Transcriptional and post-transcriptional regulation of the miR171-LaSCL6 module during somatic embryogenesis in Larix kaempferi

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Abstract

Key message Expression analysis of Larix kaempferi mature miR171s and their primary transcripts and target gene LaSCL6 during somatic embryogenesis revealed the transcriptional and post-transcriptional regulation of the miR171-LaSCL6 module.

Abstract Somatic embryogenesis provides a useful experimental system for studying the regulatory mechanisms of plant development. The level and activity of microRNA171 (miR171) fluctuate during somatic embryogenesis in Larix kaempferi, but the underlying mechanisms are still unclear. Here, in L. kaempferi we identified five members of the miR171 family, which cleave LaSCL6 mRNA at different sites. In addition, we improved the method of measuring miRNA activity in a more direct way. Furthermore, we measured the expression patterns of mature miR171s and their primary transcripts during somatic embryogenesis in L. kaempferi and found that their patterns differed, indicating that the transcription of MIR171 genes and the subsequent cleavage of their intermediate products are regulated. Taken together, our findings not only offer a means to study the regulation of miRNA activity, but also provide further insight into the regulation of L. kaempferi somatic embryogenesis by miR171-LaSCL6.

Keywords Gene expression · Japanese larch · LaSCL6 · microRNA171 · microRNA activity · Embryogenic cultures

Introduction

Somatic embryogenesis is not only a valuable technique in clonal propagation and genetic improvement, but is also an ideal experimental system in which to study the mechanisms that regulate plant development (Dobrowolska et al. 2017; Godee et al. 2017; Zhang et al. 2019). The induction of embryogenic cultures and the maintenance of embryonic potential are important for clonal plant production through somatic embryogenesis. However, after certain subculture period, the conifer embryogenic cultures may become non-embryogenic and this prevents the formation of somatic embryos and limits utilization of this technique (Klimasze-wska et al. 2016; Zhang et al. 2010). Thus, studying the molecular events controlling various stages of somatic embryogenesis could lead to a better understanding of the whole process and potentially improve the efficiency of this technique.

Many genetic factors, including microRNAs (miRNAs), have been found to control the process of somatic embryogenesis, such as AGAMOUS-LIKE (Gao et al. 2020), FUSCA (Liu et al. 2018), LEAFY COTYLEDON (Wang et al. 2020), miR156 (Long et al. 2018), miR166 (Li et al. 2016), and miR171 (Li et al. 2017). MiRNAs repress gene expression through mRNA degradation and translational inhibition (Awan et al. 2018; Djuranovic et al. 2012; Yang et al. 2019). Recently, the function of miR171 in somatic embryogenesis of angiosperm and gymnosperm has attracted increasing attention (Li et al. 2017, 2014; Zang et al. 2019). The miR171 family is highly conserved, and functions via regulating its target gene
**Materials and methods**

**Plant materials**

The induction and proliferation of cell cultures and somatic embryo maturation were performed according to the previous studies (Zhang et al. 2010, 2012, 2019). The immature seeds of *L. kaempferi* were collected in the middle of June from Dagujia seed orchard (42° 22′ N, 124° 51′ E), Liaoning Province, in northeast China. The embryonal-suspensor mass was induced from immature embryos on induction medium and then cultured on proliferation medium. The subculture was performed every 3 weeks. During subculture, some embryogenic cultures transformed into non-embryogenic cultures. Embryogenic and non-embryogenic cultures were isolated, cultured separately on proliferation medium, and harvested after culture for 14 days. After growth for 3 weeks on proliferation medium, the embryogenic cultures were then transferred to maturation medium and cultures were collected at 0, 7, 14, 21, 28, 35, and 42 days. All the samples were stored at −80 °C until DNA and RNA extraction.

**Sequence cloning and analysis of MIR171 genes**

The genomic DNA of *L. kaempferi* was isolated from the embryonal-suspensor mass with the CTAB plant genome DNA rapid extraction kit (Aidlab, China) according to the manufacturer’s protocols. *MIR171* sequences were amplified from genomic DNA with Platinum® Taq DNA polymerase (Invitrogen, USA) with specific primers (Table S1), which were designed based on the annotation of miRNA (Liu et al. 2016; Zhang et al. 2012) of *L. kaempferi*. The PCR products were purified with the Gel Extraction kit (Tiangen, China), ligated into the pEASY®-T1 simple cloning vector (Transgen, China), and sequenced. The stem-loop secondary structures of *miR171* precursors were analyzed with the Mfold web server (https://unafold.rna.albany.edu/?q=mfold) using the default parameters.

**Sequence alignment and cleavage site analysis**

The mature sequences of *miR171s* from *Arabidopsis thaliana* (Ath-miR171a, Ath-miR171b, and Ath-miR171c) and *Picea abies* (Pab-miR171a, Pab-miR171b, and Pab-miR171c) were obtained from PmiREN (https://www.pmiren.com/) (Guo et al. 2019). The sequence of *miR171a* from *Pinus densata* (Pde-miR171a) was identified by Hai et al. (2017). Together with *L. kaempferi* miR171s (Lka-miR171s), these *miR171* sequences were aligned with ClustalX (Thompson et al. 1997). The relationships between
the sequences of Lka-miR171s and LaSCL6 mRNA were analyzed using the psRNATarget web server (https://plantgrn.noble.org/psRNATarget/analysis?function=3) (Dai and Zhao 2011).

**Expression patterns of miR171s and LaSCL6 detected by qRT-PCR**

Total RNA was isolated with the RNAiso Plus reagent kit (TaKaRa, Japan) according to the manufacturer’s protocols and then reverse-transcribed into cDNA with the Mir-X miRNA First Strand Synthesis Kit (Clontech, USA) and TransScript® II one-step gDNA removal and cDNA Synthesis SuperMix kit (Transgen, China). TB Green™ Advantage® qPCR Premix (Clontech, USA) was used to assess the expression levels of mature miR171s, and TB Green® Premix Ex Taq™ (Tli RNase H Plus) (Takara, Japan) was used to assess the expression levels of the initial and non-cleaved transcripts of LaSCL6 and primary transcripts of miR171s. The primers, which are located downstream from the miR171 cleavage sites, were used for initial (both non-cleaved and cleaved) transcripts of LaSCL6; the primers, which span the miR171 cleavage sites, were used for non-cleaved transcripts of LaSCL6 (Li et al. 2014). *L. kaempferi* translation elongation factor-1 alpha 1 (*LaEF1A1*) served as the internal control (Li et al. 2014). All the primer sequences are listed in Table S1. The qRT-PCR was performed with three biological replicates and data are shown as the mean ± SD. Analysis of variance (ANOVA) was performed and the P value was generated between two samples.

**Results**

**Relationships between miR171 and LaSCL6**

Five *MIR171* genes were identified in *L. kaempferi*. After analysis of the stem-loop secondary structures of the precursors of miR171s, we found that all the mature sequences of the miR171 family were located on the 3′ arm of the stem-loop structure and the miR171a and miR171c sequences were the same (Fig. 1). *L. kaempferi* miR171s
shared high sequence identity with those from the other three species (Fig. 2). The sequence of Lka-miR171b was the same as Pab-miR171c, and one nucleotide change was found in Lka-miR171d and Ath-miR171a (Fig. 2).

The predicted target sequences of the miR171s were present in the LaSCL6 mRNA sequence (Fig. 3). When counting G:U pairs as 0.5 mismatches, LaSCL6 had 1.5 mismatches to miR171a/c, 0.5 mismatches to miR171b/d, and 2.5 mismatches to miR171e (Fig. 3). Further analysis showed that the cleavage sites 1, 2, and 3 (S1, S2, and S3) were located between the nucleotides paired to positions 10 and 11 of miR171d, miR171a/b/c, and miR171e, respectively (Fig. 3).

Expression patterns of LaSCL6 during somatic embryogenesis

The expression patterns of initial and non-cleaved transcripts of LaSCL6 during somatic embryogenesis were analyzed by qRT-PCR with LaEF1A1 as the internal control. The level of LaSCL6 initial transcripts in embryogenic cultures was about 16.2 times higher than that in non-embryogenic cultures \((P < 0.05)\) (Fig. 4a), while the level of non-cleaved transcripts of LaSCL6 was about 7.6 times higher than that in non-embryogenic cultures \((P < 0.05)\) (Fig. 4a). During the maturation of somatic embryos, the levels of initial and non-cleaved transcripts of LaSCL6 were higher at days 21, 28, and 42 with different fold-changes \((P < 0.05)\) (Fig. 4c).

We further calculated the ratio between the expression levels of non-cleaved and initial LaSCL6 transcripts in each sample using the relative quantitative analysis method. The ratios were lower in embryogenic cultures than in non-embryogenic cultures \((P < 0.05)\) (Fig. 4b) and were lower at day 0 than at other days in the maturation of somatic embryos \((P < 0.05)\) (Fig. 4d).

Fig. 3 Relationships between miR171s and LaSCL6. Relationships between the sequences of miR171s and LaSCL6 mRNA. The matches are indicated by straight lines and G:U wobbles are represented by circles. The numbers above indicate the nucleotides paired to the positions of miRNA. Arrows \((S1, S2, and S3)\) indicate the positions of the cleaved mRNA fragments identified by RNA ligase-mediated amplification of cDNA ends \((RLM-5′ RACE)\), and the numbers below refer to the frequency of RLM-5′ RACE clones corresponding to the cleavage sites (Li et al. 2014)

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**Fig. 2** Multiple sequence alignment of mature miR171s with ClustalX. Identical residues are indicated by asterisks. Pde, Pinus densata; Ath, Arabidopsis thaliana; Pab, Picea abies; Lka, Larix kaempferi

| Tree          | miR171s     | Position | 5′ Sequence | 3′ Sequence |
|---------------|-------------|----------|-------------|-------------|
| LaSCL6        | 5′...AACUCGGCGGGUUUAUACGG...3′ | 955 bp   | S1, S2, S3  |

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**Fig. 4**

- **Expression patterns** of **LaSCL6** during somatic embryogenesis.
- **qRT-PCR** with **LaEF1A1** as the internal control.
- Initial transcripts in embryogenic cultures were 16.2 times higher than non-embryogenic cultures \((P < 0.05)\) (Fig. 4a).
- Non-cleaved transcripts were 7.6 times higher in embryogenic cultures \((P < 0.05)\) (Fig. 4a).
- Maturation of somatic embryos showed higher levels at days 21, 28, and 42 (Fig. 4c).
- Ratios between non-cleaved and initial transcripts were lower in embryogenic cultures \((P < 0.05)\) (Fig. 4b).

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**Table:**

| Tree          | miR171s     | Position | 5′ Sequence | 3′ Sequence |
|---------------|-------------|----------|-------------|-------------|
| Pde-miR171a   | 5′...UGAUUGAGGGCCGCGGCAAUAUC...3′ | 954 bp   | S1, S2, S3  |
| Ath-miR171a   | 5′...UGAUUGAGGGCGGCAAUAUC...3′ | 954 bp   | S1, S2, S3  |
| Ath-miR171b/c | 5′...UGAUUGAGGGCGGCAAUAUCAC...3′ | 954 bp   | S1, S2, S3  |
| Pab-miR171a/b | 5′...UGAUUGAGGGCGGCAAUAUCAC...3′ | 954 bp   | S1, S2, S3  |
| Pab-miR171c   | 5′...UGAUUGAGGGCGGCAAUAUCAC...3′ | 954 bp   | S1, S2, S3  |
| Lka-miR171a/c | 5′...UGAUUGAGGGCGGCAAUAUCAC...3′ | 954 bp   | S1, S2, S3  |
| Lka-miR171b   | 5′...UGAUUGAGGGCGGCAAUAUCAC...3′ | 954 bp   | S1, S2, S3  |
| Lka-miR171c   | 5′...UGAUUGAGGGCGGCAAUAUCAC...3′ | 954 bp   | S1, S2, S3  |
| Lka-miR171d   | 5′...UGAUUGAGGGCGGCAAUAUCAC...3′ | 954 bp   | S1, S2, S3  |
Expression patterns of mature miR171s and their primary transcripts during somatic embryogenesis

The expression patterns of mature miR171s and their primary transcripts during somatic embryogenesis were measured by qRT-PCR with LaEF1A1 as the internal control. The levels of primary transcripts of miR171a/b/c/e were higher in embryogenic cultures ($P < 0.05$) (Fig. 5a, b, c, e), while that of miR171d showed almost no difference in embryogenic and non-embryogenic cultures ($P > 0.05$) (Fig. 5d). The levels of mature miR171b/d/e were higher in embryogenic cultures ($P < 0.05$), but their fold-changes (64.4, 5.1, and 17.0) (Fig. 5g, h, i) differed from those for their primary transcripts (6.2, 1.1, and 6.1) (Fig. 5b, d, e). During the maturation of somatic embryos, higher levels of mature miR171s were detected at day 0 ($P < 0.05$) (Fig. 5o–r), but the expression of their primary transcripts showed different patterns (Fig. 5j–n).

Discussion

Regulation of LaSCL6 by miR171 during L. kaempferi somatic embryogenesis

Identification of precursor is the prerequisite of identifying mature miRNA (Jones-Rhoades et al. 2006; Voinnet 2009). In the previous studies, the mature miR171s’ sequences were obtained by sequencing without identifying their precursors (Li et al. 2014; Zhang et al. 2012). Here, five members of the miR171 family were identified in L. kaempferi after analyzing their precursors, which contributes to study the relationships between miR171 members and LaSCL6 and the function of miR171-LaSCL6 module during somatic embryogenesis. There were few mismatches ($\leq 2.5$) between five miR171 sequences and the LaSCL6 mRNA sequence (Fig. 3). This near-perfect complementarity facilitated the
Fig. 5  Expression patterns of mature miR171s and their primary transcripts during somatic embryogenesis with LaEF1A1 as the internal control.
binding of miR171s to LaSCL6 mRNA to cleave it. The miRNA-directed cleavage of its target mRNA generally occurs between the nucleotides paired to positions 10 and 11 of the miRNA (Mallory and Bouche 2008). Considering the occurrence of three cleavage sites in LaSCL6 mRNA and the results of sequence alignment analysis of the miR171s and LaSCL6 mRNA (Fig. 3), miR171a/b/c, miR171d, and miR171e might cleave LaSCL6 mRNA at the S2, S1, and S3 sites, respectively. Notably, the frequencies of these three cleavage sites (Li et al. 2014) were different, suggesting that the roles played by the miR171s also differed. The frequency of the S1 site was higher (Fig. 3), indicating that miR171d might be more active in cleaving LaSCL6 mRNA.

**The post-transcriptional regulation of LaSCL6 by miR171 participates in the maintenance of embryogenic potential**

The levels of initial and non-cleaved transcripts of LaSCL6 were higher in embryogenic cultures (P < 0.05), but the fold-changes of the levels of the initial transcripts were greater than those of the non-cleaved transcripts (Fig. 4a), indicating that more transcripts of LaSCL6 were cleaved and higher miR171 activity occurred in embryogenic cultures (Li et al. 2014). Notably, in embryogenic and non-embryogenic cultures, the expression patterns of initial transcripts of LaSCL6 differed from those reported in our previous study (Li et al. 2014), indicating that there is no tight relationship between LaSCL6 transcription and the maintenance of embryogenic potential, while the same pattern of miR171 activity was detected in both studies, further indicating that it is the post-transcriptional regulation of LaSCL6 by miR171 that participates in the maintenance of embryogenic potential (Li et al. 2014).

**Dynamic change in miR171 activity during somatic embryogenesis**

We assumed here that if the transcripts of a gene were not cleaved by miRNA, the levels of its initial and non-cleaved transcripts in one sample would be the same, whereas if the transcripts of a gene were cleaved by miRNA, the level of initial transcripts would be higher than that of its non-cleaved transcripts in one sample. Therefore, the activity of miRNA could be indicated intuitively by the ratio between the expression levels of the non-cleaved and initial transcripts of target gene in one sample. When the ratio in one sample is lower, it means that more transcripts are cleaved and higher miRNA activity occurs in this sample.

Using this method, we re-analyzed the changes in miR171 activity during somatic embryogenesis. Higher activity of miR171 occurred in embryogenic cultures during subculture (P < 0.05) (Fig. 4b) and in the cultures at day 0 during the maturation of somatic embryos (P < 0.05) (Fig. 4d).

In the previous studies, the activity of miRNA was assessed by analyzing the levels of the target mRNA with another gene as internal control (Li et al. 2014; Tsuji et al. 2006). In this paper, we adjusted the method and used the initial transcript of the target gene as the internal control without using another gene, so that the miRNA activity was shown more simply and intuitively. This method not only offers a powerful tool to assess miRNA activity, but also helps in investigations of the mechanisms of miRNA-directed gene regulation in plant development.

**Regulation of miR171 is involved in L. kaempferi somatic embryogenesis**

The processes of miRNA biogenesis can affect its activity (Viswanathan et al. 2008; Yue et al. 2011), but little attention has been paid to the control of miR171 biogenesis during somatic embryogenesis (Li et al. 2017, 2014; Zang et al. 2019; Zhang et al. 2012). Here we measured the expression of mature miR171s and their primary transcripts during somatic embryogenesis and analyzed their relationships to clarify the regulation of miR171s.

Higher levels of miR171a/b/c/e primary transcripts were found in embryogenic cultures (P < 0.05) (Fig. 5a, b, c, e), indicating that the transcription of miR171a/b/c/e was stronger and involved in the maintenance of embryogenic potential. Higher levels of mature miR171b/d/e occurred in embryogenic cultures (P < 0.05) (Fig. 5g–i), but their fold-changes were higher than those for their pri-miRNAs (Fig. 5b, d, e), indicating that more pri-miR171b/d/e was cleaved into mature miR171b/d/e and that their cleavage was regulated. Notably, the level of pri-miR171d in embryogenic and non-embryogenic cultures showed almost no difference (P > 0.05) (Fig. 5d), but that of mature miR171d was higher in embryogenic cultures (P < 0.05) (Fig. 5h), indicating that the regulation of miR171d occurred in steps (2) and (3) of its biogenesis but not in step (1).

During the maturation of somatic embryos, the levels of all mature miR171s were higher at day 0 (P < 0.05) (Fig. 5o–r), indicating that miR171s are more active in post-embryogenic development (Fig. 4d). Meanwhile, the levels of their primary transcripts were not always higher at day 0 (Fig. 5j–n), indicating that the regulation of their transcription and cleavage were different and complex.

The cleavage of MIR171 transcripts was regulated during their biogenesis, and this might result from the differential expression of the activity of DCL1 and its partners (Kurihara et al. 2006). Of course, other factors involved in miRNA biogenesis such as transcription factors (Mediator 17/18 and Cell Division Cycle 5) (Zhang et al. 2015), RNA-binding proteins (Ren and Yu 2012), and decapping proteins
(Motomura et al. 2012), might also participate in the regulation of miR171 biogenesis.

Embryogenic culture differs from non-embryogenic culture in the mode of cell division, cell structure and morphology, histological structure, and cell fate (Quiroz-Figueroa et al. 2002). The abundance and activity of miR171 were different between the embryogenic and non-embryogenic cultures, suggesting that miR171-LaSCL6 functions in the maintenance of embryogenic potential. Notably, SCL6 interacts with WUSCHEL and SQUAMOSA PROMOTER BINDING PROTEIN LIKE to regulate meristem activity (Zhou et al. 2018) and flowering (Xue et al. 2014), respectively, adding more information about the function of miR171-SCL6 module and helping to reveal the molecular mechanism of somatic embryogenesis.

Author contribution statement Q-LZ carried out the study, analyzed the data, and wrote the manuscript. YZ and S-YH helped to culture the plant materials. W-FL designed the study, helped to analyze the data, and revised the manuscript. L-WQ provided suggestions on the experimental design and analyses. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Data archiving statement The sequences of MIR171albicidle in L. kaempferi have been submitted to GenBank with the accession numbers MN790738, MN790739, MN790740, MN790741, and MN790742, respectively.

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