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RESEARCH NOTE

Regulation of *LaMYB33* by miR159 during maintenance of embryogenic potential and somatic embryo maturation in *Larix kaempferi* (Lamb.) Carr.

Wan-Feng Li · Shou-Gong Zhang · Su-Ying Han · Tao Wu · Jun-Hong Zhang · Li-Wang Qi

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Abstract During the process of subculture of embryogenic cultures, sometimes they may become non-embryogenic, which is not desirable. However, this offers an opportunity to explore the mechanisms underlying cell fate determination and the maintenance of embryogenic potential of explants during the process of somatic embryogenesis. In a previous study, differential expression of microRNAs (miRNAs) has been detected between embryogenic and non-embryogenic cultures as well as during somatic embryo maturation of *Larix kaempferi* (Lamb.) Carr. However, little is known about the target genes of these miRNAs during these cellular differentiation processes. In this study, full-length cDNA of the *MYB* homologue from *L. kaempferi*, *LaMYB33*, was cloned. Sequence analysis showed that the miR159 target sequence is present in *LaMYB33*. The isolation of the miRNA-guided cleavage products of *LaMYB33* further suggested that this gene is regulated by miRNA. *LaMYB33* transcript levels between embryogenic and non-embryogenic cultures and during the late stage of somatic embryo maturation were measured and the results showed opposite patterns in the expression of *LaMYB33* and mature miR159. Based on the relationships between the expression patterns of *LaMYB33* and mature miR159, we concluded that the post-transcriptional regulation of *LaMYB33* by miR159 participates in the maintenance of embryogenic or non-embryogenic potential and somatic embryo maturation, providing new insights into the regulatory mechanisms of somatic embryogenesis.

Keywords Somatic embryogenesis · *LaMYB33* · miR159 · *Larix kaempferi* (Lamb.) Carr.

W.-F. Li · S.-G. Zhang · T. Wu · J.-H. Zhang · L.-W. Qi (✉)
Laboratory of Cell Biology, Research Institute of Forestry, Chinese Academy of Forestry, Beijing 100091, People's Republic of China
e-mail: lwqi@caf.ac.cn

W.-F. Li
e-mail: liwanfeng99@163.com

S.-Y. Han
Key Laboratory of Research Institute of Forest Ecology and Protection, Chinese Academy of Forestry, Beijing 100091, People's Republic of China

Introduction

Somatic embryogenesis provides a useful experimental system to investigate the regulatory mechanisms of plant development (Cairney and Pullman 2007; Quiroz-Figueroa et al. 2006; Zimmerman 1993). Global changes in gene expression occur during the formation of a somatic embryo (Vestman et al. 2011; Zhang et al. 2009). In previous work, we found that members of the miR159 family show higher levels in non-embryogenic than in embryogenic cultures of *Larix kaempferi* (Lamb.) Carr. (Zhang et al. 2010a), and the same result was found in *Citrus sinensis* L. Osb (Wu et al. 2011). In addition, our recent work showed that the expression levels of members of the miR159 family are differentially regulated during the late stage of somatic embryo maturation in *L. kaempferi* (Zhang et al. 2012). Generally, miRNAs control gene expression at the post-transcriptional level through both mRNA degradation and translational inhibition (Chen 2010; Zhang et al. 2006). Taken together, these results suggest that regulation of gene expression at both the transcriptional and post-transcriptional levels is involved in somatic embryogenesis, especially in cell fate determination, the maintenance of embryogenic or non-embryogenic potential, and somatic embryo maturation.

Regulation of MYB transcription factor by miR159 participates in many developmental processes, and *AtMYB33* and its homologues from other species have been verified as the target genes of miR159 (Achard et al. 2004; Allen et al. 2007, 2010; Alonso-Peral et al. 2010, 2012; Lu et al. 2005, 2006, 2007; Millar and Gubler 2005; Palatnik et al. 2003; Reyes and Chua 2007; Schwab et al. 2005; Tsuji et al. 2006). Analyzing the expression patterns of miRNA and its target genes contributes to understanding the functions of miRNA. In view of this, investigating the relationships between the expression patterns of miR159 and its target genes is essential to elucidate the regulatory mechanism of miR159 in *L. kaempferi* somatic embryogenesis.

Materials and methods

Database search, cloning full-length cDNA, and sequence analysis

First, we cloned the full-length cDNA sequence of the *MYB33* homologue, *LaMYB33*, from *L. kaempferi*. According to the cDNA sequence of *AtMYB33* (GenBank accession no. NM_180448.1) and its homologues from *Pinus taeda* L. (TC60689, DFCI-Pine Gene Index, http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=pine), *Oryza sativa* (GenBank accession no. X98355.1) and *Hordeum vulgare* (GenBank accession no. X87690.1) (Gubler et al. 1995), the degenerate primers 5′-AA(C/T)A(A/G)(A/G)TGGGC(T/A)CG(G/T)ATGGC-3′ and 5′-GAAGGGAGCTCCA(T/G)CTTC-3′ were used to clone the cDNA sequence fragment of the *MYB33* homologue from *L. kaempferi*, then rapid amplification of cDNA ends (RACE) experiments were performed to get the 3′ and 5′ end cDNA sequences using a SMARTer^{TM} RACE cDNA amplification kit (Clontech). Total RNA isolated from embryogenic cultures was used for cloning. The gene-specific outer primer 5′-ATGCTTCAAGGCTGCTCG-3′ and inner primer 5′-CTATCAACGCCCTTTGGG-3′ were used for 3′ RACE, and outer primer 5′-ATGGGTTGCTGATTCTGTTGATTCTC-3′ and inner primer 5′-GGGCGGGTAAAGAGGTAAT-3′ for 5′ RACE. The primers 5′-TGGTGGTGTAATGGTGATG-3′ and 5′-TGCAATGAAATAGGGTTCTA-3′ were used to amplify the full-length cDNA sequence to confirm the RACE results. The PCR products were purified using Bioteke PCR purification columns and subsequently cloned into the pGEM-T easy vector (Promega) and sequenced. The full-length cDNA sequence was submitted to GenBank with the accession no. JX157847 for *LaMYB33*. Multiple protein sequence alignments were made using ClustalX software (Thompson et al. 1997).

RNA ligase-mediated amplification of cDNA ends (RLM-5′ RACE)

Second, we identified the cleavage products of *LaMYB33* mRNAs to confirm the regulation of *LaMYB33* by miR159 in vivo using RNA ligase-mediated amplification of cDNA ends (RLM-5′ RACE) (Liu and Gorovsky 1993), using a 5′ RACE kit (Invitrogen). Total RNA was isolated from embryogenic cultures and RNA oligo adapters were directly ligated to the purified total RNA without calf intestinal phosphatase and tobacco acid pyrophosphatase treatment. The gene-specific outer primer 5′-CATGGACTGGTGCTCAAA-3′ and inner primer 5′-CTGAAGCAGCAGGACCAC-3′ were used for RLM-5′ RACE. The PCR products with the expected sizes were purified as above, then cloned and sequenced.

Quantitative RT-PCR (qRT-PCR)

Finally, we assayed the expression patterns of *LaMYB33* in embryogenic and non-embryogenic cultures and during the late stage of somatic embryo maturation in *L. kaempferi* to investigate the relationships between the expression patterns of mature miR159 and *LaMYB33*. To further confirm miR159 regulation in somatic embryogenesis, the expression patterns of one of its target genes, *LaMYB33*, in embryogenic and non-embryogenic cultures and during the late stage of somatic embryo maturation in *L. kaempferi*, were compared using quantitative reverse transcription-PCR (qRT-PCR). The same RNA from embryogenic or non-embryogenic cultures as for miRNA hybridization was used for qRT-PCR analysis (Zhang et al. 2010a). In our previous study, three pairs of embryogenic and non-embryogenic cultures were used for miRNA hybridization: E430 and H430, E017 and H017, and E375 and H375 (E stands for embryogenic callus; H stands for non-embryogenic callus) (Zhang et al. 2010a). These calli were initially all embryogenic, but during subculture, some became non-embryogenic. Embryogenic and non-embryogenic cultures from the same calli were isolated and subcultured separately in fresh proliferation medium. The materials were harvested after subculture for 3 or 14 days. The maturing somatic embryos after maturation culture for 3, 4, 5 or 7 weeks, which were at developmental stages 5, 6, 7 and 8, respectively, based on morphological observation (Zhang et al. 2012), were used to assay the expression patterns of *LaMYB33* during the late stage of somatic embryo maturation. Total RNA was extracted with a plant RNA extraction kit (R1050, Applygen) according to the manufacturer's protocol. A 5-μg aliquot of total RNA was reverse-transcribed into cDNA with the RevertAid^{TM} H Minus First Strand cDNA Synthesis kit (K1631, Fermentas, Life Sciences), and then diluted for gene isolation and qRT-PCR analysis.

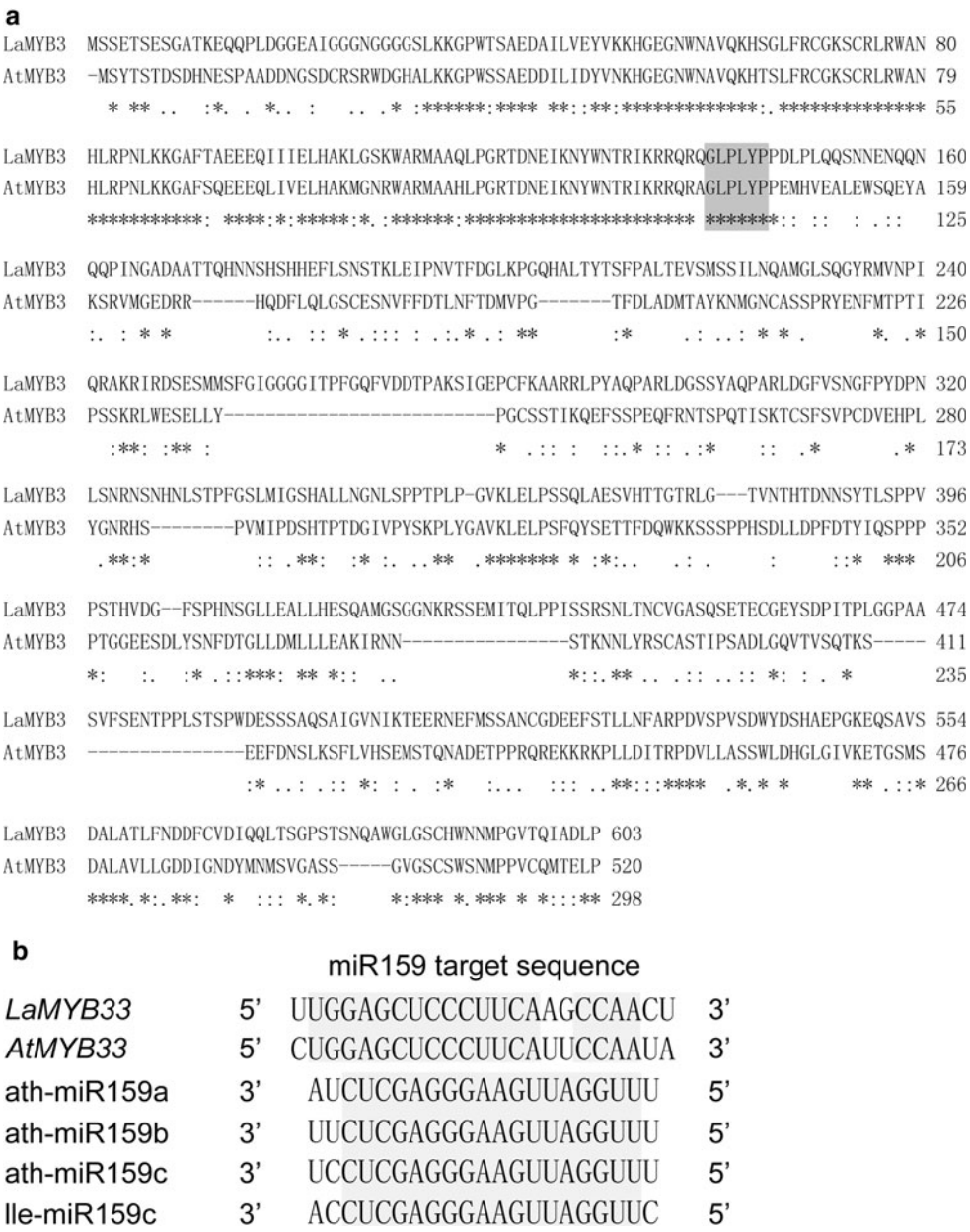

Fig. 1 Sequence analysis of *LaMYB33*. **a** Multiple protein sequence alignment of LaMYB33 and AtMYB33 (GenBank accession no. NP_850779.1) amino-acid sequences. Subgroup-specific motif signatures are *shaded grey*. Identical residues are indicated by *asterisks*. **b** Alignment of miR159 target sequences in *AtMYB33* and *LaMYB33* transcripts. Identical nucleotides between target sequences or mature miR159 sequences are *shaded grey*. Mature sequences of ath-miR159a, ath-miR159b and ath-miR159c were from the miRBase database (http://www.mirbase.org/cgi-bin/mirna_summary.pl?org=ath) and the mature sequence of lle-miR159c, one member of the *L. kaempferi* miR159 family, was identified by Zhang et al. (2012)

The qPCR analysis was performed on an Applied Biosystems 7500 Real-Time PCR System using the SYBR Premix EX Taq kit (TaKaRa Biotechnology) with *LaEF1A1* (GenBank accession no. JX157845) as internal control. Each reaction was carried out on 5 μl of diluted cDNA sample, in a total reaction system of 20 μl. The reaction procedure was set up according to the manufacturer's protocol. The primers 5′-TGGGAGTTTGATGATTGG-3′ and 5′-AAGTTGGCTTGAAGGGAG-3′ were used for non-cleaved *LaMYB33* transcripts, 5′-AGACAGAATGTGG CGAATA-3′ and 5′-CATGGACTGGTGCTCAAA-3′ for both non-cleaved and cleaved *LaMYB33* transcripts (Fig. 3a), and 5′-GACTGTACCTGTTGGTCGTG-3′ and 5′-CCTCCAGCAGAGCTTCAT-3′ for *LaEF1A1*. To check the specificity of amplification, the melting curves of the PCR products were determined. The expression levels were standardized to the constitutive expression level of *LaEF1A1*. The ratio between the expression levels of *LaMYB33* and *LaEF1A1* for each sample was calculated using the relative quantitative analysis method. The qRT-PCR was performed with three biological replicates. Data are shown as mean ± SD.

Results and discussion

Cloning and analyzing the sequence of *LaMYB33* in *L. kaempferi*

The deduced amino-acid sequence for *LaMYB33* displayed high homology with AtMYB33 from *Arabidopsis* (Fig. 1a). The sequence was 603 amino-acids long and contained a GLPLYP motif characteristic of subgroup 18 of the R2R3-MYB genes from *Arabidopsis* (Stracke et al. 2001) (Fig. 1a). Further analysis showed that the miR159 target sequence was present in the *LaMYB33* transcript (Fig. 1b).

Determination of the cleavage sites within *LaMYB33* sequence

To verify the cleavage of *LaMYB33* mRNA by miR159 in vivo and identify the cleavage sites within *LaMYB33* mRNAs, RLM-5′ RACE was performed. The RLM-5′ RACE analysis showed cleavage products for *LaMYB33* transcripts in vivo (Fig. 2a) and determined the cleavage sites within the predicted miR159 target sequence (Fig. 2b), indicating that *LaMYB33* mRNA is an in vivo miR159 cleavage target and miR159 regulation of *LaMYB33* occurs in *L. kaempferi*.

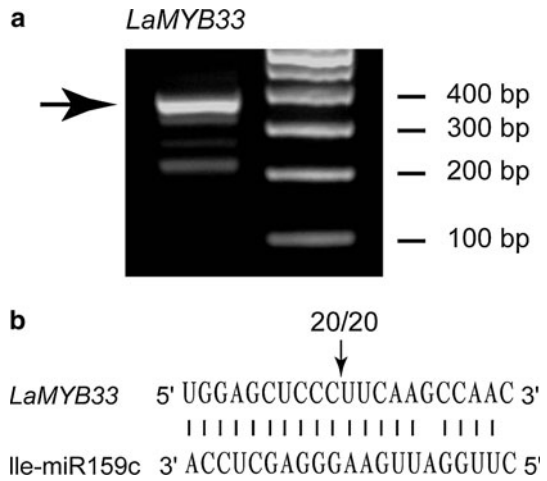

Fig. 2 miR159 cleavage sites in *LaMYB33* mRNAs determined by RLM-5′ RACE. **a** Agarose gel electrophoresis showed the nested PCR products that were cloned and sequenced for *LaMYB33*. The band with the expected size indicated by the *arrow* was cut out for purification. **b** Base-pairing interaction between miR159 and the *LaMYB33* transcript. *Vertical arrow* indicates the 5′ positions of the cleaved mRNA fragments identified by RLM-5′ RACE and the numbers refer to the frequency of RLM-5′ RACE clones corresponding to cleavage sites

LaMYB33 expression patterns and their relationships with mature miR159 expression during somatic embryogenesis

The qPCR analysis showed that the *LaMYB33* transcripts were expressed strongly in the embryogenic cultures and weakly in the non-embryogenic cultures (Fig. 3b, c), reflecting a negative correlation between the expression patterns of mature miR159 and its target gene between embryogenic and non-embryogenic cultures (Zhang et al. 2010a). During the late stage of *L. kaempferi* somatic embryo maturation, the *LaMYB33* transcript levels decreased (Fig. 3d, e), while the expression levels of mature miR159 increase (Zhang et al. 2012), also reflecting a negative correlation between the expression patterns of mature miR159 and its target gene. These results suggest that maintenance of the embryogenic or non-embryogenic potential and somatic embryo maturation involve miR159 regulation of *LaMYB33* post-transcriptionally.

During the process of subculture, transformation of embryogenic cultures to non-embryogenic cultures always means a change of cell fate and loss of embryogenic potential of explants (Quiroz-Figueroa et al. 2002; Zhang et al. 2010a). High expression of mature miR159 in non-embryogenic cultures indicates that miR159 functions in this transformation (Wu et al. 2011; Zhang et al. 2010a), and this is further supported by low expression of *LaMYB33* in non-embryogenic cultures.

Environmental and hormonal signals and some other chemicals play regulatory roles in somatic embryogenesis (Abrahamsson et al. 2012; Fehér et al. 2003; Hakman et al. 2009; Larsson et al. 2008; Lu et al. 2011; Milojević et al. 2012; Ramakrishna et al. 2012; Rodríguez-Sahagún et al. 2011; Uddenberg et al. 2011). For example, abscisic acid (ABA) promotes somatic embryo maturation (Gutmann et al. 1996; Rai et al. 2011). To understand the molecular basis of ABA regulation in somatic embryogenesis, many genes associated with ABA signaling have been identified (Fehér et al. 2003; Guan et al. 2009; Schlögl et al. 2012; Shiota et al. 2008; Vestman et al. 2011; Zhang et al. 2010b). Interestingly, miR159, which is induced by ABA (Reyes and Chua, 2007), has also been detected during somatic embryogenesis (Wu et al. 2011; Zhang et al. 2010a, 2012), indicating that interaction of miR159 and its target gene is involved in this process. In this study, we identified a target gene of miR159, *LaMYB33*, from *L. kaempferi* and confirmed the miR159 regulation of *LaMYB33* in *L. kaempferi* somatic embryogenesis, providing new evidence of miRNA-mediated ABA regulation in somatic embryogenesis.

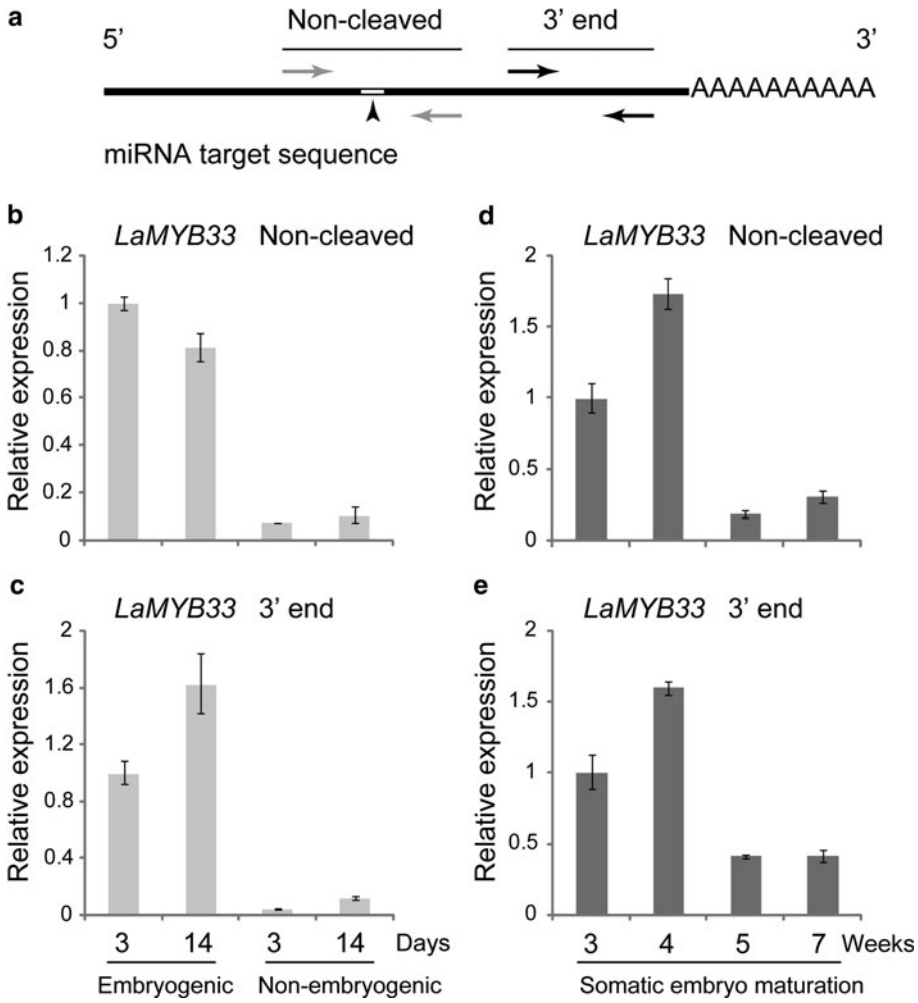

Fig. 3 Expression patterns of *LaMYB33* assayed by qRT-PCR using *LaEF1A1* as the internal control. **a** Schematic of primer design. Primers indicated by *two grey arrows*, which amplified sequences including the miR159 target site, were used to measure the non-cleaved mRNAs, and primers indicated by *two black arrows* were used to measure both non-cleaved and cleaved mRNAs. **b**, **c** Expression patterns of *LaMYB33* in embryogenic and non-embryogenic cultures sampled at 3 and 14 days of subculture. **d**, **e** Expression patterns of *LaMYB33* during the late stage of somatic embryo maturation. Samples were harvested after maturation culture for 3, 4, 5 or 7 weeks

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