Quantification of expression of miR156 precursors for evaluation of juvenility in silver birch

Precursor miRNA identification and primer design

Mature miR156 sequences and precursors were selected from other species (based on previous reports that these miRNAs and genes are the main regulators of juvenility) using the miRBase database version 22 (www.mirbase.org) and publicly available mature miRNA sequences from different plant species (Kozomara and Griffiths-Jones 2011, 2014). The CoGe database (https://genomevolution.org/coge/CoGeBlast.pl) was used to identify potential precursor miRNAs in the silver birch genome (*Betula pendula* (id 35079) scaffold assembly, vv1.2 scaffolds unmasked), containing the mature miRNA region. Precursor stem loop secondary structures were predicted using the Mfold program (http://mfold.rna.albany.edu/?q=mfold) web server (Zuker 2003). To distinguish miRNAs from other RNAs, minimum free-folding energy index (MFEI) (Zhang et al. 2006) was calculated to confirm that the precursor sequences conformed to the requirements for forming the miRNA precursor structures as reported previously (Zhang et al. 2006; Axtell and Meyers 2018; Krivmane et al. 2020). The location of the mature miRNA sequence on the stem-loop was taken into account to identify potential pre-miRNAs (Figures 1-3).

Sequence used for miR156_511 primer design:

ACTTTTTTTTTTGTTCTTTCTTCTTTGAGTTTGTTCGCTGAATCCTTTGGTTTCCACATCTGGGA
ATAAGCTGTCTTTATTATTCCTTTTCTAATTTTCTGTTTGTTTCCCTGGGACATAGAAATTGAC
AGAAGAGAGTGAGCACACAGAGGCCATATGGTATAA]AGTCTATACTAATGCTTTTGCGTG
CTCACTTCTCTTTCTGTCAGATTCCAGTCCCGGAGGAGTCCTGCCATTGTTCCAGATCCTATC
TTTTTTCTTTTTTGATGAATATTTTTCTCCTTCAACTTCAACAGC

Red colour indicates mature miRNA sequence

Green colour indicates primer sequences

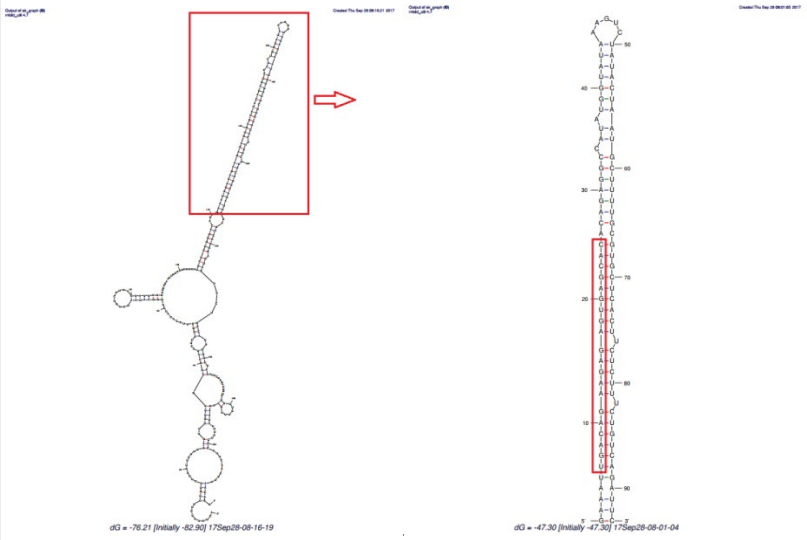

Figure 1. mir156_511 precursor sequence and hairpin-loop structure

Sequence used for miR156_789 primer design:

GCCCCACCCCCAACTCTCTCTCCTCTCTCCACAGTTATCTCCTCAGCTTTTCTCCCATATATAG
GATCGAAAAGGGTTGGAGTCCTCTAGCTAGGCTATCCGAAGCTAGCTTTTCAAATGTGTAA
ATCTTTGAAATAATTAATTAGTTTTAAAGTAGAGGAGGCACTGGTGATGTTGTTGACAGAA
GATAGAGAGCACAGATGATGAAATGCATGGAACTGCTGCATCTCACTCCTTTGTGCTCTCTA
GCTCCTGTCATCACCCTCAGCCCCTCACTTCAACTTCATACTAACATAT

Red colour indicates mature miRNA sequence

Green colour indicates primer sequences

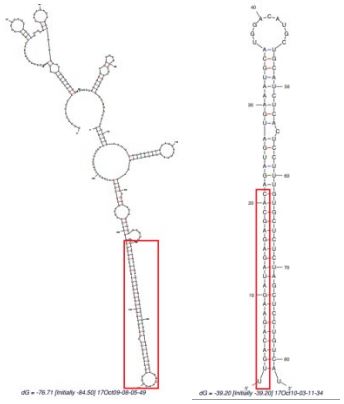

Figure 2. mir156_789 precursor sequence and hairpin-loop structure

Sequence used for miR156_374 primer design:

AAATCAAGAAAATGATTCACATGAGAAAGAAGGTAAGCACAAATATATAGTAAAGGAATG
CAATAATTAATATAGGGAGTAAAGGTGGGGGAGGGGTAGGGGGATGAGGGGCTGAAGGT
GATGACAGAAGCATAGAGAGCACAAAGGAGTGAGATGCAGGAGTTTGTTGCATATCATCA
TCT**GTGCTCTCTATCTTCTGTCAA**CACATCACCAGTGCTTCCTCTTAATTTCCCCCCGGCCCTA
TTGTCCTCTACCATATGGTCGATCTCTTTTTAAAGTAGAAAAGCTTTAAACGTA

Red colour indicates mature miRNA sequence

Green colour indicates primer sequences

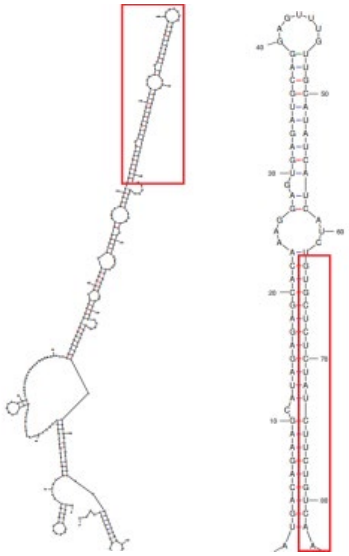

Figure 2. mir156_374 precursor sequence and hairpin-loop structure

Primers for pre-miRNA amplification were designed and selected using predicted pre-miRNA sequences (Primer 3 version 0.4.0; Table 1).

Table 1. Primers used for pre-miRNA and target gene quantification

Primer	**CDS Accession Nr from GoGe database**	**Primer Sequences (5' -3')**	**Length of RT-PCR product (bp)**
miR156_511	FID:1261039049, Bpev01.c0511.g0001.m0001	F: TGTTTCCCTGGGACATAGAA R: GTGAGCACGCAAAAGCATTA	91

miR156_789	FID:1261053161, Bpev01.c0789.g0006.m0001	F: GGCACTGGTGATGTTGTTGA R: GGGTGATGACAGGAGCTAGA	103
miR156_374	FID:1261027051, Bpev01.c0374.g0009.m0001	F: ACAAAGGAGTGAGATGCAGGA R: GTAGAGGACAATAGGGCCGG	114

RNA extraction and quality control

Total extracted RNA from all samples was treated with the Turbo DNA-free kit (ThermoFisher Scientific, Cat. No. AM1907) following the manufacturer's instructions. RNA concentration was measured with a Qubit and Quant-iT™ RNA BR Assay Kit (ThermoFisher, Cat. No. Q10210). RNA purity (DNA contamination) was tested by polymerase chain reaction (PCR) with an RNA stock solution as template and three birch genomic microsatellite locus primers L7.8, L7.4 and L1.10 (Kulju, Pekkinen, and Varvio 2004). Each forward primer was labelled with a different fluorophore (6-FAM, HEX, or TMR) to facilitate visualization using capillary electrophoresis. The PCR reactions for the microsatellite markers were carried out in a 10 µL solution containing a final concentration of 1x HOT FIREPol® Blend Master Mix with 10 mM $MgCl_2$ (Solis Biodyne, Cat.No. 04-27-00120), 0.3 mM of each primer, 1 µL RNA solution. PCR cycling conditions consisted of an initial denaturation step of 95 °C for 15 min; 35 cycles of 95 °C for 20 s, 55 °C for 30 s, and 72 °C for 45 s; followed by a final extension step of 72 °C for 10 min. PCR reaction were carried out in an Eppendorf Mastercycler gradient thermal cycler. Amplification fragments were separated on an ABI Prism 3130xl Genetic Analyzer (Life Technologies) and genotyped with GeneMapper 3.5. If no PCR amplification fragments were detected, RNA samples were considered to be free of DNA contamination. If PCR amplification fragments were detected, RNA samples were again treated with the Turbo DNA-free kit, and RNA concentrations purity were reanalysed prior to reverse transcription and real time PCR analysis.

Reverse transcription and real-time PCR

The Taqman Reverse transcription kit (Thermo Fisher Scientific, Cat. no. 4304134) was used for reverse transcription of 1 µg RNA with Oligo d(T)16 (Thermo Fisher Scientific Cat. no. 4304134). The obtained cDNA was diluted to 10 ng/µl with nuclease-free water and 2 µl of cDNA was used for RT-PCR analysis.

Comparative Ct RT- PCR was performed with the Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific, Cat. No. K0221) using a standard protocol on a StepOnePlus thermocycler (Thermo Fisher Scientific, PN 4376785). For each sample, three technical replications were done. Four reference genes (endogenous controls) were used: α-tubulin (Keinänen et al. 2007; Žiarovská et al. 2013; Fernández-Fuego et al. 2017), Peptidyl-prolyl isomerase (cyclophilin) and transcription factor CBF1 (Žiarovská et al. 2013). Real-time PCR conditions were: 10 min at 95 °C, followed by 40 cycles of denaturation for 15 s at 95 °C, 15 s at 95 °C, 60 s at 60°C, 15 s at 95°C. Data analysis was done using the StepOne ™ Software v.2.3. Relative expression levels were determined and represented using the Relative quantitation (RQ) method after normalization with the endogenous control levels.

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