRNAs were identified in each sample, and 7731 miRNAs were identified in all samples (Table 2). The pre-miRNA sequence and structure of all miRNA are shown in Additional File 6. The occurrence frequency and TPM of all miRNAs are shown in Additional File 7.

3.3. DE miRNA analysis of wheat seedlings after mowing

We combined the DE miRNAs from all samples (T0, T1, T2, and T3) and identified 714 DE miRNAs that were expressed in all regeneration stages of wheat, and 715 miRNAs whose expression was altered in the regeneration stage of mowed wheat (Fig. 4 and Additional file 8). Moreover, 142 DE miRNAs (56 up and 86 down), 119 DE miRNAs (52 up and 68 down), and 187 DE miRNAs (115 up and 72 down) were observed at 2, 24, and 72 h after the wheat seedlings were mowed, respectively. On comparing the DEs with those from 2 h after mowing (T1 treatment), 176 DE miRNAs (100 up and 76 down) and 200 DE miRNAs (129 up and 71 down) were identified during the T2 and T3 sampling times. Furthermore, 65 up-DE miRNAs and 73 down-DE miRNAs were identified between the T3 and T2 regeneration stages. These findings suggest that miRNAs play important roles in the rapid regeneration of mowed wheat seedlings.

3.4. Expression trend cluster analysis of DE miRNA in different regeneration stage

To investigate the expression profiles of miRNAs during the different regeneration stages, trend cluster analysis was used. A total of 26 expression profiles of miRNAs in the regeneration stage are shown in Fig. 5**a**. Profiles 7, 6, 8, 24, 10, and 16 are significant expression profiles, and profiles 7 and 18 are classified as class I (Fig. 5**a**). The expression of miRNAs in this class was changed at 2 h, but not at 24 or 72 h after mowing. The expression of miRNAs in profile 7 decreased after 2 h of mowing, whereas the expression of miRNAs in profile 18 increased 2 h after mowing. Profiles 9–16 were classified as class II, and the expression of miRNAs in this class did not change at 2 h after mowing, but was different at 24 or 72 h after mowing (Fig. 5**a**). The expression of miRNAs in profiles 11 and 17 was altered at 24 h after mowing, whereas the expression of miRNAs in profiles 12 and 13 was only changed 72 h after mowing. However, the expression of miRNAs in profiles 9, 10, 15, and 16 showed changes at 24 and 72 h after mowing. Profiles 0–4 and 21–24 were classified as class III, and the expression of miRNAs in this class was altered at 2, 24, and 72 h after mowing (Fig. 5**a**); the expression of

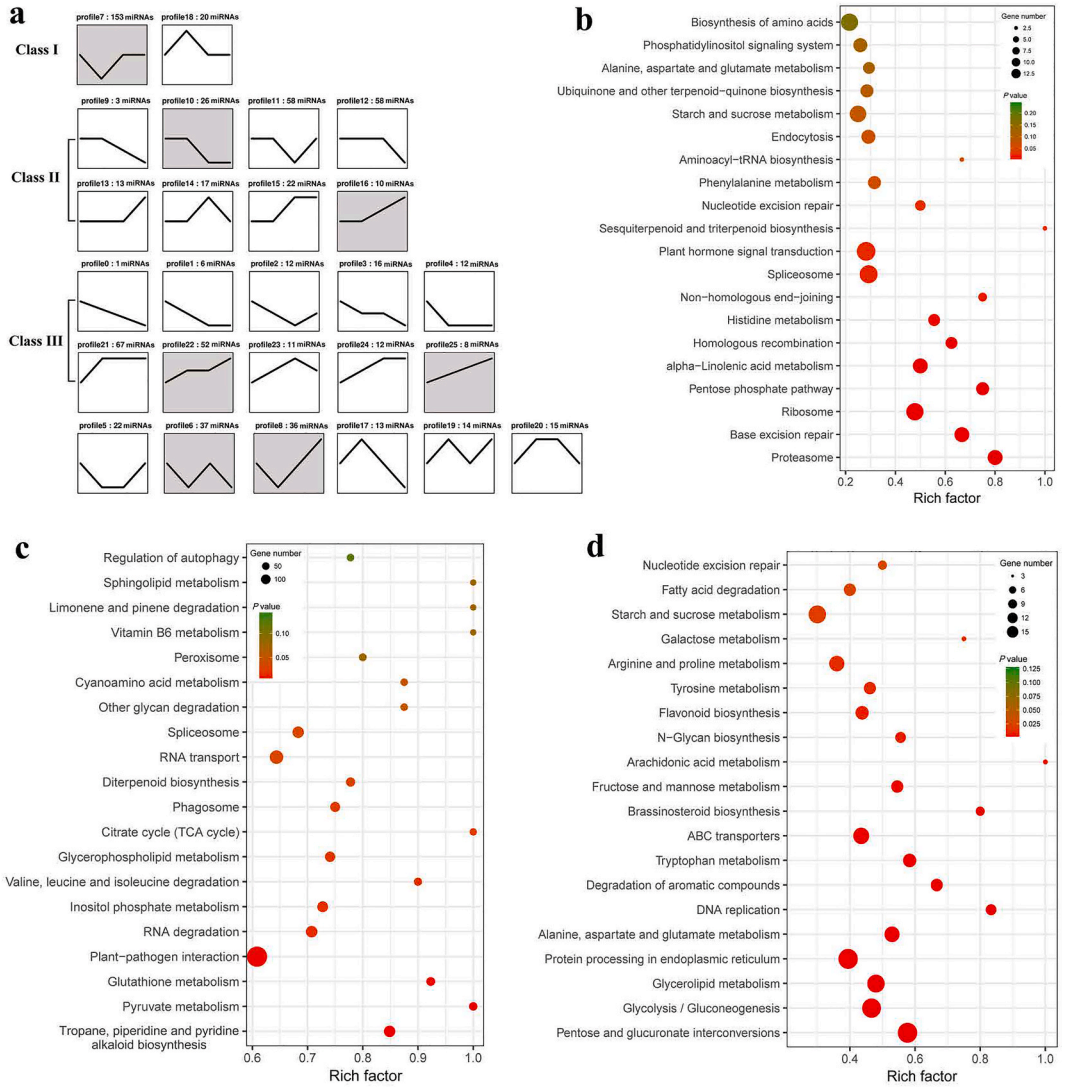

Fig. 5. Expression trend cluster of differentially expressed miRNAs and KEGG enrichment of target genes. **(a)** Cluster analysis of expression trend of differentially expressed miRNAs (DE miRNAs). All DE miRNAs were classified into 26 categories according to their expression level. Class I: miRNA expression changed at 2 h after mowing, but did not change at 24 h and 72 h; Class II: miRNA expression did not change at 2 h after mowing, but changed at 24 h or 72 h; Class III: miRNA expression changed at 2, 24 and 72 h after mowing compared to 0 h **(b, c,** and **d)** KEGG enrichment of target genes of miRNAs in class I, II, and III. The target genes of miRNAs in Class I, II, and III were predicted and enriched by KEGG.

miRNAs in profiles 0–4 was lower at 2, 24, and 72 h after mowing while the expression in profiles 21–25 was higher at 2, 24, and 72 h after mowing.

3.5. *Target gene prediction of miRNAs and function analysis*

Based on target gene prediction, more than 4000 miRNAs in each sample, and 6666 miRNAs in all samples were predicted (Table 3). And 84,378 target genes were present in all samples and 17,987 target genes were present for DE miRNAs (Table 3 and Additional file 9). KEGG analysis of the target genes of miRNAs in classes I, II, and III are shown in Fig. 5. The preferential pathways of target genes in class I were significantly concentrated in the following: proteasome, ribosome, base excision repair, pentose phosphate pathway, alpha-linolenic acid metabolism, homologous recombination, histidine metabolism, non-homologous end-joining, spliceosome, plant hormone signal transduction, sesquiterpenoid and triterpenoid biosynthesis, and nucleotide excision repair (Fig. 5**b**). The preferential pathways of target genes in class II were significantly concentrated in the following: tropane, piperidine and pyridine alkaloid biosynthesis, pyruvate metabolism, glutathione metabolism, plant-pathogen interaction, RNA degradation, inositol phosphate metabolism, valine, leucine and isoleucine degradation, glycerophospholipid metabolism, citrate cycle, phagosome, diterpenoid biosynthesis, RNA transport, and spliceosome (Fig. 5**c**). The preferential pathways of target genes in class III were significantly concentrated in the following: pentose and glucuronate interconversions, glycolysis and gluconeogenesis, glycerolipid metabolism, protein processing in endoplasmic reticulum, alanine, aspartate and glutamate metabolism, DNA replication, degradation of aromatic compounds, tryptophan metabolism, ABC transporters, brassinosteroid biosynthesis, fructose and mannose metabolism, arachidonic acid metabolism, *N*-Glycan biosynthesis, flavonoid biosynthesis, tyrosine metabolism, arginine and proline metabolism, galactose metabolism, starch and sucrose metabolism, fatty acid degradation, and nucleotide excision repair (Fig. 5**d**).

3.6. *Interaction analysis of miRNAs and target genes related to auxins and cis-zeatin*

Among all the DE miRNAs, 18 miRNAs interacted with target genes

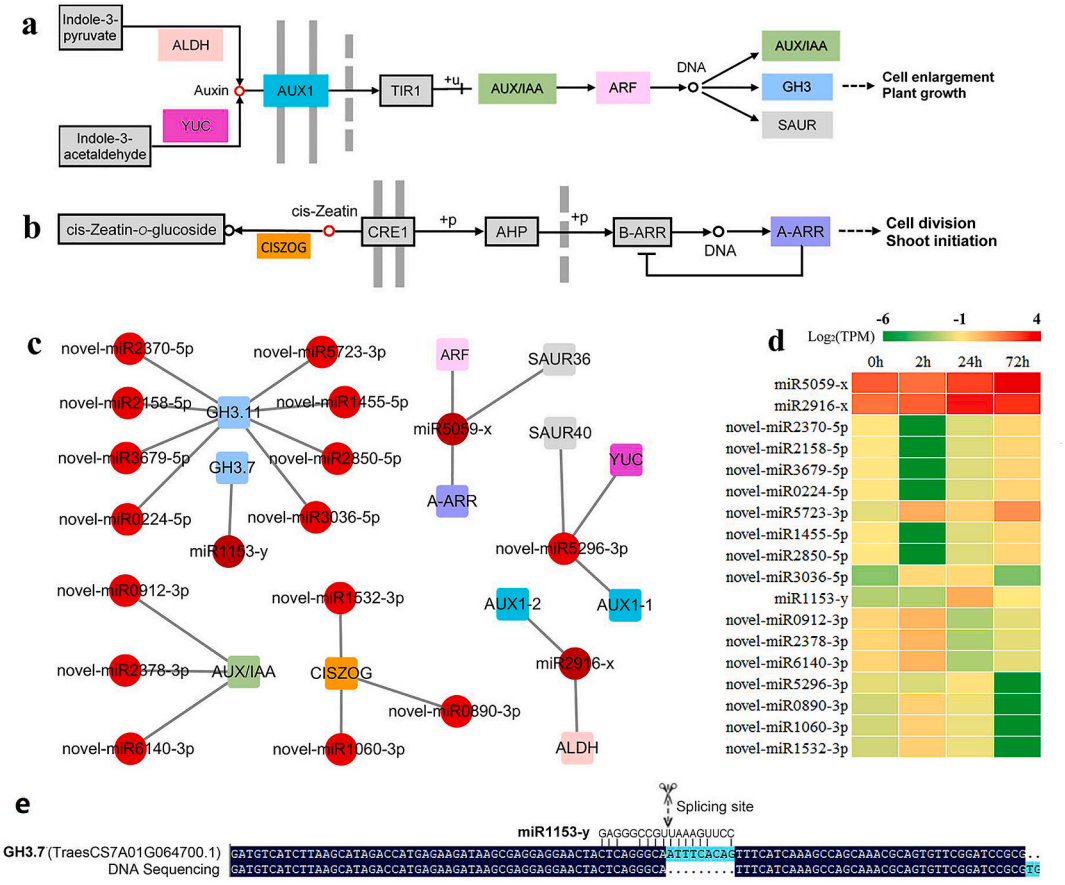

Fig. 6. Interaction analysis of miRNAs and their target genes related to auxin and cytokinin signal transduction. **(a, b)** The signal transduction pathway of auxin and. **(c)** Interaction network of miRNAs and their target genes. **(d)** Heat map of miRNAs targeted to genes related to auxin and CK signal transduction. **(e)** miR1153-y induce the specific cleavage site in target *GH3.7*.

Table 1
Number of identified total tag, clean tag, and unique tag.

Sample	Total tag number	Clean tag number	Unique tag number
T0-1	15,412,064 (100%)	13,943,505 (93.15%)	4,351,959 (28.23%)
T0-2	13,409,848 (100%)	12,052,223 (92.31%)	4,518,654 (33.69%)
T0-3	19,372,964 (100%)	17,959,333 (95.19%)	5,133,577 (26.49%)
T1-1	14,515,377 (100%)	13,229,067 (93.60%)	4,398,440 (30.3%)
T1-2	16,008,404 (100%)	14,528,209 (93.22%)	4,618,487 (28.85%)
T1-3	17,603,520 (100%)	16,489,178 (96.15%)	5,752,981 (32.68%)
T2-1	17,031,248 (100%)	15,731,691 (94.88%)	5,775,145 (33.9%)
T2-2	16,966,593 (100%)	15,746,608 (95.25%)	5,683,729 (33.49%)
T2-3	16,588,116 (100%)	15,122,151 (93.65%)	4,286,433 (25.84%)
T3-1	16,885,703 (100%)	15,393,159 (93.55%)	5,157,076 (30.54%)
T3-2	18,772,019 (100%)	17,029,554 (94.03%)	5,927,629 (31.57%)
T3-3	20,409,399 (100%)	18,878,465 (95.27%)	6,121,636 (29.99%)

Mowed winter wheat.

involved in auxin synthesis and signal transduction (Fig. 6). Nine miRNAs interacted with the auxin responsive GH3 gene family (*GH3.7* and *GH 3.11*); this included eight newly identified miRNAs. According to RACE assay, sequencing results of more than 80% clones (25 clones) showed that miR1153-y was identified as mediating the cleavage of *GH3.7* and the cleavage site is between the 9th and 10th bases of the miRNA (Fig. 6e). And other miRNAs were not validated in the RACE assay though we tested 30 clones for each target genes. A known miRNA, named miR5059-x, interacted with *auxin response factor (ARF)* and *SAUR auxin-responsive protein* (*SAUR*) *36*. A known miRNA, named miR2916-x, interacted with *auxin influx carrier* (*AUX1-2*) and *aldehyde dehydrogenase* (*ALDH*). A newly identified miRNA, named novel-miR5296–3p, interacted with *AUX1-1, SAUR40*, and *indole-3-acetaldehyde oxidase* (*YUC*). In addition, three newly identified miRNAs interacted with *auxin-responsive protein* (*AUX/IAA*), including novel-miR0912–3p, novel-miR2378–3P, and novel-miR6140–3p. Four miRNAs were determined to be involved in interactions with gene involved in cytokinin synthesis and signal transduction (Fig. 6). Three newly identified miRNAs, novel-miR1060–3p, novel-miR1532–3p, and novel-miR0890–3p interacted with *CISZOG*. One known miRNA, miR5059-x interacted with *type-A two-component response regulator* (*A-ARR*).

3.7. Expression analysis of target genes related to auxin and cis-zeatin

The expression of several target genes related to CKs was altered in response to mowing. The expression of *A-ARR*, a negative regulator of CK signal transduction, was significantly inhibited during the regeneration of mowed wheat seedlings. The expression of *CISZOG*, an important gene for cytokinin transformation, significantly increased within 72 h after mowing (Fig. 7**a** and **l**). The expression levels of 10 other genes associated with auxin synthesis and signal transduction were significantly altered. The expression levels of *ALDH* and *ARF* decreased continuously for 72 h after mowing, while the expression of *YUC* was lower at 2 h and 24 h after mowing (Fig. 7**b**, **c** and **k**). The expression levels of two *auxin influx carrier genes*, including *AUX1-1* and *AUX1-2*, were also lower at 72 h after mowing (Fig. 7**e** and **f**). In addition, the expression levels of *AUX/IAA* and *SUAR40* were significantly lower 2 and 24 h after mowing (Fig. 7**d** and **i**) and *SUAR36* expression also decreased 2 h after mowing (Fig. 7**i** and **j**). The expression of *GH3.11* was significantly higher 2 h and 24 h after mowing, while the expression of *GH3.7* was higher 2 h after mowing but lower at 24 and 72 h after mowing (Fig. 7**g** and **h**). These results indicate that the expression of

Table 2
Identified known and novel miRNAs.

Sample	Known miRNAs in wheat			Known miRNAs in databases			Novel miRNAs		
	Number	Unique tags	Total tags	Number	Unique tags	Total tags	Number	Unique tags	Total tags
T0-1	85	1377 (0.03%)	159,307 (1.14%)	282	30,617 (0.70%)	2,016,637 (14.46%)	4399	3068 (0.07%)	30,529 (0.22%)
T0-2	90	1784 (0.04%)	161,204 (1.34%)	303	33,401 (0.74%)	1,238,468 (10.28%)	4360	3090 (0.07%)	36,715 (0.30%)
T0-3	84	1436 (0.03%)	193,807 (1.08%)	276	34,713 (0.68%)	2,630,076 (14.64%)	4706	3408 (0.07%)	36,298 (0.20%)
T1-1	86	1487 (0.03%)	139,065 (1.05%)	297	29,452 (0.67%)	2,055,523 (15.54%)	4438	3225 (0.07%)	32,341 (0.24%)
T1-2	85	1672 (0.04%)	193,278 (1.33%)	330	36,077 (0.78%)	2,233,624 (15.37%)	4520	3243 (0.07%)	51,788 (0.36%)
T1-3	82	1598 (0.03%)	208,292 (1.26%)	286	36,612 (0.64%)	2,397,527 (14.54%)	4994	3645 (0.06%)	34,541 (0.21%)
T2-1	91	1932 (0.03%)	212,992 (1.35%)	321	42,918 (0.74%)	2,045,199 (13.00%)	5318	3804 (0.07%)	50,862 (0.32%)
T2-2	84	1619 (0.03%)	177,102 (1.12%)	318	38,891 (0.68%)	2,260,243 (14.35%)	4999	3658 (0.06%)	36,210 (0.23%)
T2-3	83	1602 (0.04%)	222,612 (1.47%)	299	34,223 (0.80%)	3,255,664 (21.53%)	4896	3447 (0.08%)	51,461 (0.34%)
T3-1	91	1803 (0.03%)	197,009 (1.28%)	331	40,531 (0.79%)	2,410,969 (15.66%)	4879	3523 (0.07%)	57,488 (0.37%)
T3-2	86	1729 (0.03%)	238,001 (1.40%)	319	40,884 (0.69%)	2,419,409 (14.21%)	5263	3836 (0.06%)	41,250 (0.24%)
T3-3	88	1726 (0.03%)	208,516 (1.10%)	301	39,809 (0.65%)	2,307,031 (12.22%)	5256	3876 (0.06%)	44,216 (0.23%)
Total	103			783			6845		

Table 3
Prediction of target gene of miRNA.

Sample	Number of miRNAs	Number of target genes	Number of target sites
T0-1	4120	40,469	101,578
T0-2	4104	42,283	102,871
T0-3	4334	41,828	105,847
T1-1	4162	42,849	103,886
T1-2	4279	46,859	113,224
T1-3	4581	38,590	100,938
T2-1	4956	49,356	126,029
T2-2	4657	41,624	105,542
T2-3	4635	41,618	108,202
T3-1	4603	48,197	118,094
T3-2	4909	46,631	117,754
T3-3	4845	37,486	100,853
total	6666	84,378	235,449

genes related to the synthesis and signal transduction of auxins, except for those belonging to the *GH3* gene family, was inhibited during the regeneration of mowed wheat.

4. Discussion

The compensatory regeneration of winter wheat after mowing or grazing is important for maintaining a certain level of nutrient accumulation and affects the grain harvest. In this study, we investigated the mechanism underlying the compensatory regeneration of mowed winter wheat and discovered that endogenous auxin concentration was decreased and *cis*-zeatin concentration was increased during the regeneration. Furthermore, several miRNAs and their target genes related to the synthesis and signal transduction of auxins and CKs were found to play important regulatory roles in the rapid regeneration of mowed wheat seedlings.

Winter wheat is been widely grown globally for forage production during winter. However, excessive harvesting of wheat during winter affects grain yield (Butchee and Edwards, 2013); the immediate compensatory regeneration of winter wheat is important to maintain yield (Arzadun et al., 2006). Therefore, studying the rapid regeneration of winter wheat after mowing has high practical value. In this study, we demonstrated the rapid regeneration of winter wheat after mowing (Fig. 1 and Additional File 5). During regeneration, the height and fresh weight of young leaves increased rapidly within 72 h after mowing (Fig. 1). The young leaves grew very rapidly to form mature leaves to initiate photosynthesis and accumulate nutrients as soon as possible (Video 1).

During the rapid regeneration of wheat, endogenous auxins and cytokinins, as well as their interactions, play key regulatory roles in the development and growth of young leaves (Gonzalez et al., 2012; Skalák et al., 2019). In this study, we found that endogenous *cis*-zeatin concentrations increased significantly, and auxin concentrations decreased significantly during the regeneration of wheat after mowing (Fig. 2). These results are in agreement with those of a previous study (Schafer et al., 2015). Moreover, the ratio of cytokinin to auxin also increased significantly (Fig. 2), which was beneficial to the growth of buds and young leaves (Müller and Leyser, 2011). These results suggest that the rapid regeneration of wheat after mowing can be attributed to changes in the concentrations of endogenous cytokinins and auxins.

miRNAs (miRNAs) are important regulators of gene expression at the transcriptional level, and they are essential for plant growth and development (Budak et al., 2015). During plant development and morphogenesis, auxins play an important role. MiRNAs regulate the expression of genes related to auxin biosynthesis, degradation, and signal transduction by post-transcriptional modification (Budak et al., 2015). In this study, two miRNAs were associated with auxin synthesis and 15 miRNAs were associated with auxin signal transduction (Fig. 6). This finding suggests that miRNAs regulate the expression of genes related to the synthesis and signal transduction of auxins.

Among all the identified miRNAs, we identified a novel miRNA, named novel-miR5296–3p, which targeted *YUC*, a gene encoding an important enzyme for IAA synthesis (Zhao, 2010). However, its concentration was not completely consistent with *YUC* expression; thus, there may be other regulatory factors involved in the transcription of *YUC*. Moreover, miR2196-x, which was previously identified in *Populus euphratica* (Li et al., 2009), showed a higher concentration in mowed forage wheat in this study (Fig. 6). The gene expression of *ALDH* showed an opposite trend, suggesting a negative regulation of *ALDH* expression by miR2196-x (Figs. 6 and 7). Furthermore, the decrease in *ALDH* expression was consistent with the trend in IAA concentration (Fig. 2). These results indicate that novel-miR5296–3p and miR2196-x act as negative regulators of IAA synthesis.

The AUX protein is an auxin influx transporter and plays an important role in auxin transportation (Vandenbussche and Jzadnikova, 2010). In this study, we identified that increased concentration of miR2916-xwas negatively correlated with the expression of *AUX1-2*, which is homologous to the AUX protein (Figs. 6 and 7). Our data suggest that miR2916-x is involved in the negative regulation of IAA transport. In addition, three novel miRNAs targeted *AUX/IAA* that codes for an important auxin-responsive protein (Liscum and Reed, 2002). Although these miRNAs targeted *AUX/IAA*, they did not completely inhibit its expression and other factors are involved in regulating the expression of *AUX/IAA* (Figs. 6 and 7). Furthermore, miR5059-x, which was first identified in barley (Schreiber et al., 2011), was found to target *ARF* and *SUAR36* (Fig. 6). Auxin regulates plant developmental processes by regulating gene expression via auxin response factors (ARFs) (Boer et al., 2014; Guilfoyle and Hagen, 2007). Notably, the concentration of miR5059-x was negatively correlated with the expression of *ARF* in mowed wheat seedlings, suggesting the negative regulation of

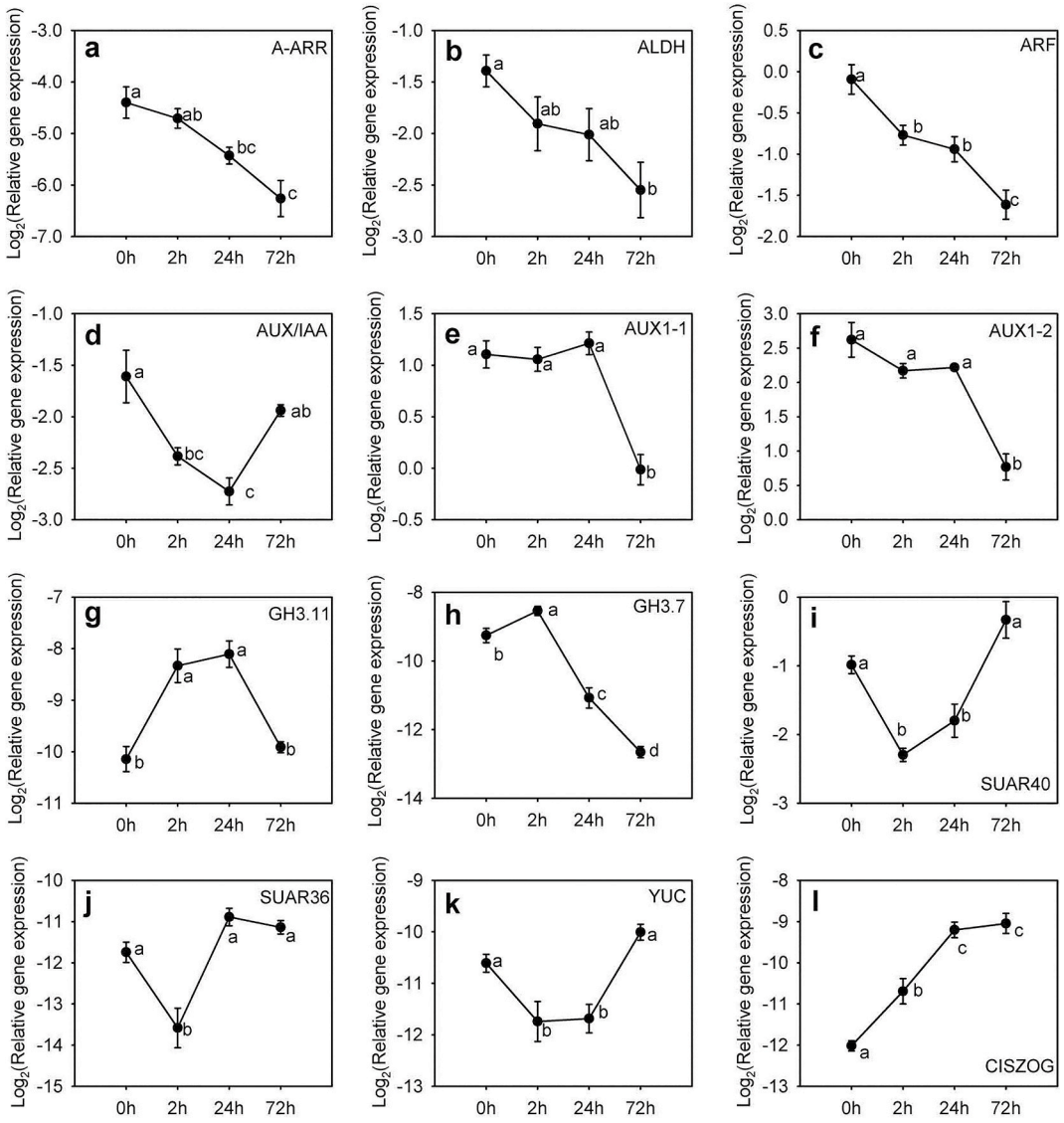

Fig. 7. The relative expression of target genes related to auxin and CK signal transduction. *A-ARR*, type-A two-component response regulator; *ALDH*, Aldehyde dehydrogenase; *ARF*, auxin response factor; *AUX/IAA*, auxin-responsive protein; *AUX1-1*, auxin influx transporter1-1; *AUX1-2*, auxin influx transporter 1–2; *GH3.11*, GH3 family protein11; *GH3.7*, GH3 family protein7; *SUAR40*, SAUR auxin-responsive protein 40; *SUAR36*, SAUR auxin-responsive protein 36; *YUC*, flavin-containing monooxygenase; *CISZOG*, *O*-glucosyltransferase.

ARF gene by miR5059-x (Figs. 6 and 7). Furthermore, *SUAR36* expression was not completely negatively corelated with the concentration of miR5059-x in mowed wheat seedlings, suggesting multiple regulation of *SUAR36* expression. GH3-mediated auxin homeostasis is an essential component in the complex network of auxin actions (Jung-Eun et al., 2007). In this study, the gene expression of two GH3 family members was analysed, and they both showed negative correlation with the concentrations of nine targeted miRNAs (Figs. 6 and 7). Furthermore, miR1153-y mediated the cleavage of GH3.7 (Fig. 6**a**), indicating that miR1153-y negatively regulated the expression of *GH3.7* and affected the regeneration of winter wheat after mowing. The plant miRNAs generally induce a site-specific cleavage in the target mRNA between 10th and 11th nucleotides (Park et al., 2014), but miR 1153-y cleaves *GH3.7* between the 9th and 10th nucleotide. Our study revealed three known miRNAs in the miRbase database, along with 12 novel miRNAs that were found to be involve in auxin biosynthesis and signal transduction, which consequently result in the rapid regeneration of winter wheat after mowing.

CKs have been reported to stimulate the regrowth of barley after mowing (Christiansen et al., 1995). Although cZ has long been considered to have little to no biological activity compared with trans-zeatin and N6-isopentenyladenine, it is also reported to be associated with growth regulation (Schafer et al., 2015), and increased cZ concentrations during the post-mowing regeneration of wheat, which our study also confirmed (Fig. 2). These results indicate that cZ plays a key role in the regeneration of winter wheat.

CISZOG is an important metabolic enzyme, which plays an important role in cZ synthesis, and its overexpression results in a decreased cZ concentration (Toru et al., 2012). However, the expression of *CISZOG* is not consistent with changes in cZ concentration, and our results do not agree with those of previous studies (Toru et al., 2012). It has been proposed that increased expression of *CISZOG* enhances the transformation of cZ into other types of CKs, and helps in maintaining high CK activity under special experimental conditions, such as mowing (Gajdosovä et al., 2011). Furthermore, the decreased expression of novel-miR1532–3p, novel-miR1060–3p, and novel-miR0890–3p targeted *CISZOG* and eliminated the expression suppression of *CISZOG* 72 h after mowing (Fig. 7), which could be attributed to miRNA-guided RNA cleavage (Llave et al., 2002). However, *CISZOG* and miRNA expression were not exactly negatively correlated within 2 h after mowing (Fig. 7),

which indicates the existence of other regulation methods (Dragomir et al., 2018; Yu et al., 2017). The two-component response regulators are important CK receptors, and they transduce signals via alteration of their own phosphorylation states (Kieber and Schaller, 2018). The type-A two-component response regulators were reported to be negative regulators of CK signalling (To et al., 2004). Our results confirmed that lower gene expression of *A-ARR* was consistent with increased CK signalling and rapid regeneration of seedlings (Figs. 1 and 2). We observed that increase in miR5059-x concentration in mowed wheat was negatively correlated with *A-ARR* expression, suggesting that miR5059-x mediated the cleavage or downregulated *A-ARR* (Fig. 7). This effect of miRNAs has been reported in other crops (Branscheid et al., 2015). Thus, our study showed that *cis*-zeatin is involved in the rapid regeneration of mowed forage wheat, and we were able to identify four miRNAs that targeted to genes involved in CK synthesis and signal transduction.

Although this study showed that auxin and *cis*-zeatin play important roles in the compensatory regeneration of mowed wheat seedlings, and revealed the interaction between miRNAs and some key genes related to their biosynthesis and signal transduction, more direct evidences is required to further clarify these interactions. Future studies will focus on these interactions, as well as on finding additional evidence for direct interactions using other methods.

5. Conclusions

This study revealed the importance of auxins and cZ in the rapid regeneration of mowed winter wheat, as demonstrated by the decreased auxin content and increased cZ content in seedlings after mowing. The increased expression of miR5059-x and miR2916-x inhibited the expression of *ARF* and *ALDH*, prevented auxin signal transduction, and thus affected wheat regeneration. Meanwhile, the increased expression of miR5059-x resulted in downregulation of expression of *A-ARR* and activation of signal transduction via CK, thereby inducing the regeneration of mowed forage wheat. Furthermore, miR1153-y was identified as mediating the cleavage of *GH3.7* by RACE assay. Moreover, a decrease in the expression of three novel identified miRNAs (novel-miR1532–3p, novel-miR1060–3p, and novel-miR0890–3p) resulted in increased expression of *CISZOG*, which contributed to the synthesis of potent cytokinins. Therefore, our study shows that miRNAs play important roles in the post-mowing regeneration of winter wheat by regulating the expression of genes related to the synthesis and signal transduction of auxins and *cis*-zeatin.

Author contributions

Y X and C Z designed and directed this study, and they are also drafted the manuscript. G C and M Z performed the experiments and analysed the data, and they are also revised the manuscript. S Z conducted the miRNA-directed cleavage analysis of target mRNAs. M M determined the levels of endogenous auxins and CKs. S L and Z W improved the data analysis and revised the manuscript. All authors have read and approved the manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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