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Integration of small RNAs, transcriptome and degradome sequencing reveals the mechanism of high photosynthetic efficiency in *Medicago ruthenica* (L.)

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Abstract

Background: *Medicago ruthenica*, a superior leguminous forage, is strongly drought-resistant and has wide adaptability. It is closely related to *Medicago sativa* (a commonly cultivated forage). Characterized by its high tolerance to environmental stress, *M. ruthenica* is a valuable genetic resource. However, its low yield limits its large-scale promotion and utilization. Leaf morphology, an important agronomic trait, is closely related to forage yield and photosynthetic efficiency.

Results: In this study, leaf width and leaf area were found significantly positively correlated with forage yield in *M. ruthenica*. Specifically, the cultivar *M. ruthenica* ‘Mengnong No.1’ (MN No.1) with a larger leaf area had higher photosynthetic efficiency. Furthermore, the key miRNA-target gene networks associated with high photosynthetic efficiency of *M. ruthenica* were identified using small RNAs and transcriptome sequencing. Totally, 63 differentially expressed miRNAs (DEMs) were identified, including some known miRNAs (miR408, miR171, and miR398, etc.). These known miRNAs corresponded to 55 target genes; 6 miRNA-target gene pairs had negatively correlated expression patterns. Degradome sequencing analysis further confirmed the authenticity of miR408-targeted cleavage of *LAC5*; namely, the miR408-*LAC5* module may mediate the mechanism of high photosynthetic efficiency formation in *M. ruthenica*.

Conclusions: This study for the first time proposed that leaf morphology as a key trait affecting the high-yielding plant type of *M. ruthenica*. The key gene modules screened in this study may provide new insights into the elucidation of photosynthetic efficiency mechanism and the bio-design breeding of legume forages in the future.

Keywords: *Medicago ruthenica*; photosynthetic efficiency; yield formation; leaf morphology; sequencing

Background

Increasing forage yield has always been a priority for the development of herbivorous animal husbandry industry. Photosynthesis has been shown to tightly affect plant yield accumulation, which can be greatly influenced by the solar radiation capture and photosynthetic efficiency [1]. Moreover, leaf shape, the important photosynthetic organ in plants, strongly affects plant architecture and yield [2, 3]. Different leaf morphology results in varied photosynthetic performance among different cultivars or the same cultivars in different environments [4]. As has been evidenced previously,

leaves must be wide enough for absorbing sufficient light energy, and flat and thin enough for facilitating gas exchange (CO_2 , O_2 , and H_2O) [5]. Leaf morphogenesis is mainly influenced by genetic and environmental factors; leaves may exhibit considerable morphological diversity among species and cultivars [6]. Additionally, light conditions can also influence leaf structure; low light intensity can produce one layer of palisade tissue with a round shape, whereas under high light conditions, palisade cells present in two or three layers and polarized in the direction of leaf thickness with column shape [7]. Therefore, the optimization of leaf shape is the key to breeding high photosynthetic efficiency forages.

MicroRNAs (miRNAs) are RNAs with 19–24 nucleotide (nt) in length in eukaryotes. miRNAs play important roles in the whole process of plant growth, which is mediated by regulating the expression of target genes at the post-transcriptional level through direct cleavage or inhibition of translation [8]. Accumulating studies have provided evidence that miRNAs participate in modulating plant morphology, yield, photoresponse, etc. [9–11]. The conserved miR408 family has been identified in many plant species [12, 13]. The basic function of miR408 in plants has been reported. As previously reported, miR408 expression can be induced in plant by different abiotic stresses, such as copper (Cu), light, and low-temperature stress [14–18].

Cu is an indispensable transition metal for plants. Several plant miRNAs can be induced by Cu deficiency, and can post-transcriptionally repress the transcripts encoding Cu proteins which are hence named as Cu-miRNAs [19–21]. It has been shown that miR408 targets several genes encoding Cu-binding proteins; these proteins are members of laccase and phytoecyanin [21]. Laccases (such as *LAC3*, *LAC4*, *LAC17*, and *LAC11*) are Cu-containing oxidase enzymes, which are closely implicated in lignin monomer synthesis [22–24]. Plastocyanin is also a Cu-binding protein, which is the reaction centre of photosystem I (PSI) and acts as an electron transport carrier in eukaryotic photosynthetic organisms [25, 26]. Currently, it has been proposed that miR408 can facilitate Cu delivery to plastocyanin, thereby regulating photosynthesis [27]. As has been evidenced, miR408 overexpression in model plants indeed improves photosynthetic performance [28]. Additionally, multiple studies have confirmed that miR408 overexpression can increase leaf area, plant height, plant biomass, seed yield, etc. [18, 29, 30].

Medicago ruthenica (L.), as a perennial legume forage [31], is closely related to alfalfa (*Medicago sativa*), which is an excellent germplasm material for cross breeding of *Medicago* genus [32, 33]. *M. ruthenica* is highly adaptable to drought, low temperature, high temperature, saline-alkali and other harsh environment, so it is considered as a valuable forage for both artificial and natural grasslands [34, 35]. However, the forage yield and seed yield of *M. ruthenica* is low, which limits its large-scale popularization and application. Therefore, high-yield breeding has always been an important direction of *M. ruthenica* breeding [36]. It has been shown that plant architecture is fundamentally important to plant growth and productivity [37, 38]. The plant architecture of *M. ruthenica* varies significantly, and its germplasm resources differ greatly in growth habit, leaf shape, plant height, and seed size [35, 39]. Recently published genome information provides a powerful reference for investigating the mechanisms of yield-related agronomic characteristics in *M. ruthenica* [40].

In this study, the forage yield, photosynthetic characteristics, and yield-related traits of three *M. ruthenica* cultivars were compared. It was found that there was a significant positive correlation between leaf morphological characteristics and forage yield in *M. ruthenica*. Namely, the cultivar with wide leaves and large leaf area showed the highest photosynthetic utilization efficiency and high yield. To reveal the mechanisms of high photosynthetic efficiency in *M. ruthenica* leaves, we

performed a multiomics analysis to identify the potential regulation of miRNA-target pairs in *M. ruthenica* leaf morphogenesis. The results of the present work explored the molecular regulatory network for the high photosynthetic efficiency of *M. ruthenica*. Moreover, this study provided important guiding significance for the bio-design of high-yield *M. ruthenica*.

Results

Leaf morphological and anatomical characteristics of three *M. ruthenica* cultivars

As shown by the varietal yield test results of three *M. ruthenica* cultivars (ZLX, MN No.1, and MN No.2), MN No.1 was outstanding in high yield ($p < 0.05$, **Table S1**). There was a significantly positive correlation between leaf morphological characteristics and yield (**Table S2**). The leaf morphology of three *M. ruthenica* cultivars was notably different (**Fig. 1A**). The leaf width of MN No.1 (8.86 mm) was remarkably higher than that of ZLX (4.75 mm) and MN No.2 (3.08 mm) ($p < 0.05$) (**Fig. 1B**). Based on the ratio of leaf length to width, the leaf morphology was discriminated. Specifically, The length-width ratio of MN No.1 had a length-to-width ratio of 1.85, showing a broad oval shape; ZLX was oval with a ratio of 3.65; MN No.2 was in the shape of a long strip with a length-to-width ratio of 5.13 (**Fig. 1B**). The leaf area of MN No.1 was 32.12% and 62.31% larger than that of ZLX and MN No.2, respectively ($p < 0.05$, **Fig. 1C**).

According to the leaf anatomic staining results, *M. ruthenica* showed typical dorsoventral leaves; the mesophyll was divided into palisade tissue and spongy tissue (**Fig. 2**). MN No.1 showed a significantly higher palisade tissue thickness than ZLX and MN No.2, while its spongy tissue thickness was notably lower than the other two cultivars ($p < 0.05$) (**Table 1**). Therefore, MN No.1 had the largest thickness ratio of palisade tissue to spongy tissue, indicating that it had developed palisade tissue. Additionally, among these three *M. ruthenica* cultivars, MN No.1 leaves had the highest cell tense ratio (CTR), and MN No.2 had the lowest CTR (**Table 1**), which indicated that the cell structure of MN No.1 leaves was the most compact, while that of MN No.2 leaves was the loosest. Moreover, MN No.1 leaves showed a dramatically higher thickness of the upper epidermis and lower epidermis than ZLX and MN No.2 ($p < 0.05$, **Table 1**).

Leaf photosynthetic characteristics of three *M. ruthenica* cultivars

Chlorophyll (Chl) plays an important role in light absorption during photosynthesis. The Chl concentration of three *M. ruthenica* cultivars with different leaf morphology was compared (**Table 2**). MN No.1 had the highest content of chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (Chl t), and carotenoids (Car), followed by ZLX and then MN No.2, with these contents of MN No.1 and ZLX significantly higher than MN No.2 ($p < 0.05$) (**Table 2**). Additionally, the ratio of chlorophyll a to chlorophyll b (Chl a/b) of MN No.1 and ZLX was also notably higher than that of MN No.2 ($p < 0.05$) (**Table 2**).

Subsequently, the effect of light intensity on the net photosynthetic rate (Pn) of three *M. ruthenica* cultivars was explored. Under low light intensity ($\text{PAR} < 200 \mu\text{mol} \cdot \text{CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), the Pn of three *M. ruthenica* cultivars increased linearly, but the increasing amplitude was gradually decreased with the increase of light intensity (**Fig. 3**). There were significant differences in Pn among these three cultivars. Based on the leaf light response curve, photosynthetic characteristic parameters were calculated (**Table 3**). It was found that MN No.1 had noticeably higher maximum Pn (P_{max}), apparent quantum efficiency (AQE), and light saturation point (LSP) than ZLX and MN No.2 ($p < 0.05$), while the dark respiration rate (R_d) and light compensation point (LCP) of MN No.1 were significantly lower than those of other two cultivars ($p < 0.05$) (**Table 3**). In conclusion, MN No.1

exhibited the strongest light energy utilization efficiency.

Small RNA sequencing and identification of differentially expressed miRNAs (DEMs) in three *M. ruthenica* cultivars

To identify miRNAs involved in the morphology of *M. ruthenica* leaves, we performed small RNA sequencing of 9 leaf samples. An overview of reads from raw data to cleaned sequences is listed in **Table S3**. After deleting low-quality sequences, the obtained sequences (18-25 nt in length) are listed in **Table S4**. The expression levels of miRNAs in three cultivars were analyzed and compared. A total of 63 miRNAs with differential expression level was identified ($p < 0.01$, **Table S5**). The numbers of DEMs among the three cultivars was identified (**Fig. 4A**). As shown by the heatmap of DEMs the identified DEMs included 24 known miRNAs (such as miR398b, miR171d, miR156h, miR160f, miR169j, and miR408), and 39 novel miRNAs (**Fig. 4B**). Members of the same miRNA family showed consistent expression patterns. For example, 5 miR408s were significantly upregulated in the cultivar MN No.1.

Annotation and enrichment analysis of the target genes for DEMs

Transcriptome libraries were constructed, and the transcriptome sequencing (RNA-seq) details of 9 samples in *M. ruthenica* are shown in **Table S6**. According to the results, a total of 5047 and 457 genes were annotated with enriched GO terms and KEGG pathways, respectively. Additionally, 816 target genes for DEMs were obtained from three libraries; 689 target genes were annotated into three main GO categories: biological process, cellular component, and molecular function. Visualization of the enrichment terms was processed using REVIGO software [41] (**Fig. 5A-5C**). Under biological processes, leaf morphogenesis-associated terms were identified, such as meristem initiation (GO:0010014), signal transduction (GO:0007165), DNA-templated transcription (GO:0006351), cell differentiation (GO:0030154), polarity specification of adaxial/abaxial axis (GO:0009944), xylem development (GO:0010089), and callose deposition in cell wall (GO:0052543) (**Fig. 5A**). Under the cellular component, SCF ubiquitin ligase complex (GO:0019005), extrinsic component of plasma membrane (GO:0019897), cytoplasmic ubiquitin ligase complex (GO:0000153) and U5 snRNP (GO:0005682) were the most significantly enriched terms ($p < 0.001$, **Fig. 5B**). Under molecular function, all terms were notably enriched, including ADP binding (GO:0043531), ATP binding (GO:0005524), miRNA binding (GO:0035198), signaling receptor activity (GO:0038023), copper ion binding (GO:0005507), etc. (**Fig. 5C**).

The top 20 most enriched KEGG pathways are shown in **Fig. 5D**. It was found that plant-pathogen interaction (ko04626), spliceosome (ko03040), autophagy-other eukaryotes (ko04136), synthesis and degradation of ketone bodies (ko00072), and plant hormone signal transduction (ko04075) were the remarkably enriched pathways ($p < 0.01$), which indicated that these pathways were tightly implicated in the leaf morphogenesis of *M. ruthenica*.

Correlation analysis of the expression profiles of the miRNAs and their target genes

For investigating the genes regulating leaf morphology in *M. ruthenica*, differentially expressed genes (DEGs) among the three cultivars were analyzed. As shown by RNA-seq results, 1540, 1956 and 3014 DEGs were identified among three comparison groups (ZLX vs MN No.1, ZLX vs MN No.2, and MN No.1 vs MN No.2), respectively (**Fig. S1, Table S7**).

In a survey, the expression level of miRNAs in miR160, miR169, miR408, miR398, miR171, miR166, and miR156 families was significantly different among three *M. ruthenica* cultivars ($p < 0.01$, **Fig. 6A**). Subsequently, a total of 55 target genes corresponding to these key known DEMs

were identified (**Fig. 6B**). A total of 8 pairs of miRNA-known target genes were significantly different among these three groups, in which 6 pairs were negatively correlated (**Table 4**). For example, ath-miR408-3p was significantly upregulated in the cultivar MN No.1, while its target gene *HSR201*, *PGR*, and *LAC5* were notably downregulated in MN No.1. mtr-miR171d was downregulated, while its target gene *SNUPN* was upregulated in MN No.1 (MN No.1 vs MN No.2). These miRNA-target gene pairs may regulate leaf morphological development.

Furthermore, the expression of these miRNA–target pairs was verified using real-time quantitative PCR (qRT-PCR). The expression trends of 3 miRNAs (miR408, miR171d, and miR398b) and 6 target genes (*HSR201*, *PGR*, *LAC5*, *SNUPN*, *ACR8*, and *At5g64700*) were similar to those obtained by deep sequencing (**Fig. 7**). The negative correlation of the expression level of these miRNA-target pairs was confirmed. Namely, miR408 family members were notably upregulated in the cultivar MN No.1 ($p < 0.01$, **Fig. 7A**), while their target genes *HSR201*, *PGR*, *LAC5*, and *ACR8* showed significant opposite expression patterns ($p < 0.01$, **Fig. 7B**). miR171d was significantly upregulated in the cultivar MN No.2 ($p < 0.01$, **Fig. 7A**), while its target genes *SNUPN* and *At5g64700* were remarkably downregulated ($p < 0.01$, **Fig. 7B**).

Identification of miRNA targets via degradome sequencing in *M. ruthenica*

After integrating transcriptome and small RNA sequencing data, the expression network of miR408, miR171, and their target genes was constructed (**Fig. 8**). In the network, 5 miR408 family members and 3 miR171 family members were identified. The network plot for miR408 and its target genes was associated with 17 known KEGG pathways (including ko00620, ko02010, ko03015, and ko00330, etc.). miR171 and its target genes involved 3 known KEGG pathways (ko03015, ko04075, and ko00100) (**Table S8**). The ko03015 was found involved both in ath-miR408-3p_R-3-*CID9* and mtr-miR171g-*CPSF100* pairs. However, the authenticity of miRNAs cleaving target genes needs to be further verified.

Degradome sequencing is currently accepted as a promising method for combining high-throughput sequencing with large-scale miRNA target screening [42, 43]. In this study, the authenticity of miRNAs and their predicted target genes in three groups of *M. ruthenica* was further verified using degradome sequencing. The miRNAs-target genes association analysis based on degradome sequencing has been summarized in **Table S9**. After transcriptome and small RNA sequencing analysis, the miRNA-target gene pairs were obtained, mainly involving miR408, miR171, and their target genes. Several key target plots (T-plots) of miR408, miR171 and their target genes validated by degradome sequencing are shown in **Fig. 9**. It was observed that ath-miR408-3p_R-3 could cleave the target gene *LAC5* (evm.model.fragScaff_scaffold_247_pilon.59) through a specific site (**Fig. 9A**), however, the other predicted target genes were not matched in the degradome sequencing. Interestingly, degradome sequencing results revealed some more plausible miRNA-target gene pairs, such as miR408-targeted cleavage *ARPN* (evm.model.fragScaff_scaffold_51_pilon.97) (**Fig. 9B**) and *NAP-1* (evm.model.fragScaff_scaffold_104_pilon.86) (**Fig. 9C**), and miR171-targeted cleavage *SCL6* (evm.model.fragScaff_scaffold_74_pilon.214) (**Fig. 9D**). These findings may provide additional clues to elucidate the mechanism of high photosynthetic efficiency in *M. ruthenica*.

Discussion

It has been widely accepted that enhancing photosynthetic efficiency is critical for advancing forage crop yield [44–46]. Plant architecture and leaf morphology can influence light intensity, thus

affecting the photosynthetic ability of plants [47]. These characteristics can be considered to play a role in dictating forage performance and yield; optimizing these developmental characteristics is essential for high-yield forage breeding. Our results showed a significant positive correlation between leaf morphology and hay yield in *M. ruthenica* ($p < 0.01$). The photosynthetic indexes of three *M. ruthenica* cultivars were compared. The broad-leaved cultivar (MN No.1) had the highest photosynthetic pigment content and Pn. Namely, the *M. ruthenica* cultivars with high photosynthetic efficiency had a large leaf area. Consistently, Tsukaya [5] has also reported that leaf morphology is the key to affecting photosynthesis; leaves must be as wide as possible for absorbing sufficient light energy. Moreover, accumulating studies have provided evidence for the relationship between leaf anatomical structure and photosynthetic characteristics in many species [48-50]. In this study, the leaves of three *M. ruthenica* cultivars were observed by paraffin sectioning to further clarify the relationship between leaf anatomical structure and high photosynthetic efficiency. The results showed that cultivar MN No.1 with high photosynthetic efficiency exhibited a greater leaf thickness than the other two cultivars. Consistent findings have also been reported by McClendon [51] and Nikolopoulos et al. [52]. Thicker leaves have relatively large mesophyll cells [53, 54], and mesophyll cells contain a large number of chloroplasts that functions as the main sites for photosynthesis in plants. The increase in mesophyll cell density leads to an enlarged leaf area, which in turn affects the photosynthetic capacity of leaves [55]. In this study, the mesophyll cells of *M. ruthenica* differentiated into palisade tissues and spongy tissues; the ratio of palisade tissues to spongy tissues was different among the three *M. ruthenica* cultivars. Consequently, MN No.1 with strong photosynthetic capacity has developed palisade tissues and high cell tightness. Taken together, engineering leaf anatomy using gene editing technology to modulate cell proliferation and expansion is a promising method for improving photosynthetic efficiency in the future.

Indeed, there remain many challenges in high-yield *M. ruthenica* breeding, including photosynthetic efficiency and molecular mechanisms controlling leaf morphology. It's well-accepted that miRNAs are essential regulators of gene expression and are involved in multiple aspects of plant architecture. For example, the miR160 family members target the ARFs genes that are involved in the auxin signal transduction pathway, which is critical for plant development and stress-induced responses [56, 57]. miR169 and its key target NFYA genes participate in plant physiological and biochemical activities through an abscisic acid-mediated hormone signaling pathway [58, 59]. miR398 has been proposed to be directly involved in the regulatory network of plant stress, which regulates plant responses to drought stress, salt stress, oxidative stress, copper, and bacterial infection, etc. [60]. Knockdown of miR166 can improve the drought resistance of plants, which can also lead to morphological changes, such as plant dwarfing, leaf curling, and xylem diameter reduction [61, 62]. The regulatory network regarding the miR156-SPL module is highly conserved in different plant species, which plays important roles in regulating plant stress resistance and production performance [63]. The expression of the above miRNAs was significantly different among the three *M. ruthenica* cultivars. Specifically, mtr-miR160f-p5 and mtr-miR169j were highly expressed in the cultivar ZLX, however, they were poorly expressed in MN No.1. ath-miR398b-3p_R-1, gma-miR166i-p5_1ss10TC, and ppe-miR156h-p3_2ss4GA19TC_1(2) showed high expression abundance in the cultivar MN No.2 (**Fig. 6A**). It is worth mentioning that the predicted target genes of these miRNAs showed no significant expression difference among the three cultivars, which suggested that these target genes may also respond to other metabolic processes in the growth and development of *M. ruthenica*.

Plant miRNAs are known to negatively regulate the expression of target genes at the post-transcriptional level through cleavage or/and translational inhibition [8, 64, 65]. In this study, 66 miRNA-target gene pairs with negative regulation were identified. mtr-miR171d showed a high expression abundance in the cultivar MN No.2, which was predicted to negatively regulate the expression of its target genes *SNUPN* (evm.model.fragScaff_scaffold_93_pilon.67) and *At5g64700* (evm.model.fragScaff_scaffold_65_pilon.357). It has been shown that miR171 regulates the SCL transcription factor family members to play important roles in plant development, stress response, and photochrome signaling, etc. [66, 67]. Software prediction and degradome sequencing identified *SCL6* (evm.model.fragScaff_scaffold_74_pilon.214) as a target gene of mtr-miR171d-p3; but *SCL6* expression showed no significant expression difference among the three cultivars (**Supplementary Fig. 2**); these findings suggested that the miR171d-*SCL* pair was not the regulator of the leaf morphogenesis of *M. ruthenica*. mtr-miR171d-*SNUPN* and mtr-miR171d-*At5g64700* pairs in *M. ruthenica* also provided a reference for the module analysis of plant architecture formation of legume forage. The other four pairs are target genes (*LAC5*, *PGR*, *HSR201*, and *ACR8*) for miR408.

miR408 is a typical multifunctional miRNA that has been identified in many plant species [68]. A previous study has shown that high-level miR408 accumulation in plants can improve photosynthesis through enhancing photosynthetic efficiency and CO₂ fixation capacity [18]. As miR408 hyper-accumulation can improve the overall photosynthetic performance, its potential of increasing the yield has also been verified in several model plants [18, 29, 30]. In the present study, miR408 was highly expressed in the cultivar MN No.1 with high photosynthetic efficiency, with its expression approximately 8-fold higher in MN No.1 than that in MN No.2 and ZLX (**Fig. 7A**).

The results of degradation sequencing and psRobot prediction indicated that the targets genes of miR408 could encode Cu proteins, including the laccase genes *LAC3* and *LAC5*. *LAC5* is a target gene negatively regulated by ath-miR408-3p, whereas *LAC3* expression was consistent with the ath-miR408-3p expression trend. As has been evidenced previously, Cu miRNAs are upregulated in the absence of Cu, which can mediate the post-transcriptional downregulation of transcripts encoding Cu proteins [19]. Additionally, it has been shown that the Cu miRNAs tightly participate in regulating various processes of plant growth and development [21]. miR397 and miR857 have been reported to regulate lignin biosynthesis in *Arabidopsis* via targeting several laccase genes [69, 70]. miR397-*OsLAC* can influence the plant architecture and yield of rice [71]. We have also previously reported that the miR397-*LAC17* module affects lignin synthesis in *M. ruthenica* [39]. In the current work, we proposed that modulating miR408-*LAC5* expression pattern may enhance the photosynthetic efficiency and thus increase the yield in *M. ruthenica*.

Conclusion

In conclusion, we identified miRNAs and their target genes involving high photosynthetic efficiency in *M. ruthenica* were identified through small RNA, transcriptome, and degradome sequencing. However, the complicated miRNAs-mediated regulatory network remains to be further clarified. It is worth mentioning that miR408 was highly expressed in the high-yield cultivar MN No.1, which negatively regulated laccase gene (*LAC5*) expression. In other words, the module miR408-*LAC5* may be closely implicated in the photosynthetic response of *M. ruthenica*. This work provide a reference for revealing the mechanism of high photosynthetic efficiency in *M. ruthenica*. Moreover, this study may lay a solid basis for further research on miRNAs and their target genes in breeding high-yield plant architecture in legume forage.

Materials and methods

Plant materials preparation

M. ruthenica ‘Zhilixing’ (hereinafter referred to as ZLX), *M. ruthenica* ‘Mengnong No.1’ (MN No.1) and *M. ruthenica* ‘Mengnong No.2’ (MN No.2) were used in this study. ZLX was registered as a new variety by China Forage Variety Registration Committee in 1993 (Registration No.: 130). MN 1 and MN 2 were approved by the Forage Variety Resources Registration Committee of Inner Mongolia Autonomous Region of China in 2019 (Registration No.: 003-2019) and 2020 (Registration No.: 020-2020), respectively. Before the experiment, all the plants were cultivated (111°23'46"E, 40°31'17"N) for more than 10 years, showing stable phenotypic characteristics. The seeds were sown at an base (111°22'30"E, 40°41'30"N) at August end, 2018 [39]. Leaves at the early flowering stage were sampled in 2020. The collected samples were then immediately frozen with liquid nitrogen, followed by storage (-80°C) for the next-step experiment.

Leaf phenotype and leaf anatomy

The 3-month-old plants were measured for leaf morphology. A total of 20 leaves were selected (10 cm below the top of each plant). Leaf length and width (the widest position) of the middle leaflet of a ternately compound leaf were determined using a vernier caliper, followed by measuring leaf area with mesh paper with an accuracy of 1 mm (1 mm² per grid).

The anatomical structure of leaves at the early-flowering stage was observed. Specifically, following procedures published previously [39], leaves at the top of each plant (sampled in four directions: east, west, north, and south) were fixed in formalin-acid-alcohol (FAA) solution and dehydrated with xylene and ethanol, followed by the dehydration using a xylene-chloroform mixture. Next, the samples were cleared, paraffin-embedded, sectioned, and dewaxed. After that, the sections underwent safranin O/ Fast green staining, and then the observation under an optical microscope.

Chlorophyll concentration

Leaf samples were collected from 10 plants for each cultivar in four directions (east, west, south, and north). Leaves (0.2 g fresh weight, FW) were prepared. Chl was extracted utilizing 10 ml (V) of 80% acetone away from light until the leaf become white. The optical density (OD) value at 663 nm (OD_{663}), 645 nm (OD_{645}), and 470 nm (OD_{470}) was measured using Alan's method for detecting Chl a and Chl b [72]. Chl t and Car were calculated as follows.

Chlorophyll concentration (mg·L⁻¹): (1)-(4).

$$Chl\ a = 12.2 \times OD_{663} - 2.81 \times OD_{645} \quad (1)$$

$$Chl\ b = 20.13 \times OD_{645} - 5.03 \times OD_{663} \quad (2)$$

$$Chl\ t = Chl\ a + Chl\ b \quad (3)$$

$$Car = (1000 \times OD_{470} - 3.27 \times Chl\ a - 104 \times Chl\ b) / 229 \quad (4)$$

Chlorophyll content (mg·g⁻¹) = (Chlorophyll concentration × Extraction volume × Dilution ratio) / FW

Photosynthetic characteristics

According to the slightly modified method of Li et al. [73], the photosynthetic light response curves of the expanded leaves in three *M. ruthenica* cultivars were measured. The photosynthetic active radiation (PAR) in leaf chamber of Li-6400 photosynthesis system was adjusted by applying a red and blue light source; the light intensity was set as 0, 50, 100, 150, 200, 400, 600, 800, 1000, 1200, 1500, and 2000 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The Pn, stomatal conductance (Gs), intercellular CO₂ concentration (Ci), and transpiration rate (Tr) of three cultivars under different light intensities were measured from 9:00 to 11:00 am. Test material selection and repeated setting in light response curve determination were similar to that in Chl concentration determination. The optical response curve was fitted by the Farquhar model [74], and the calculation formula was as follows:

$$P_n = \frac{\text{light} \times \text{AQE} + P_{\max} - \sqrt{(\text{light} \times \text{AQE} + P_{\max})^2 - 4 \text{light} \times \text{AQE} \times P_{\max}}}{2K} - R_{\text{day}} \quad (6)$$

In the above formula, AQE is the apparent quantum efficiency, R_{day} is the dark respiration rate, K is the bending angle of the light response curve, and $P_{\max}$ is the maximum Pn. The LSP and LCP parameters were calculated using the Richardson method [75].

RNA extraction and cDNA synthesis

Total RNA extraction and cDNA synthesis from leaves of three *M. ruthenica* cultivars were conducted as previously published [39].

Transcriptome sequencing and data analysis

RNA-seq analysis was performed on Illumina Novaseq™ 6000 (Lianchuan Biotechnology Co., Ltd., Hangzhou, China) using three cultivars of high-quality RNA in three cultivars following a set protocol. For obtaining the information on reads, Hisat2 [76] was used to perform reference genome alignment on the valid data after preprocessing. Genome information of *M. ruthenica* (accession WNNG00000000) was obtained by Wang [40].

Fragments per kilobase million (FPKM) values served for gene expression normalization. DE-Seq software was employed for identifying DEGs among different groups [77]. Genes with a corrected *P*-value of < 0.05 and |log₂ FC| of > 1 were considered as DEGs. Afterwards, Gene Ontology (GO) (<http://www.geneontology.org>) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (<https://www.genome.jp/kegg/>) enrichment analyses were carried out on all DEGs; significant enrichment was identified with $p \leq 0.05$ as a threshold in hypergeometric tests.

Small RNAs sequencing and miRNA basic analysis

Small RNA libraries of 9 *M. ruthenica* samples were constructed using TruSeq Small RNA Sample Prep Kits (Illumina, San Diego, USA), which were then sequenced by Illumina Hiseq 2500 (LC Sciences, USA) with single-end (SE50) sequencing (50 bp). Clean reads were obtained using an in-house tool designated ACGT101-miR (LC Sciences). Subsequently, unique sequences containing 18-25 bases were mapped to miRBase 22.0 by a BLAST search for miRNAs identification. miRNA family classification, miRNA frequency in other species, miRNA precursor number in different species and miRNA base preference were statistically analyzed. The specific calculation method was referred to the previous report [39].

Degradome libraries construction and analysis

The construction of degradome libraries was simplified and optimized based on previous report [78]. A NanoDropND-1000 (NanoDrop, Wilmington, DE, USA) was employed for the quantification of total RNA and the purity. After that, Poly (A) RNA was purified from ~20 µg RNA of three cultivar groups using poly-T oligo-attached magnetic beads. The first strand cDNA was synthesized using a 3-adaptor random primer. Next, PCR was used for amplifying degradome libraries; Illumina HiSeq2500 (LC Bio, China) was applied for sequencing (single-end sequencing read with 1×50 bp length).

The CleaveLand 4.0 program was used for degradome analysis [79]. We identified mRNAs that matched the degradome sequence using oligomap short reading frame calibrator. GSTAr.pl, Generic Small RNA Transcript Aligner was used for predicting the mRNA sequences of target genes paired with the miRNA sequences. Common mRNA (the target gene of miRNA) was identified after combining the predicted miRNA-mRNA pairs with mRNA in the generated degradome density file. The predicted results were shown in T-plots.

qRT-PCR analysis

Following the previous method [39], TB Green® Premix Ex Taq™ GC (Perfect Real Time) and a CFX Connect Real-Time PCR Detection System (Bio-RAD, USA) were used for qRT-PCR analysis. *MrACTIN* and U6 snRNA served as an internal reference for mRNA and miRNA expression analysis, respectively. All the primers were shown in Table S10.

Supplementary Information

The following supporting information can be downloaded at: XXX.

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Authors' contributions

F.S., C.G., and Y.Z. conceived and designed the study. Y.Z., W.Y., Y.Q., and F.T. performed the experiments. Y.Z., X.G., and F.Y. carried out the data analysis. Y.Z. wrote the manuscript. F.S. and C.G. revised the manuscript. All authors participated in this work and agreed to submit the manuscript.

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Data Availability

Raw sequencing data can be found at <https://www.ncbi.nlm.nih.gov/sra/PRJNA962259>.

Declarations

Competing interests

All authors declare that they have no competing interests.

Ethics approval and consent to participate

All plant materials and methods complied with relevant institutional, national, and international

guidelines and legislation.

Consent for publication

Not applicable.

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612

Figure legends

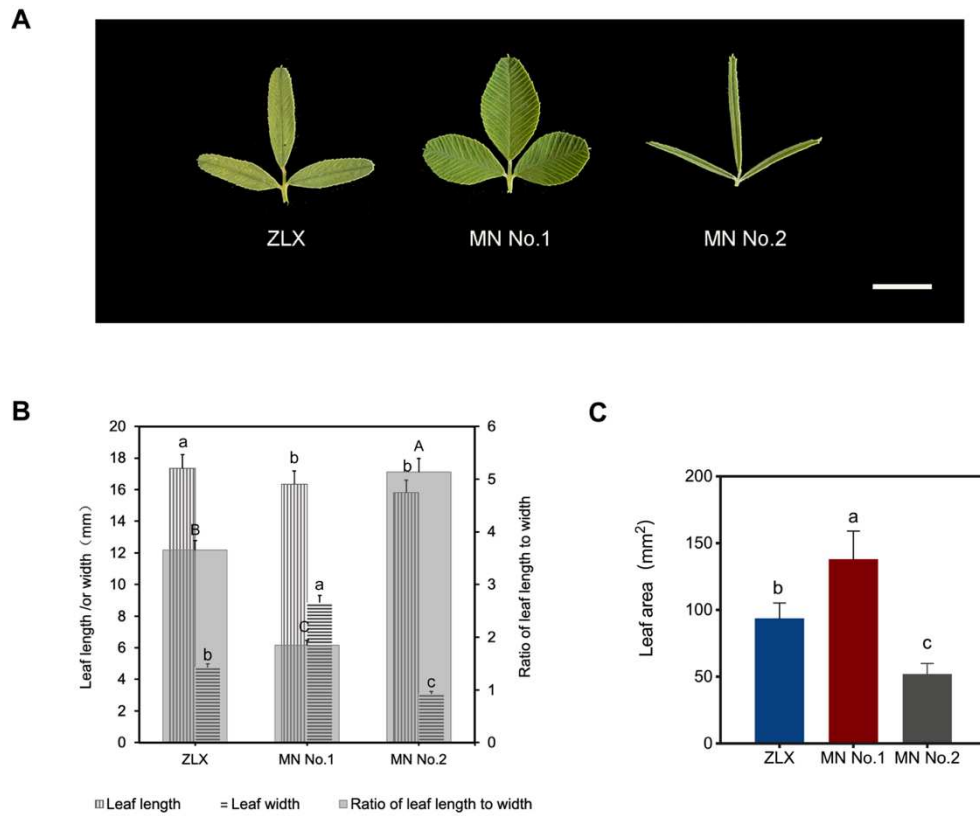


Fig. 1 Leaf morphology of three *M. ruthenica* cultivars was analyzed. **(A)** Leaves of three *M. ruthenica* cultivars at the early flowering stage. Bar = 1 cm. **(B)** Comparison of the leaf length, leaf width, and the ratio of leaf length to width among three *M. ruthenica* cultivars. Different lowercase letters indicated significant differences in the leaf length (or width) of the three cultivars, and different uppercase letters indicated significant differences in the ratio of leaf length to width of the three cultivars ($p < 0.05$). **(C)** Comparison of leaf area among three *M. ruthenica* cultivars. In **(B)** and **(C)**, values are means $\pm$ S.D. of twenty biological replicates.

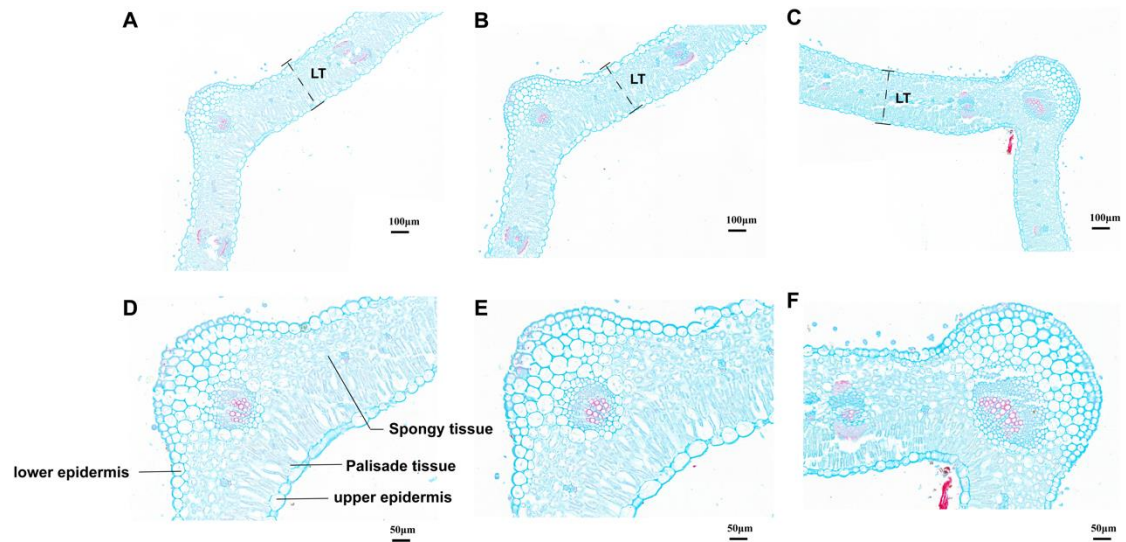


Fig. 2 Leaf cross-section of three *M. ruthenica* cultivars was stained with Safranin O/Fast Green. (A) and (D), ZLX. (B) and (E), MN No.1. (C) and (F), MN No.2. (A)-(C), at 15.0x magnifications, bar=100 μm . (D)-(F), at 35.0x magnifications, bar=50 μm . The leaf thickness was measured according to the "LT" of (A)-(C) labels. The schematic diagram of the leaf structure is marked in (D), (E), and (F) are the same.

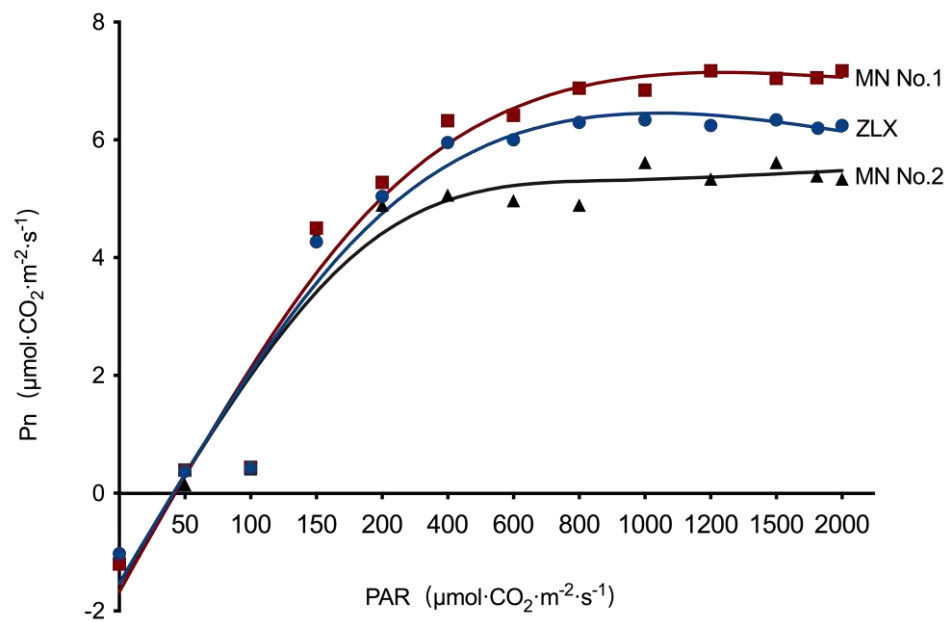


Fig. 3 The effect of light intensity on the Pn of three *M.ruthenica* cultivars was detected. Pn, net photosynthetic rate. PAR, photosynthetically active radiation.

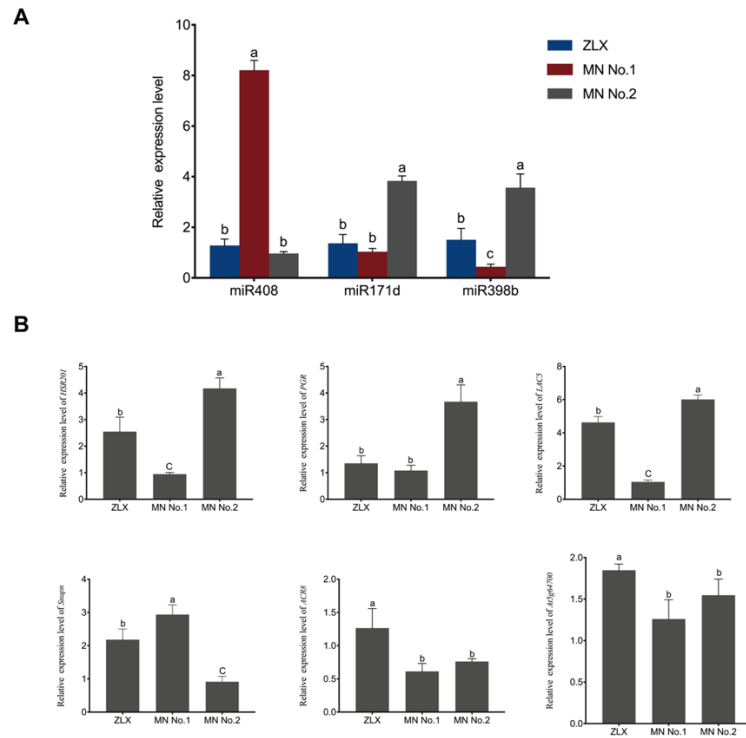


Fig. 7 Relative expression level of miRNAs and their targets in three *M. ruthenica* cultivars were verified using qRT-PCR. MrU6 SnRNA and *MrACTIN* served as the internal control for miRNAs and mRNAs, respectively. In (A) and (B), values are means $\pm$ S.D. of three technical replicates from three biological replicates. Different lowercase letters indicate significant differences ($p < 0.01$).

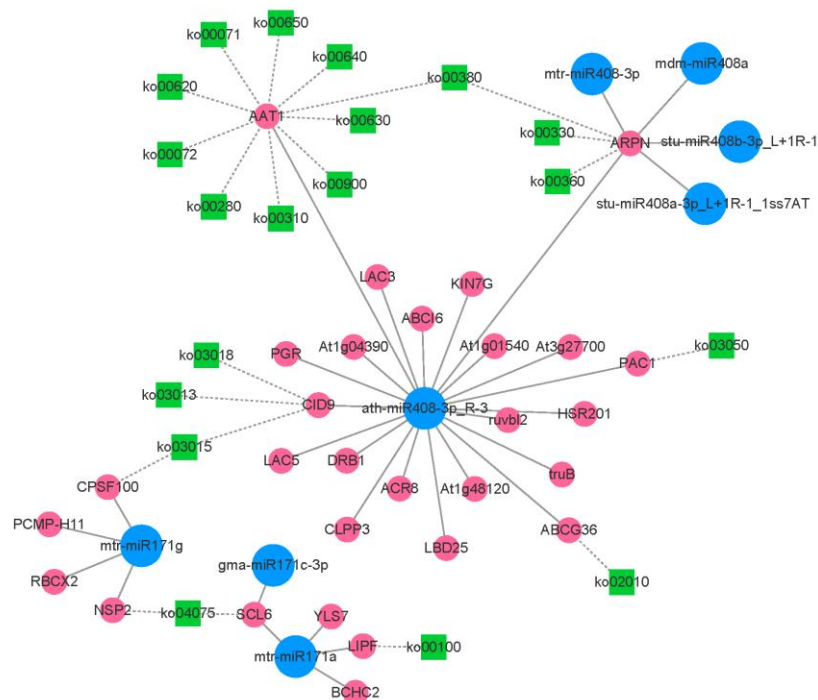


Fig. 8 The network plot of the miR408, miR171 and their target genes.

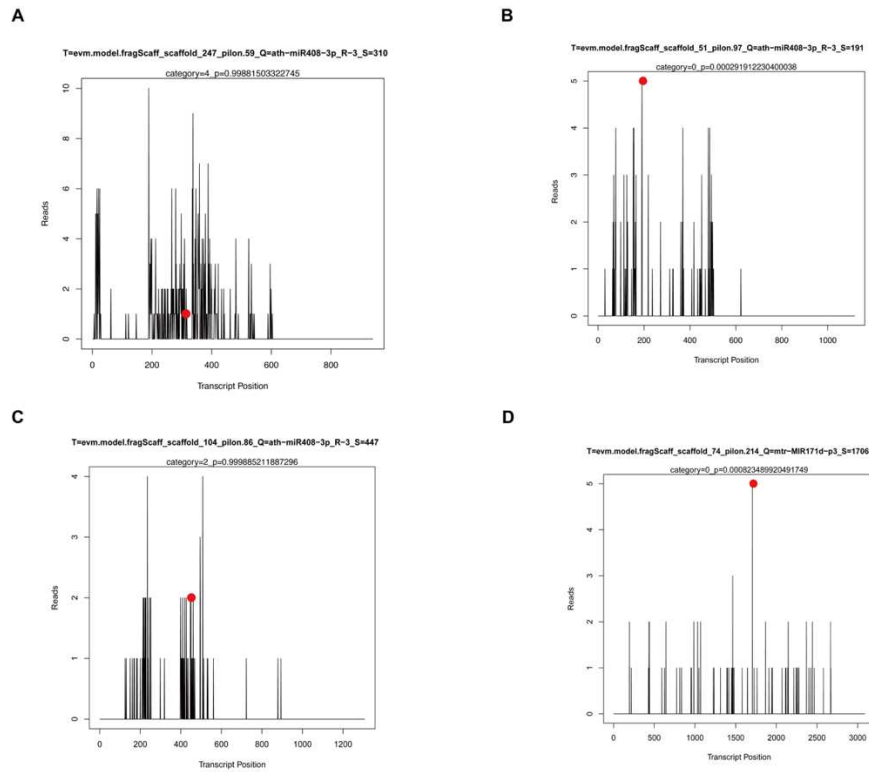


Fig. 9 The T-plots for the target genes of miRNAs by degradome sequencing. X-axis represents the site position of target transcript. Y-axis represents the normal abundance of raw tags. Red circle represents the cleavage site.

Table 1. Statistics of anatomic characteristics of leaf cross-section of three *M.ruthenica* cultivars

Leaf structure	Cultivar		
	ZLX	MN No.1	MN No.2
Upper epidermis thickness / μm	13.43 $\pm$ 0.32b	16.52 $\pm$ 0.53a	14.57 $\pm$ 0.40b
Lower epidermis thickness / μm	13.16 $\pm$ 0.51ab	14.93 $\pm$ 0.58a	14.06 $\pm$ 0.32b
Leaf thickness/ μm	381.72 $\pm$ 6.66ab	418.71 $\pm$ 27.8a	350.34 $\pm$ 4.88b
Palisade tissue thickness/ μm	96.46 $\pm$ 1.10ab	103.85 $\pm$ 3.52a	89.15 $\pm$ 4.28b
Spongy tissue thickness/ μm	78.32 $\pm$ 1.94a	58.76 $\pm$ 2.50c	74.43 $\pm$ 2.75a
Thickness ratio of palisade tissue to spongy tissue	1.23 $\pm$ 0.03b	1.76 $\pm$ 0.07a	1.20 $\pm$ 0.05b
Cell tense ratio (CTR)	40.83 $\pm$ 0.53ab	42.43 $\pm$ 1.32a	39.54 $\pm$ 0.69b
Spongy ratio (SR)	37.37 $\pm$ 0.80ab	35.78 $\pm$ 0.74b	38.48 $\pm$ 0.85a

Note: Completely different lowercase letters on the same line indicate significant differences ($p < 0.05$). CTR= (palisade tissue thickness/leaf thickness) $\times$ 100%, SR = (sponge tissue thickness/leaf thickness) $\times$ 100%.

Table 2. Photosynthetic pigment content in leaves of three *M.ruthenica* cultivars

Cultivar	Chl a	Chl b	Chl t	Chl a/b	Car
ZLX	1.76±0.03a	0.43±0.02a	2.19±0.17a	4.09±0.10a	0.37±0.11ab
MN No.1	1.88±0.06a	0.48±0.03a	2.36±0.16a	3.92±0.07a	0.42±0.02a
MN No.2	1.20±0.02b	0.38±0.03b	1.58±0.08b	3.16±0.04b	0.27±0.02b

Note: Completely different lowercase letters on the same column indicate significant differences ($p < 0.05$).

Table 3. Photosynthetic response parameters of three *M.ruthenica* cultivars

Cultivar	LSP ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	LCP ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	AQE ($\mu\text{mol}\cdot\mu\text{mol}^{-1}$)	R _{day} ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	P _{max} ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)
ZLX	208.5±10.67b	26.64±0.22a	0.0345±0.0002b	1.24±0.020b	6.13±0.19b
MN No.1	224.3±2.33a	21.56±0.31b	0.0453±0.0010a	1.06±0.001c	7.29±0.21a
MN No.2	198.23±2.61b	26.88±0.36a	0.0246±0.0003c	1.43±0.010a	5.43±0.07c

Note: Different lowercase letters on the same column indicate significant differences ($p < 0.05$).

Table 4. The miRNA-known target gene pairs showed significantly differentially expressed in the three comparison groups

Comparison	miRNA name	Target name	Target ID	Annotation
MN No.1 vs MN No.2	↑ ath-miR408-3p	↓ <i>HSR201</i>	evm.model.fragScaff_scaffold_118_pilon.20	putative benzyl alcohol O-benzoyltransferase [<i>Medicago truncatula</i>]
	↑ ath-miR408-3p	↑ <i>LAC3</i>	evm.model.original_scaffold_920_pilon.22	laccase-3 [<i>Medicago truncatula</i>]
	↑ ath-miR408-3p	↓ <i>PGR</i>	evm.model.fragScaff_scaffold_189_pilon.215	protein PGR [<i>Medicago truncatula</i>]
	↑ ath-miR408-3p	↓ <i>LAC5</i>	evm.model.fragScaff_scaffold_247_pilon.59	putative laccase [<i>Medicago truncatula</i>]
	↓ mtr- miR171d	↑ <i>Snupn</i>	evm.model.fragScaff_scaffold_93_pilon.67	snurportin-1 [<i>Medicago truncatula</i>]
ZLX vs MN No.1	↓ ath-miR408-3p	↓ <i>HSR201</i>	evm.model.fragScaff_scaffold_118_pilon.20	putative benzyl alcohol O-benzoyltransferase [<i>Medicago truncatula</i>]
	↓ ath-miR408-3p	↑ <i>ACR8</i>	evm.model.original_scaffold_637_pilon.94	uncharacterized protein LOC112421360 [<i>Medicago truncatula</i>]
ZLX vs MN No.2	↓ ath-miR398b-3p_R-1	↓ <i>At5g56260</i>	evm.model.fragScaff_scaffold_49_pilon.33	putative 4-hydroxy-4-methyl-2-oxoglutarate aldolase 3 [<i>Medicago truncatula</i>]
	↓ mtr-miR171d	↑ <i>At5g64700</i>	evm.model.fragScaff_scaffold_65_pilon.357	WAT1-related protein At5g64700 [<i>Medicago truncatula</i>]

Note: ↑, upregulation; ↓, downregulation.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryMaterial.zip](#)