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ARTICLE

The Regulatory Roles of microRNAs and Associated Target Genes during Early Somatic Embryogenesis in *Liriodendron Sino-Americanum*

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ABSTRACT

Somatic cells respond to considerable stress, and go through a series of phytohormone pathways, then forming an embryo. The developmental process is recorded as somatic embryogenesis (SE). One of the key components regulating SE are the microRNAs (miRNAs). Despite previous studies, it is still not clear exactly how miRNAs exert their function of regulating targets during conditionally activated early SE. Here, we use *Liriodendron sino-americanum* as a model system and perform a combined analysis of microfluidic chips and degradome sequencing to study this process. We identified a total of 386 conserved miRNAs and 153 novel miRNAs during early SE. According to the ANOVA test, 239 miRNAs showed 12 distinct expression patterns. Through degradome sequencing, 419 targets and 198 targets were identified for 136 known miRNAs and 37 novel miRNAs, respectively. Gene Ontology (GO) and metabolism pathway enrichment analysis revealed that these targets were significantly involved in oxidation-reduction processes, calmodulin-mediated signal transduction pathways and carbohydrate metabolism. The genes that were related to stress responses, phytohormone pathways and plant metabolism were identified within the targets of miR319, miR395, miR408, miR472, miR482, miR390, miR2055, miR156, miR157, miR171, miR396, miR397, miR529, miR535 and miR159. According to promoter analysis, various cis-acting elements related to plant growth and development, phytohormones response and stress response were present in the promoter of the miRNAs. The differential expression patterns of 11 miRNA-target modules were confirmed by real-time quantitative PCR. The study demonstrated that the miRNA plays an important role in the early SE process by regulating its target and then participating in carbohydrate metabolism and stress response. It also provided a valuable resource for further research in determining the genetic mechanism of SE, and then facilitating breeding programs on plants.

KEYWORDS

Liriodendron sino-americanum; early somatic embryogenesis; miRNAs; targeted transcriptions

1 Introduction

Somatic embryogenesis is a process that allows somatic cells to induce formation of an embryo, finally regenerating a complete plant [1]. During the process, cells go through a series of characteristic

events: activation of cell division, reprogramming of the physiological metabolism, and the gene expression patterns [2–4]. Previous research has demonstrated that microRNAs (miRNAs) are essential for previous cell differentiation and development processes during early embryogenesis [5–7].

MiRNAs are a class of endogenous, noncoding, small RNAs [8], of only 17–25 nuleotides (nt) in length [9–10]. The first miRNAs, *lin-4* and *let-7*, were identified in *Caenorhabditis elegans* through forward genetic screens [11–12]. After a decade, the first plant miRNAs were discovered in *Arabidopsis* [13–14]. Since then, identification and functional analysis of plant miRNAs have become one of the hottest research fields in plant biology [15]. As a result, a large number of miRNA genes have been identified in many plants species, and have been found to be critical for proper embryonic patterning and differentiation [14]. Mutations of genes involved in miRNAs biogenesis, such as Dicer-like1 (DCL1), cause defects in embryogenesis and meristem development [12,16,17]. In Arabidopsis miR156 was up-regulated during the SE process [18]. And overexpression of miR156 could accelerate the induction of SE from callus in citrus [19]. MiR157, another member of miR156, was also involved in the initial phases of SE induction in cotton [20] and in cucumber [21]. Furthermore, several miRNAs were involved in the regulation of phytohormones during plant SE. In *Larix leptolepis*, miR166 was involved in auxin biosynthesis and signaling, and then affected the development of the somatic embryo to a mature stage [22]. In *Arabidopsis thaliana*, miR160 repressed callus formation via the direct interaction between auxins and cytokinins [23]. Also, miR393 was verified to contributes to the embryogenic transition via modification of the sensitivity to an auxin treatment [24]. Moreover, miR156/157 and miR159 contribute to the ethylene response [20] and ABA responses involved in SE, respectively [25]. MiRNAs have been reported to play an important role in somatic embryogenesis in many plants: loblolly pine [26], Arabidopsis [27], Sweet orange [28], larch [29], longan [30], cotton [31], radish [32] and lilium [33]. The reported miRNAs have been involved in plant metabolism, signal transduction, cell cycle, stress response and plant development.

Liriodendron, a member of the Magnoliaceae, is regarded as a basal angiosperm. *Liriodendron sino-americanum*, derived from the sexual hybridization between *Liriodendron tulipifera* and *Liriodendron chinense,* is characterized by a high ornamental, ecological and commercial value. Compared with its parents, *Liriodendron sino-americanum* has obvious heterosis. It has strong drought and cold resistance, tall tree shape, straight trunk, high timber rate, good material, and has been significantly improved in its flower shape and ornamental color. It is an excellent woody tree species and garden afforestation tree species. Therefore, it is crucial to improve the quality and yield of *Liriodendron sino-americanum*. In view of previous studies, the somatic embryogenesis system represents a very powerful tool for massive propagation and trait improvement of higher plants [34]. As a result, determination of the mechanism of somatic embryogenesis in *Liriodendron sino-americanum* is urgently needed. Previously, according to deep sequencing and microarray hybridization, an overview of miRNAs during somatic embryogenesis in *Liriodendron sino-americanum* was reported [35]. However, the mechanism of miRNA-mediated target gene inhibition during early somatic embryogenesis remains largely unknown. In this study, we use chip hybridization and degradome sequencing to reveal the transcriptional regulation of miRNAs and their targets at different stages of early somatic embryogenesis.

2 Materials and Methods

2.1 Plant Materials

Synchronized cultures of somatic embryos at differentl developmental stages were obtained from an improved *Liriodendron sino-americanum* (*L. tulipifera* × *L. Chinese*) system established in our lab. Three cultures were immediately frozen in liquid nitrogen and stored at −80°C for further use. We established a synchronized somatic embryogenesis system using an embryogenic cell suspension. The embryogenic cell suspension was dark cultured at 23°C, and 95 rpm with 3/4 MS medium supplemented with 2,4-D 2 $mg \cdot L^{-1}$, BA 0.2 $mg \cdot L^{-1}$, CH 0.5 $g \cdot L^{-1}$, VC 5 $mg \cdot L^{-1}$, and sucrose 30 $g \cdot L^{-1}$ in a shaker. Embryonic

single cells were collected by sifting the suspension through 150 and 400 mesh sieves. The cell mass which was larger than 106 μm, and the cell debris which was smaller than 38 μm, were discarded. The cell diameters between 38 μm and 106 μm, which passed through 150 mesh sieves, but collected by 400 mesh sieves, were considered to be embryonic single cells. After dark cultures during 2 days at 23°C, and 95 rpm with 3/4 MS medium supplemented with NAA 0.2 $mg \cdot L^{-1}$, KT 0.5 $mg \cdot L^{-1}$, BA 0.2 $mg \cdot L^{-1}$, LH 0.5 $g \cdot L^{-1}$, VC 5 $mg \cdot L^{-1}$, and sucrose 30 $g \cdot L^{-1}$ in a shaker, embryonic single cells presented thickened cell walls and contained an increased inclusion. Then the above embryonic single cells were transferred to a somatic embryo-inducing medium, which contained 3/4 MS medium supplemented with ABA 2 $mg \cdot L^{-1}$, LH 0.2 $mg \cdot L^{-1}$, VC 5 $mg \cdot L^{-1}$, activated carbon 2 $g \cdot L^{-1}$, sucrose 40 $g \cdot L^{-1}$, and agar 7 $g \cdot L^{-1}$. After a 2 day-dark-culture in an incubator at 23°C, the dense embryonic tissues were observed. After a 7 days post-differentiation culture in a somatic embryo-inducing medium, early globular embryos were formed. The samples were harvested at four developmental stages as follows: (1) embryonic suspension cells after 7 days on sub-culture, labeled as ES1; (2) embryonic single cells after 2 days on sub-culture, labeled as ES2; (3) embryonic tissues after a 2 days post-differentiation culture, labeled as ES3, and (4) globular embryos after 7 days post-differentiation culture, labeled as ES4. Total RNAs of every sample were extracted using the Total RNA Purification kit (Norgen Biotek Corporation, Canada).

2.2 Microarray Hybridization and miRNA Identification

The microarray was synthesized and then hybridized *in situ* by LC Science. The microarray contained probes complementary to 1117 miRNAs, and control probes were used as quality controls. Each probe was represented by two replicates in each chip. Hybridization images were collected using a laser scanner (GenePix 4000B, Molecular Device) and digitized using Array-Pro image analysis software (Media Cybernetics). Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression). Two criteria were used to affirm a miRNA signal as detectable: first, signal intensity higher than three times the background standard deviation; second, spot coefficient of variation (CV) less than 0.5 (where the CV was calculated by signal standard deviation/signal intensity). To discover the expression profiles of miRNAs, an ANOVA test was performed, with the significance threshold set to 0.01.

2.3 Degradome Sequencing, Target Identification and Analysis

RNA extracted from flowers, leaves, buds and the tissues from four developmental stages of somatic embryogenesis were used for degradome sequencing. Equal amounts of all RNA samples were mixed together and then used to prepared the degradome library. The method differed considerably from past efforts [36,37] and with some modifications. The extracted sequencing reads were analyzed by a public software package, CleavaLand 3.0, to identify potentially cleaved targets. The degradome data was mapped to the *Liriodendron sino-americanum* transcriptome data (still unpublished), and t-plots were generated for the potential miRNA targets. Then the target transcripts were subjected to a Gene Ontology (GO) database and mapped to the referenced canonical pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG). As a result, the targets were allocated to various functional categories on the basis of GO and KEGG analysis. The promoter cis-element analysis was performed in Plantcare.

2.4 Quantitative Real-Time PCR Validation

To validate the miRNA-target expression patterns, quantitative real-time PCR (qRT-PCR) on miRNAs and their targets was performed on a Roche Applied Science LightCycler 480. The RNA samples used for qRT-PCR were identical to those used for the experiments mentioned above. The reverse transcription reactions for miRNAs and cDNA were carried out using the miRNA 1st strand cDNA Synthesis kit (by stem-loop) (Vazyme, China) and the HiScript II Q RT SuperMix for qPCR (Vazyme, China), respectively. The stem-loop primers of miRNAs were designed based on the sequences of the respective miRNAs, and

primers for the targets were designed using the Primer 5 program. 18S and Actin were selected as reference genes. qRT-PCR was performed using the Ace Q qPCR SYBR Green Master Mix kit (Vazyme, China). Each reaction was repeated three times independently.

3 Results

3.1 Somatic Embryogenesis-Responsive miRNAs in Liriodendron Sino-Americanum

Our small RNA library was assembled from four batches of sequencing data sets to increase coverage. To identify the miRNAs during somatic embryogenesis in *Liriodendron sino-americanum*, we first collected embryonic tissues at different stages [ES1, ES2, ES3, ES4], and then prepared separate microfluidic chips for each stage. The microarray contained 1117 probes, including 1024 miRNA sequences detected in a mixed RNA pool microarray and 93 miRNA sequences predicted by deep sequencing, as described in a previous study [35]. After background subtraction and normalization, a total of 539 miRNAs were detected. Of these miRNAs, 386 belonged to 148 known miRNA families. Most of the known miRNA families had more than one member, such as the miR171 family, which contained 27 genes and it was the biggest, followed by the miR166, miR159 and miR156 families (Fig. 1). In addition, we detected 153 species-specific miRNAs (Supplementary Tab. 1).

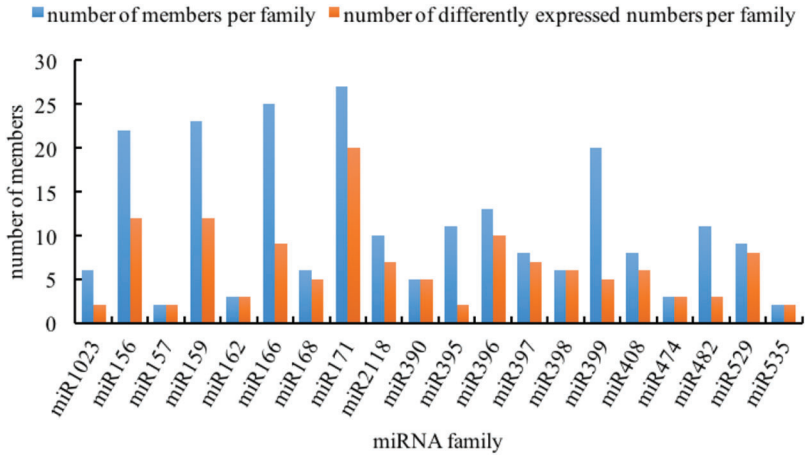

Figure 1: Number of members in differently expressed miRNAs families which had more than one member

The differential expression of miRNAs in the four libraries (derived from successive embryonic stages) was analyzed to identify miRNAs in *Liriodendron sino-americanum* that participated in somatic embryogenesis. In total, 239 miRNAs showed differential expression patterns. Out of these miRNAs, 177 belonged to 68 previously identified miRNA families, while the remaining 62 were species-specific miRNAs (Supplementary Tab. 2). 20 miRNA families contained more than one member. The miR171 family was the largest with 20 miRNAs, followed by the miR159, miR156 and miR396 families. Twelve distinct expression patterns were observed ($P < 0.01$) (Fig. 2). It was observed that 86 miRNAs were the most abundant at the stage ES1(Cluster I, II and III), including miR157, miR390, miR397, miR408, miR472 and miR535. At the stage ES2, 26 miRNAs were detected with the highest signal and down-regulated subsequently (Clusters IV and V), such as most members of miR159. 43 miRNAs showed a maximum expression at the stage ES3 (Clusters VI, VII and VIII), such as miR319, while 84 miRNAs showed a maximum expression at the stage ES4 (Clusters IX, X, XI and XII), including miR395, miR529 and miR2055. In conclusion, numerous miRNAs were detected with differential expression.

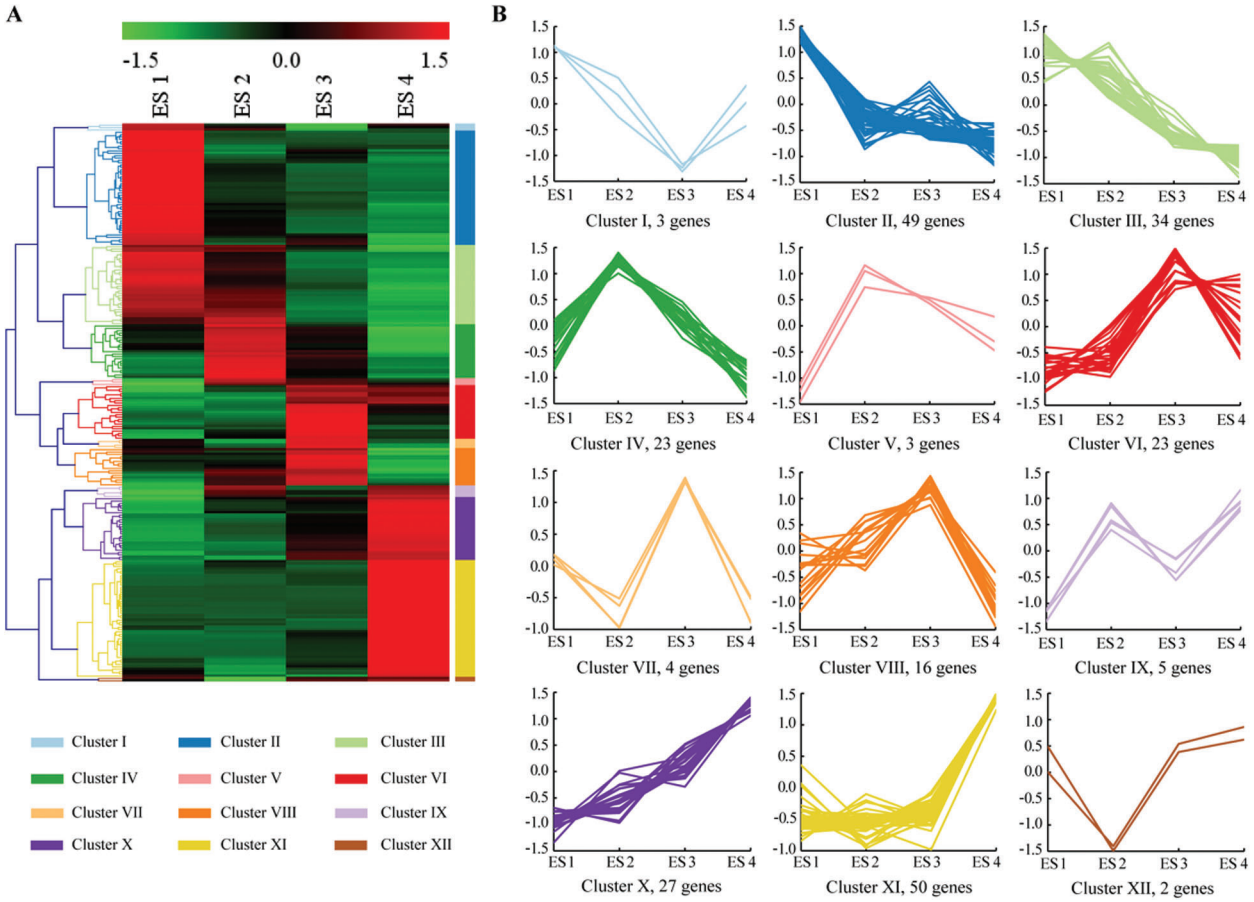

Figure 2: Hierarchial clustering of the profiles of 239 miRNAs differentially expressed in the early somatic embryogenesis. (A) Heat map displaying the changes in the different stages of somatic embryogenesis, (B) Twelve patterns (I–XII) of miRNA expression and the relevant number or miRNA

3.2 Target Prediction of the Known and Novel miRNAs by Degradome Sequencing

To better understand the biological functions of somatic embryogenesis-responsive miRNAs identified in our study, degradome sequencing was employed. We generated 15,888,537 raw reads representing 6,413,098 unique reads from mixed degradome pools. 15,774,245 reads were successfully mapped to cDNA sequences. We then used CleaveLand 3.0, a public software package, to identify degraded targets for each of the miRNAs families [36,37]. On the base of signature abundance at each occupied transcript position,all of the cleaved transcripts were categorized into 0, 1, 2, 3 and 4, respectively (Supplementary Tab. 3) [38,39]. Among the identified miRNA targets, 217, 8, 221, 33 and 138 fell into the categories 0, 1, 2, 3 and 4, respectively.

Based on the analysis, 419 targets were identified for 136 known miRNAs from the mixed degradome. In addition, we also identified 198 targets for 37 species-specific miRNAs. If we only considered differentially expressed miRNAs, we found 412 targets for 107 miRNAs, out of which, 268 were targeted by 82 known miRNAs, while 144 were targets for 25 species-specific miRNAs (Supplementary Tab. 4). Most identified miRNAs were capable of regulating multiple targets; while some transcripts were cleaved by more than one miRNA. For example, PN-3p-139853 was able to cleave 26 targets, the highest number of transcripts cleaved by a single miRNA; while 38 miRNAs were found to cleave just one target transcript. This shows that there was a complex genetic regulation on the basis of the miRNA and

transcript interaction during somatic embryogenesis. In plants, the cleavage always took place between nucleotide pairs 10 and 11 of the miRNA. Therefore, a discrete peak would appear at the sliced site of the cleavage products. In this study, we detected the cleavage sites of identified targets degraded by the corresponding miRNAs and generated a 'target plot' (t-plots) (Fig. 3).

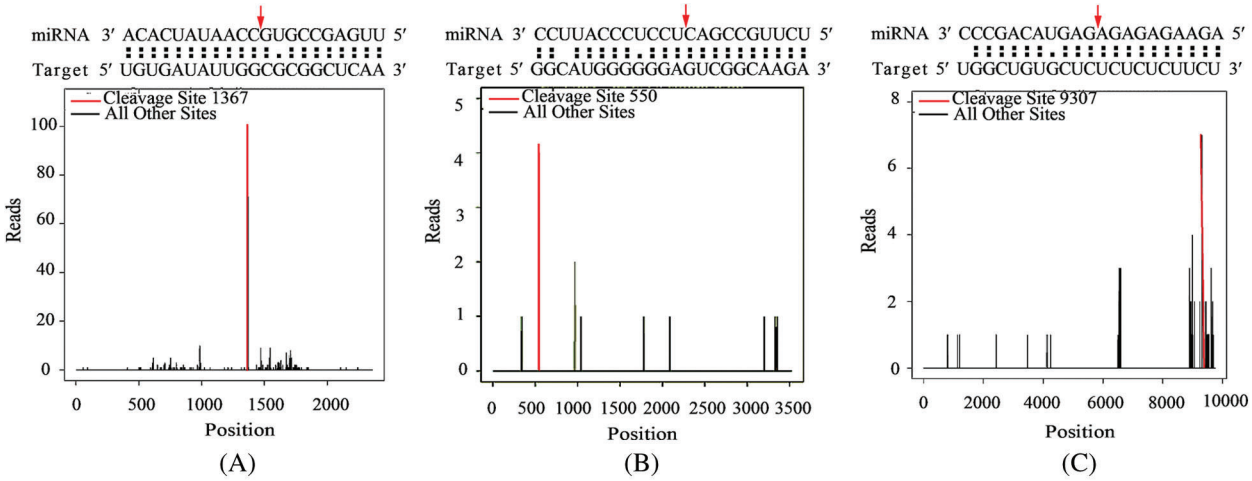

Figure 3: Target plot (t-plot) of representative miRNAs targets validated through degradome sequencing. The red line and arrow indicated cleavage sites (x-axis) and the y-axis represent the detected reads. The alignment sequence of miRNA and target are shown on the upper position of the t-plots. (A) miR171 and its target. (B) miR482 and its target. (C) miR529 and its target

3.3 GO Annotation and Enrichment Analysis of miRNAs Targets during Somatic Embryogenesis

To better understand the biological role and potential genetic regulation of the identified miRNAs and their targets during *Liriodendron sino-americanum* early somatic embryogenesis, we annotated the 617 identified target transcripts using Gene Ontology (GO) categories (Fig. 4; Supplementary Tab. 5). According to the ontological descriptions of the GO term, the target genes were divided into three categories: biological process, cellular components, and molecular function. 25 transcripts were categorized with the GO biological processes; the two most highly represented were the oxidation-reduction and metabolic processes. Of the 15 cellular component categories, the frequent categories were integral components of membrane, membrane and nucleus. Finally, there were 10 molecular function categories, with the two most abundant being the ATP and metal ion bindings. Somatic embryogenesis is induced by certain stress levels [40], and involves changes in cellular metabolism and division rate [41,42]. Consistently, according to the GO annotation, various transcripts involved in stress response, plant metabolism and cell division were identified in the early somatic embryogenesis in *Liriodendron sino-americanum*.

Further analysis of GO functional enrichment indicated that abundant biological pathways were involved in early somatic embryogenesis (Fig. 5). Significantly enriched GO terms were visualized using the reviGO tool [43] and plotted. Terms related to the tricarboxylic acid cycle enzyme complex (GO: 0045239), fumarate metabolism (GO: 0006106), response to salt stress (GO: 0046872), calcium ion transmembrane transport (GO: 0070588) and ATP binding (GO: 0005524) were found to be enriched; all of these processes may be involved in somatic embryogenesis [4,29,44].

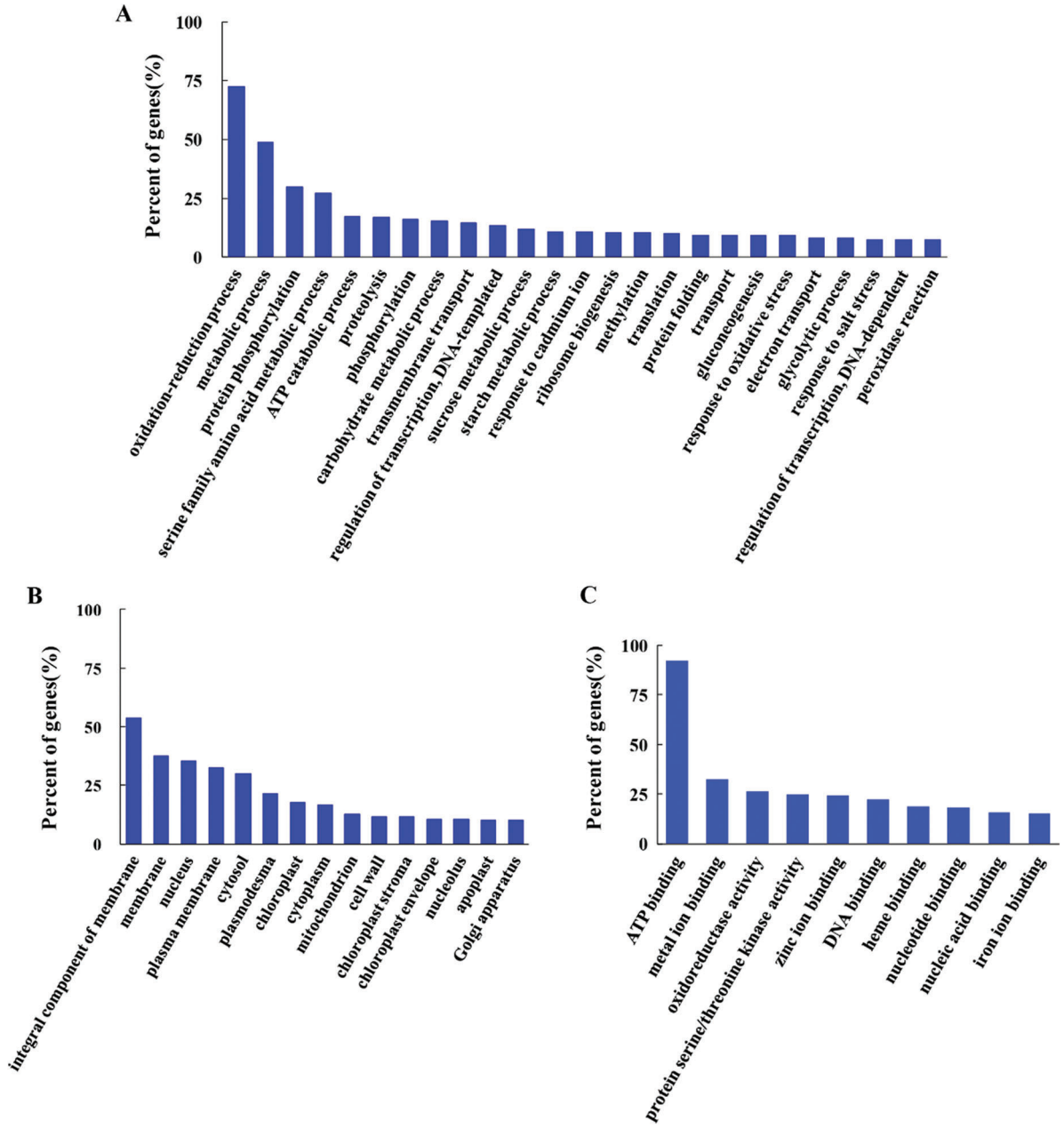

Figure 4: Gene ontology (GO) classification of target transcripts for identified miRNAs. (A) Biological process. (B) Cellular compent. (C) Molecular function. The x-axis indicates the GO term, and the percent of genes in each term is shown in the y-axis

Figure 5: GO enrichment of identified targets. The size of the circle represents the number of genes, and the color of the circle represents the *p*-value

3.4 Functional Classification Using the KEGG Database

To further characterize the functions of miRNAs-targets during early somatic embryogenesis, we performed KEGG pathway analysis and enrichment (Fig. 6). According to our analysis, identified target transcripts were assigned to 229 unique ko numbers and they were mostly involved in metabolism (85.5%), genetic information processing (27.4%), cellular processes (16.0%), organismal systems (16.3%) and environmental information processing (6.7%). The most important enriched metabolic pathway categories among the miRNA targets were carbohydrate metabolism, amino acid metabolism, biosynthesis of other secondary metabolites, energy metabolism and lipid metabolism. The major pathway in organismal systems was environmental adaptation. Analysis of KEGG pathway enrichment identified that various metabolic pathways were potentially involved in early somatic embryogenesis, including the citrate cycle (ko00020), the reductive carboxylate cycle (ko00720), pentose and glucuronate interconversions (ko00040), glycerolipid metabolism (ko00561) and cysteine and methionine metabolism (ko00270).

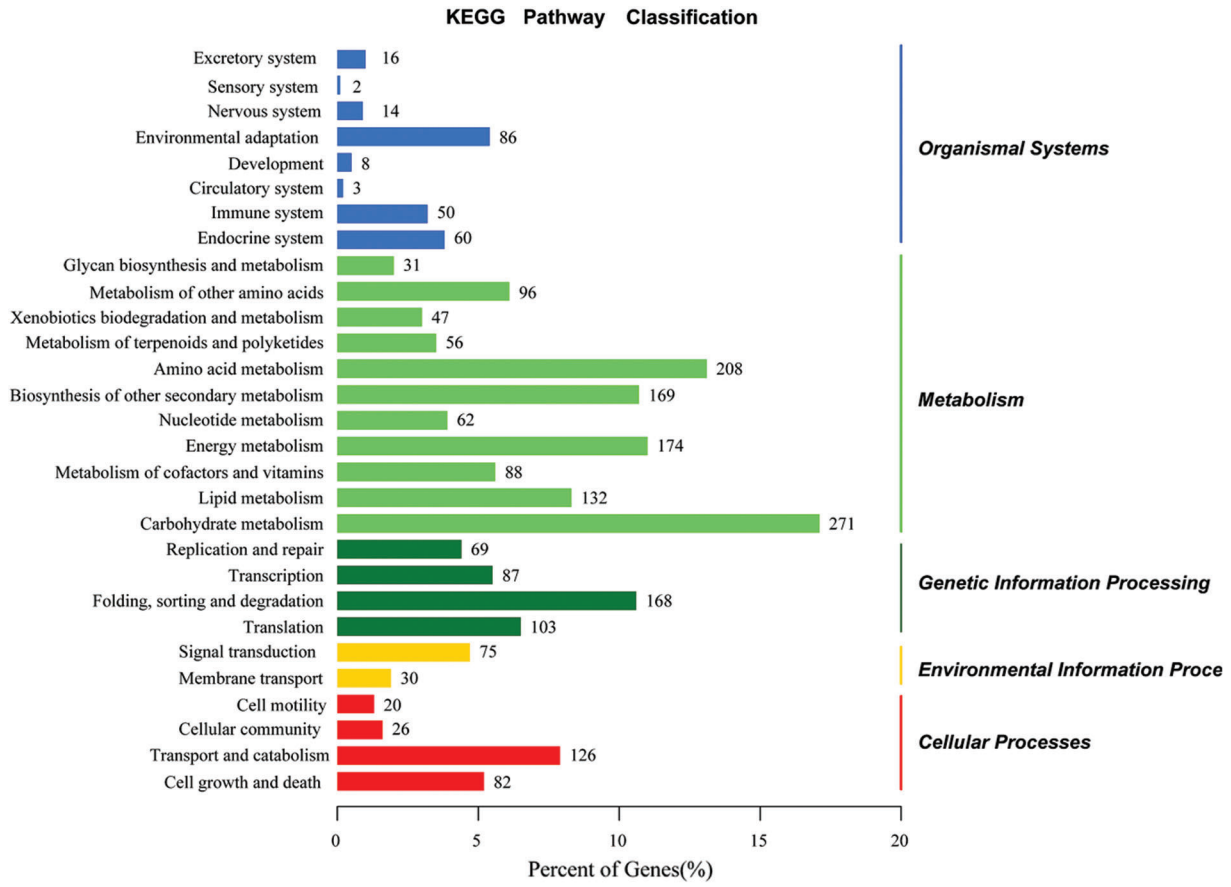

Figure 6: KEGG pathway classification of identified targets

3.5 Identification of Cis-Acting Elements in the Promoter of miRNAs

Cis-elements in the promoter are closely related to gene expression and function. To further identify the regulated role of miRNAs during the early somatic embryogenesis, we analyzed the cis-acting elements of the promoter sequence of several different-expressed miRNAs using PlantCARE (Fig. 7). The result showed that cis-acting elements such as phytohormone -response elements, stress-response elements and several metabolism-related elements were present in the promoter. The cis-acting elements related to meristem expression such as CAT-box, CCGTCC-box, as-1, MYC were present in almost all promoter regions of the miRNA. Only miR156 and miR157 consisted of the MBSI element, and E2Fb, and re2f-1 just existed in the miR535 and miR171, respectively. Elements involved in salicylic acid responsiveness, abscisic acid responsiveness, MeJA-responsiveness, like the TCA-element, ABRE, CGTCA-motif, TGACG-motif, AAGAA-motif were identified in most promoters of miRNA. Otherwise, auxin-responsive elements and gibberellin-responsive elements were also identified. Besides, wound response elements (like WUN-motif, WRE3), drought-related elements (like MBS, DRE), anoxic specific inducible elements (like GC-motif, ARE), and defense and stress responsiveness elements (like TC-rich repeats, STRE) were commonly present in the promoters. LTR, defined as an element involved in low-temperature responsiveness, was also found in the part promoter.

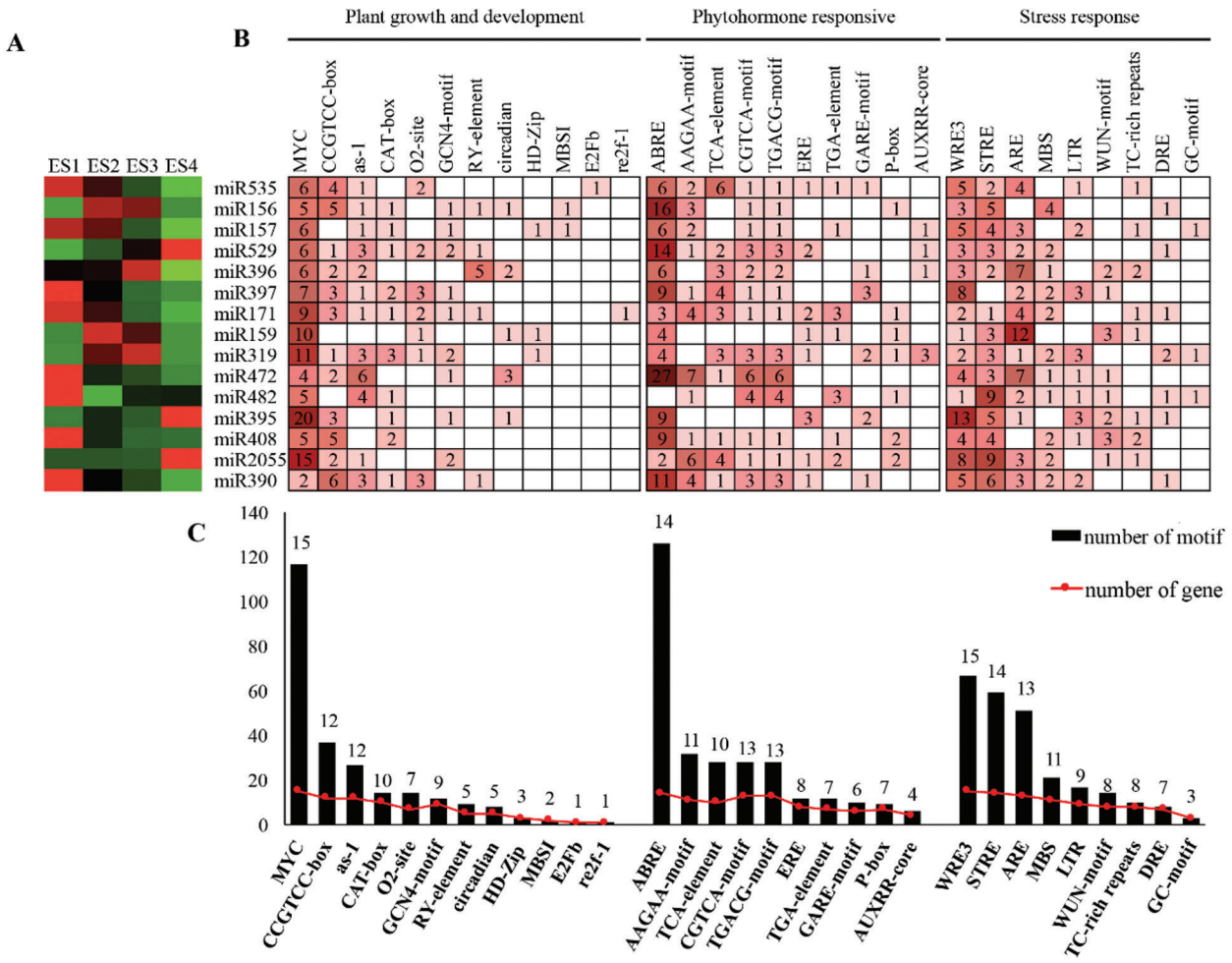

Figure 7: Cis-acting element analysis of the promoter of miRNAs. (A) Heat map displaying the expression pattern of miRNAs. (B) Description of cis-acting elements in the promoter. (C) Statistics of the number of miRNA that contained specific elements (red dots) and total number of cis-acting elements (black boxes)

3.5 Validation of MiRNA Expression Profiles and Their Targets by qRT-PCR

MiRNAs regulate the expression of their corresponding targets and perform modulating roles during metabolism, processing of genetic information, cellular processes, organismal systems, and processing of environmental information. To confirm the dynamic expression of miRNA-target modules, a real-time quantitative PCR (qRT-PCR) analysis was performed (Fig. 8). Templates for microfluidic chip expression analysis were derived from the same four stages of somatic embryogenesis. Primers used for the miRNAs and their targets are shown in the supplement (Supplementary Tab. 6).

The expression profiles of the randomly chosen miRNAs analyzed by qRT-PCR were consistent with those derived from the microfluidic chips. Consistently, the expression level of their targets were negatively correlated with the miRNAs. The elevated accumulation of miR156 and miR529 were opposite to the decreased expression of their corresponding targets. While the miR171, miR482, miR472, miR390, miR408, and miR396 were down regulated, their corresponding targets were up regulated, respectively. The expression of miR159 varied with the stage of somatic embryogenesis and showed the maximum expression at the stage SE2, while its target showed the minimum expression at SE2. In particular, miR2055, a miRNA that had not been previously detected during the early somatic

embryogenesis in other plants, was detected with an up-regulated expression in *Liriodendron sino-americanum*. It was opposite to the expression of its target, which showed a down-regulated expression. In addition, we validated the dynamic expression pattern of a novel miRNA, PN-3p-139853, that participated in the carbohydrate metabolism. It displayed a significant accumulation accompanied by the decrease of its target.

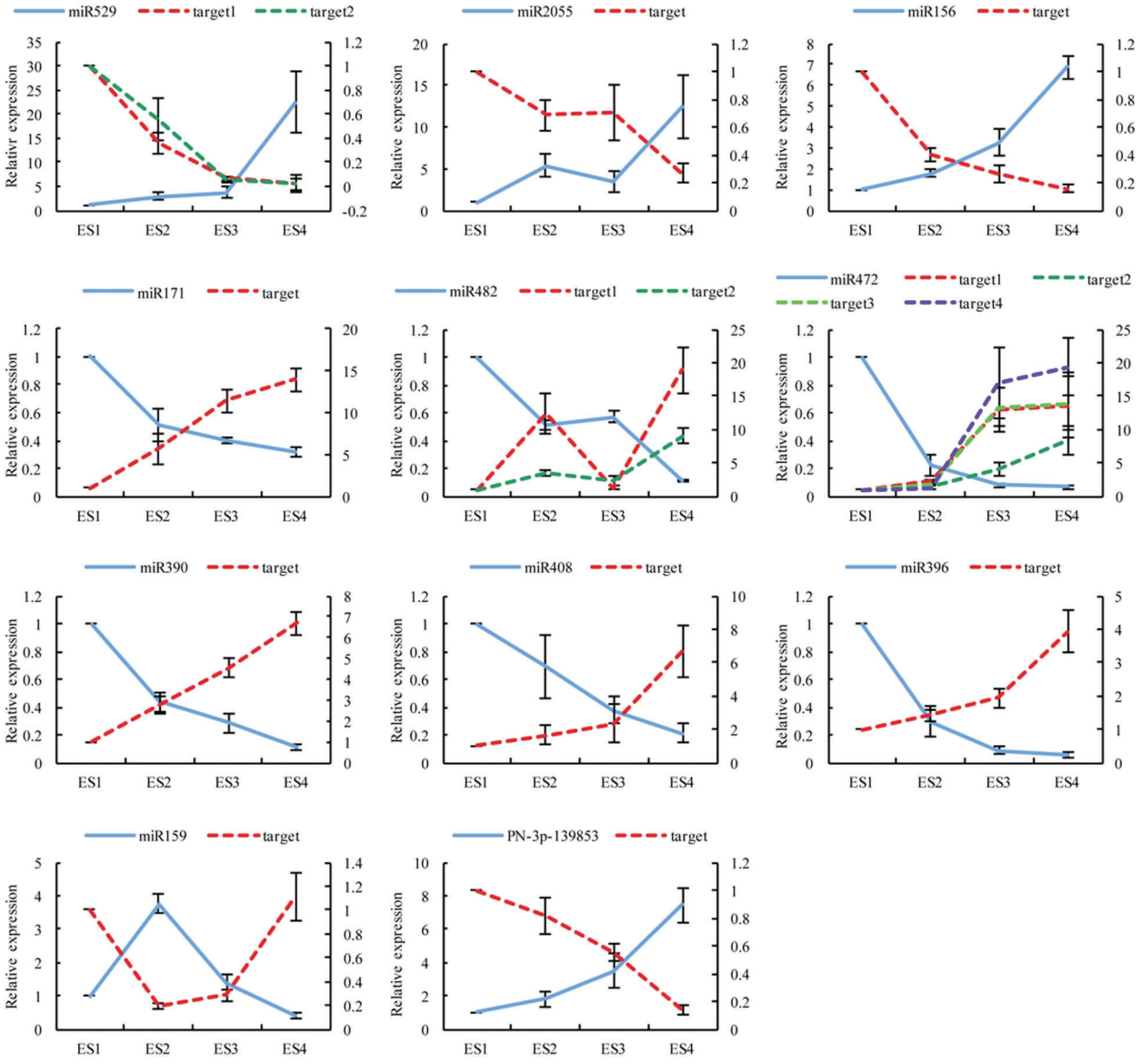

Figure 8: qRT-PCR analysis of the relative expression pattern of miRNAs and their targets. The x-axis represents the stage, and the relative expression level is shown in the y-axis

Our qRT-PCR assay confirmed that the expression level of miRNAs was regulated during early somatic embryogenesis, resulting in complementary patterns of their target transcripts. The expression mode was consistent with the microfluidic chip data, indicating the fidelity of the chip results.

4 Discussion

Embryogenesis, representing a key stage in the life cycle of plants [45,46], consists of two distinct phases: early morphological events and late maturation [29,47]. Previous research has identified that the key developmental processes during early embryogenesis could be considered as a miniature model of plant development [48,49]. So far, however, no study on miRNA-regulated transcripts during early somatic embryogenesis has been conducted in *Liriodendron sino-americanum*. We identified, through microarray analysis, 539 unique miRNAs in successive stages of *Liriodendron sino-americanum* somatic embryo, of which 386 were known miRNAs and 153 were species-specific miRNAs. After analysis, 239 miRNAs were confirmed to be differentially expressed. Furthermore, we identified 617 targets for 173 miRNAs in total using degradome sequencing. Among these, 419 targets and 198 targets were identified for 136 known miRNAs and 37 novel miRNAs, respectively. If we only considered differentially expressed miRNAs, we found 412 targets for 107 miRNAs, out of which, 268 were targeted by 82 known miRNAs, while 144 were targets for 25 species-specific miRNAs. GO annotation and KEGG analysis showed that target transcripts were involved in various processes, including plant metabolism, signal transduction, and stress response (Fig. 9). Promoter analysis identified several cis-elements, such as growth and development-related elements, hormone-response elements, and several stress-response elements. Besides, in this study, we identified the targets of miR535 and miR2055. These miRNAs were previously unknown to regulate transcripts in somatic embryogenesis, which may have a regulatory role during early somatic embryogenesis in *Liriodendron sino-americanum*.

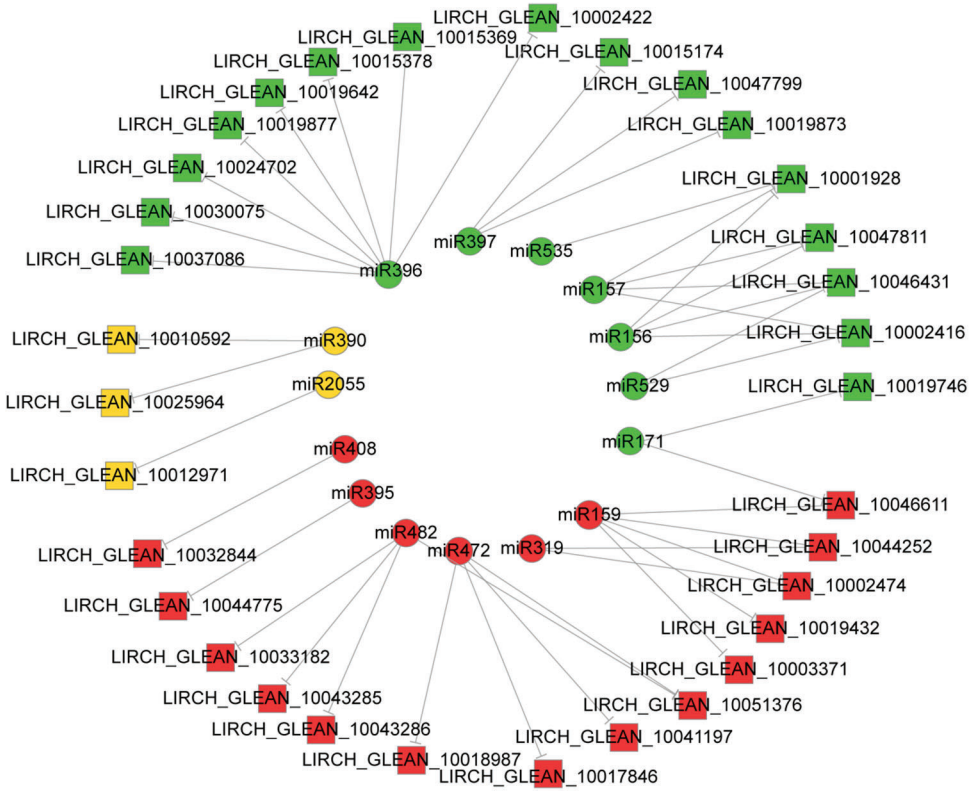

Figure 9: miRNAs targeting transcripts regulating the early somatic embryogenesis process. The circle represented the miRNAs, and the square represented the targets. The red circle and square indicated the miRNAs and the role that targets play under stress response during the early somatic embryogenesis. The yellow circle and square indicated the miRNAs and targets participating in hormone pathways in early somatic embryogenesis. The green circle and square indicated the miRNAs and the function that targets exertin plant metabolism in early somatic embryogenesis

4.1 Stress Related miRNA-Targets in Early Somatic Embryogenesis

Induction of somatic embryogenesis always requires a series of experimental steps that exerts considerable stress in the explant [50]. Stress-regulated miRNA-targets have been related to callus embryogenic potential in sweet orange [28,51]. In *Rorippa indica* [52], salt and drought stress led to high-efficiency regeneration via somatic embryogenesis. According to previous studies, miR319, miR395, miR408, miR472 and miR482 are involved in stress response and have different expression patterns. In addition, their targets are related to the oxidation-reduction process. A recent research reported that flavonoids, acting as antioxidants, may affect the auxin levels during early somatic embryo developmental stages [53]. In cotton, the homeostasis of reactive oxygen species is closely related to somatic embryogenesis [54,55]. miR395 regulates sulphate assimilation and distribution by negatively regulating genes encoding ATP sulphurylases (APs) and sulphate transporters, and it was found to be up-regulated in response to drought stress [56–58]. Similarly, we found that miR395 was upregulated during early somatic embryogenesis in *Liriodendron sino-americanum*.

miR159, which targets MYB transcription factors, has an important role to enable plant response to environmental stress [59]. In wheat, it was reported to be down-regulated in the root, but up-regulated in the leaf under drought stress conditions [60]. In larch, miR159 accumulates at the cotyledon embryo stage [29], while in citrus, miR159 is most strongly expressed in globular-shaped embryos [28]. In *Liriodendron sino-americanum*, miR159 showed a differential expression pattern during the early stages of the somatic embryogenesis, possibly indicating a regulatory function for this gene. miR472 and miR482, which have been linked with drought tolerance response in wheat, were down-regulated in this study. This may be because the early stages of somatic embryogenesis are involved in the activation of stress responses in plants.

4.2 Phytohormones Pathway Related miRNA-Target in Early Somatic Embryogenesis

The process of somatic embryogenesis involves a series of dedifferentiation events controlled by various plant growth regulators (PGRs) [61,62]. Auxin is a critical PGR involved in the control of cell division and differentiation [4]. miR390 participate in the process of somatic embryogenesis through the regulation of targets involved in auxin signaling [63,64]. In Arabidopsis, it was verified that miR390 modulates the formation of ta-siRNAs, which target ARF2, ARF3 and ARF4 genes [63]. In *Dimocarpus longan*, this process is involved in regulating somatic embryogenesis, where miR390 directs the production of tasiRNA-*ARF3/4* [65]. In *Liriodendron sino-americanum*, miR390 was down-regulated during development. This suggested that miR390 might be responsible in the somatic embryogenesis.

4.3 Plant Metabolism Related miRNA-Targets in Early Somatic Embryogenesis

Early somatic embryogenesis is a developmental process by which somatic cells in suitable conditions undergo restructuring through a set of characteristic events. It was reported that miR156, miR397, and miR171 are differentially expressed in the callus after it has started to differentiate toward an early somatic embryo [66]. By mediating suppression of *Squamosa Promoter Binding Like* (*SPL*) genes, miR156 gene plays critical regulatory roles in the plant developmental process, such as during the juvenile to adult transition [67]. In *Liriodendron sino-americanum*, different members of the miR156 family were strongly expressed throughout early somatic embryogenesis, suggesting that miR156 plays an important regulatory role in the developmental process. This is consistent with the fact that miR156-mediated target inhibition is necessary for early somatic embryogenesis [7].

In citrus, miR171 exerts a regulatory function during the somatic embryo induction process through targeting the *Scarecrow-like* (*SCL*) gene family [28]. Similarly, in *Larix kaempferi*, miR171 might regulate the mode of cell division and participate in the maintenance of somatic embryogenic potential [68]. In *Liriodendron sino-americanum*, most members of the miR171 family were down-regulated

during early somatic embryogenesis, which is consistent with the expression pattern of miR171 during radish somatic embryogenesis [32].

miR397 has been previously confirmed to target laccases, which are associated with lignification and thickening of the cell wall [30]. In rice, comparing with other organs, miR397 is specifically expressed in the embryogenic callus, indicating that it might be important in maintaining the cell in a meristematic state [66]. Consistently, in this study, the expression of miR397 was decreased during early development. At early stages, high expression of miR397 might be involved in maintaining the cell in a meristematic state. At later stages, when cells start to differentiate, a low expression level of miR397 potentially allows the accumulation of laccases, regulating the development of the cell wall.

4.4 Unique miRNAs in Somatic Embryogenesis

miR535 is a unique miRNA that has not been previously described to have a regulatory function during the plant developmental processes. It has not been found in Arabidopsis or in *Populus*, which both have been completely sequenced genomes [69]. In *Liriodendron sino-americanum*, miR535 kept a high expression level throughout early somatic embryogenesis. Interestingly, it targets the same transcripts as miR156, suggesting that miR535, like miR156, may play a key role in regulating early somatic embryogenesis.

Up to date, miR2055 has not been found to be involved in somatic embryogenesis. The only report on miR2055 comes from a study on grain development in indica rice [70], where the actual function of miR2055 was not confirmed. In *Liriodendron sino-americanum*, miR2055 shows a significantly differential expression pattern, and reached its peak at stage 4. After GO term analysis, we found that miR2055 may play a key role in calcium-mediated signal transduction by regulating its target. Calcium plays a significant role in the regulation of various cellular and physiological processes in higher plants [71,72]. Research in carrot has shown that calcium enhances somatic embryogenic frequency, and that its absence suppresses somatic embryo formation [4]. This suggested that miR2055 could play an intermediary role by regulating calcium mediated signal transduction during early somatic embryogenesis.

5 Conclusions

Somatic embryogenesis is known as the developmental process by which somatic cells undergo activation of cell division, and reprogramming of physiology, metabolism, and presenting dynamic gene expression patterns. It is widely used in the biomass production of economically important plants. Since *Liriodendron sino-americanum* is characterized by a high ornamental, ecological and commercial value, it is crucial to improve its quality and yield. In this study, we employed miRNA and degradome sequencing to uncover a complex regulatory mechanism during early somatic embryogenesis. The microarray hybridization revealed 239 miRNAs showing dynamic expression patterns. Among these miRNAs, several miRNAs have been reported in other plants to show a regulatory function during SE processes, such as miR319, miR395, miR408, miR472, miR482, miR159, miR390, miR156, miR171, and miR397. Besides, miR535 and miR2055 showed a significantly different expression pattern during *Liriodendron sino-americanum* SE. These two miRNAs have not been described to have a regulatory function during plant developmental processes previously. Furthermore, the degradome sequencing corroborated the targeted transcriptions of miRNAs. As a result, we identified 617 targets for 173 miRNAs. Function annotation reflected that the transcripts targeted by miRNAs were involved in stress response, signal transduction, and plant metabolism. The regulatory roles of microRNAs associated with target genes during early somatic embryogenesis in *Liriodendron sino-americanum* provided a useful resource for further studies on the genetic mechanisms during SE in other plants.

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