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Original Paper

Expression of highly conserved developmental phase change regulating miR156 and miR172 microRNAs in silver birch using real-time PCR and high-throughput sequencing methodologies

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Abstract

Silver birch (*Betula pendula* Roth) is the most significant deciduous tree species in Latvia. Efficient vegetative reproduction methods are crucial for shortening breeding cycles. In many woody tree species, success of vegetative propagation sharply decreases as individuals mature. Development of molecular genetic markers related to phase change has the potential to increase the efficiency of vegetative reproduction methods. Studies in annual model plant species have identified evolutionary highly conserved miRNAs that are involved in phase change – miR156 and miR172. This study compared expression levels of these miRNA families using two approaches – real-time PCR (RT-PCR) and high-throughput sequencing, in silver birch *in vitro* shoot samples. High expression of miR156 in juvenile samples was identified by both methods, but increased expression of miR172 was only observed by RT-PCR. Further studies in long-lived perennial species are needed to fully elucidate the miRNAs involved in developmental phase transition processes.

Key words: *in vitro* propagation, juvenility, miR156, miR172, molecular markers.
Abbreviations: HTS, high-throughput sequencing; miRNA, micro ribonucleic acid; mRNA, messenger ribonucleic acid; pre-miRNA, precursor micro ribonucleic acid; RT-PCR. real time polymerase chain reaction.

Introduction

Silver birch (*Betula pendula* Roth) is the most significant deciduous tree species in Latvia. Forest stands with birch as the dominant species occupy 27% of the total area of Latvian forests (http://www.silava.lv/petijumi/nacionlais-mea-monitorings.aspx). In addition, it is a significant silvicultural species, and birch timber is processed for sawn wood, plywood and panelling manufacture, as well as for biomass production (Dubois et al. 2020). A Latvian silver birch breeding programme was established in the mid-1990s, and analysis of results from progeny tests indicate that breeding can enable simultaneous improvement of both growth traits and stem quality (Gailis et al. 2020). In addition, initial results from the silvicultural analysis of a 40-year-old clonal birch plantation indicate that the use of selected clonal material can increase the final value of obtained timber, suggesting that low-density short-rotation plantations can also be effective for birch silviculture in Latvia. Due to the possibility to combine both additive and non-additive gene effects, vegetative propagation of mature birch trees enables capture of genetic gain more effectively compared to generative reproduction (George et al. 2008). Efficient clonal or vegetative reproduction methods are crucial for shortening breeding cycles, enhancing deployment of improved germplasm, as well as being indispensable for the establishment of clonal plantations.

In many woody tree species, including silver birch, as individuals age and transition to the mature phase, their capacity for vegetative propagation sharply decreases. This can pose a significant hurdle, as assessment of silviculturally important traits is most often undertaken in mature trees. Explants obtained from mature trees are often exhibit low morphogenic ability, characterised by slow growth and reduced responsiveness to hormones regulating plant growth (McCown 2000). However, this situation can at least partially be alleviated by the use of micropropagation techniques, whereby mature plant material undergoes rejuvenation via axillary or adventitious shoot proliferation, resulting in juvenile characteristics including a high multiplication and rooting rate (Welander 1993; Sánchez et al. 1997). Rejuvenation of mature shoots can be facilitated by a range of *in vitro* methods, such as composition of growth media and regulation of *in vitro* propagation and maintenance conditions. Responsiveness to these techniques varies between birch individuals, with

some genotypes able to be successfully rejuvenated, while others remain in the mature phase, gradually lose vitality, and are not able to be propagated (Ewald et al. 2001; O'Dowd 2004).

Methods to assess the juvenility or maturity of *in vitro* shoots would enable evaluation of different genotypes and *in vitro* techniques, increasing the efficiency of silver birch micropropagation. Assessment of phase change using morphological and physiological characteristics has been utilised, for example leaf and stem morphology, cellular and sub-cellular anatomy etc. However, in a number of species, these changes are minor, and juvenility state is often difficult to be unambiguously determined (Wang et al. 2011; Feng et al. 2016; Xu et al. 2016). The development of molecular genetic markers related to phase change has the potential to increase the accuracy of assessment of the juvenility or maturity of *in vitro* shoots, and to investigate factors influencing *in vitro* morphogenic properties of silver birch genotypes.

MicroRNAs (miRNAs) are short (usually 20 to 24 nucleotides), non-protein coding RNA molecules that regulate gene expression by binding to messenger RNAs (mRNAs) and supressing their translation to proteins. MiRNAs are involved in many biological and metabolic processes by post-transcriptional regulation of gene expression. After binding to mRNA molecules, mature miRNAs associate with members of the Argonaute (AGO) family of proteins to form a miRNA-induced silencing complex (miRISC), leading to cleavage of mRNAs, thus inactivating them, or interfering with translation processes, thus preventing protein production (Carrington, Ambros 2003). MiRNAs are initially produced as longer (they can be up to several hundred nucleotides long) primary miRNA molecules (pri-miRNAs), which have a characteristic stem-loop structure. These are then processed by various enzymes to precursor miRNAs (pre-miRNAs), which are subsequently enzymatically cleaved to produce mature miRNAs, which are usually 20 to 24 nucleotides in length (Neutelings et al. 2012). MiRNAs are classified into families, based on sequence similarity. MiRNA family members can diversify their functionality, either by sequence divergence (leading to diversification of target mRNAs), or by regulation of expression (pre-miRNAs are expressed in different tissue types or in differing conditions) (Li, Mao 2007).

Studies in annual model plant species have identified evolutionary highly conserved miRNAs that are involved in phase change – miR156 and miR172 (Ahsan et al. 2019a; Ahsan et al. 2019b; Wu et al. 2009; Wang et al. 2011; Xing et al. 2014; Jia et al. 2017). MiR156 represses the expression of *SQUAMOSA* promoter binding protein-like (*SBP*/*SPL*) transcription factor genes, promoting the transition to maturity by up-regulating key MADS-box genes, such as *APETALA* (*AP1*), *LEAFY* (*LFY*) and *FRUITFULL* (*FUL*). Increased expression of miR156 prolongs the juvenile phase and delays phase change for both herbaceous and woody plants (Wu, Poethig 2006; Wu et al. 2009; Wang et al. 2011; Zhang et al. 2015). MiR156 also suppresses expression of miR172, leading to elevated levels of miR172 target genes, which are AP2-like transcription factor family genes: *APETALA2* (*AP2*), *TARGET OF EAT1* (*TOE1*), *TOE2* and *TOE3, SCHLAFMUTZE* (*SMZ*), and *SCHNARCHZAPFEN* (*SNZ*).

The aim of this study was to assess expression levels of these highly conserved miR156 and miR172 microRNAs using two complementary methodological approaches – real-time PCR (RT-PCR) and high-throughput sequencing (HTS), in silver birch *in vitro* shoot samples with differing morphogenic properties. These *in vitro* samples were either rejuvenated explants, which were able to be propagated, or explants showing signs of maturity, which were recalcitrant to propagation. Samples from seedlings and mature trees were used as juvenile and mature controls, respectively. Expression of miR156 is expected to be high in juvenile samples, decreasing as the tissues transition to maturity. Conversely, expression of miR172 is expected to be low in juvenile tissues, and higher in mature samples.

Materials and methods

Four types of sample types were analysed with both analysis techniques – rejuvenated *in vitro* shoots (REV), which can be propagated, *in vitro* mature shoots (IVM), which exhibited typical signs of maturity, and did not proliferate, leaves from two month old seedlings were used as a juvenile control (JUV), and leaves from a mature (~20 years old) silver birch were used as a mature control (MAT).

RNA extraction, PCR primers and real-time PCR methods are described in Krivmane et al. (2022). Briefly, total RNA was extracted from samples using a standard phenol/chloroform/isoamyl alcohol protocol, and checked for DNA contamination by PCR. Comparative Ct RT-PCR was performed with the Maxima SYBR Green/ROX qPCR Master Mix (2 ×) (Thermo Fisher Scientific, Cat. No. K0221) using a standard protocol on a StepOnePlus thermocycler (Thermo Fisher Scientific, PN 4376785) using PCR primers amplifying conserved miRNA precursor sequences. For each sample, three technical replications were done. The actin (Ruonala et al. 2006) and cyclophilin (Žiarovská et al. 2013) genes were used as endogenous controls for normalisation of precursor miRNA expression. The REV sample was set as the reference, therefore having a relative quantity (RQ) value of one. The minimum and maximum RQ values indicate the error associated with the RQ value for the analysed precursor miRNAs and target genes. These values were computed using $RQ_{min} = 2 - (RQ - SE)$, $RQ_{max} = 2 - (RQ + SE)$, where SE is the standard error for the RQ.

For high-throughput sequencing of small RNAs from birch, essentially the same protocol from a previous study in our laboratory of miRNA expression in Scots pine

(Krivmane et al., 2020) was followed. RNA extraction was as for the RT-PCR analyses. Total RNA samples were enriched for small RNA as outlined in the Ion Total RNA-Seq Kit v2 for Small RNA Libraries Preparation guide (Thermofisher Scientific Manual 4475936 revision B.0) and 15 small RNA barcoded libraries were prepared using the CleanTag™ Small RNA Library Preparation Kit (TriLink Biotechnologies Catalog # L-3206) according to the manufacturer's protocol. Libraries were sequenced on an Ion GeneStudio™ S5 System with two Ion 530 sequencing chips using the manufacturer's protocols and kits. The obtained sequences were analysed using the CLC Genomics Workbench software version 21.0.5 (QIAGEN), conserved mature miRNAs sequences were identified by comparison to sequences in the miRBase (v22) database (Kozomara, Griffiths-Jones 2014).

Results

Analysis of specific precursor miRNA expression patterns was done using real-time PCR (Krivmane et al. 2022). Of seven miR156 precursors tested, three showed the expected transcript levels. Transcription of miR156_789, miR156_511 and miR156_374 was highest in juvenile controls, and lowest in mature controls. Correspondingly, expression of these precursor miRNAs was higher in rejuvenated *in vitro* shoots compared to mature *in vitro* shoots (Fig. 1). Expression analysis of three miR172 precursor transcripts indicated that two (miR172_1931 and miR172_42 had the expected pattern – high expression in the mature control, and low expression in the juvenile control. Compared to the mature control, expression in the *in vitro* shoot samples was low, but was higher in the mature *in vitro* shoots compared to rejuvenated *in vitro* shoots (Fig. 2).

Preliminary analysis of the small RNA sequencing data was done to identify short RNA transcripts corresponding to mature miR156 and miR172 sequences. A total of 117 unique miR156 sequences (isomiRs) were identified from the entire sequence dataset. Of these, only four were statistically significantly differentially expressed with an absolute fold change of ≥ 1.5 between the mature and juvenile control samples, and p-values < 0.05. For the *in vitro* samples, two different clones were used for both the *in vitro* mature and *in vitro* juvenile sample types (REV1 and IVM1 were derived from clone VKA and REV2 and was derived from clone IVM2 54-257). Expression of these four miR156 isomiRs differed between clones of the same *in vitro* sample type, in particular between REV1 and REV2, the two rejuvenated *in vitro* samples. Expression of the four miR156 isomiRs was high in REV2, as expected, but was low in REV1. Expression of bpe-miR156a-3p and bpe-miR156a-5p was lower in the IVM2 (*in vitro* mature) sample, as expected, but higher in the IMV1 sample (Fig. 3).

The normalised expression levels were significantly different ($p < 0.05$) for all four miRNAs between the mature and juvenile control samples. In addition, expression of bpe-miR156a-3p was significantly different between the sample pair MAT vs IVM-1. Expression of bpe-miR156a-5p was significantly different between the sample pairs MAT vs IVM-1 and MAT vs REV-2. Expression of bpe-miR156x was significantly different between the sample pairs MAT vs IVM-1, MAT vs IVM-2, MAT vs REV-2 and REV1 vs JUV. Expression of bpe-miR156ab was significantly different between the sample pairs MAT vs IVM-1, MAT vs IVM-2, MAT vs REV-2, REV-1 vs IVM2 and REV1 vs JUV.

The normalised expression values varied between the two clones in the *in vitro* mature (IVM) samples and in particular between the two *in vitro* rejuvenated (REV) clones. Expression values of the four miRNAs was lower in the REV-1 sample compared to the REV-2 sample, but this difference was only significant ($p < 0.05$) for bpe-miR156ab.

Only nine unique miR172 sequences (isomiRs) were found in the entire HTS data set, which were grouped into seven isomiRs groups based on mature miRNA

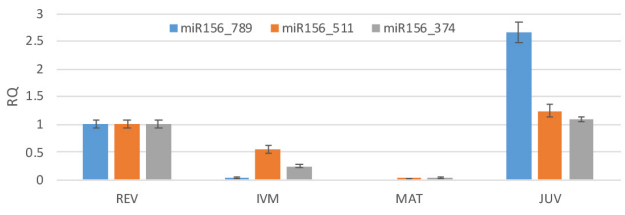

Fig. 1. Relative expression (RQ) of the miR156 family miRNA precursors in samples from leaves of rejuvenated *in vitro* shoots (REV), typical mature *in vitro* shoots (IVM), mature controls (MAT), juvenile controls (JUV). The REV sample was set as the reference sample (RQ = 1). Bars indicate minimum and maximum RQ values. Expression of each miR156 precursor was significantly different ($p < 0.05$) between all sample types.

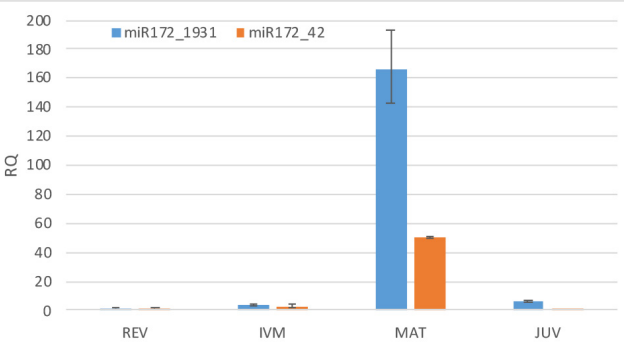

Fig. 2. Relative expression (RQ) of the miR172 family miRNA precursors in samples from leaves of rejuvenated *in vitro* shoots (REV), typical mature *in vitro* shoots (IVM), mature controls (MAT), juvenile controls (JUV). The REV sample was set as the reference sample (RQ = 1). Bars indicate minimum and maximum RQ values. Expression of miR172_1931 was significantly different ($p < 0.05$) between all sample types. Expression of miR172_42 was significantly different ($p < 0.05$) between all sample types except REV and JUV.

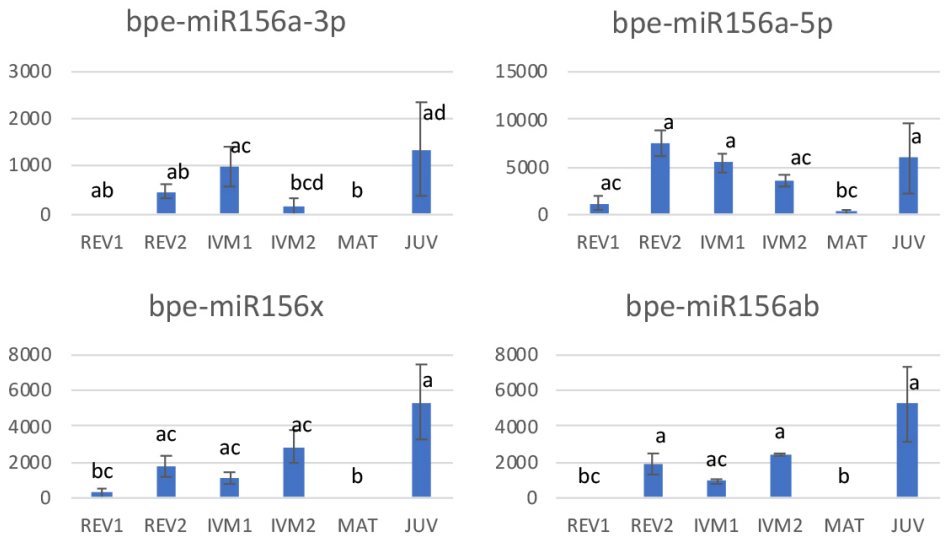

Fig. 3. Mean expression values (read counts) of miR156 family members in samples from leaves of rejuvenated *in vitro* shoots (REV1, REV2), typical mature *in vitro* shoots (IVM1, IVM2), mature controls (MAT), juvenile controls (JUV). Bars indicate standard errors. Samples labelled with the same letter are not statistically different ($p > 0.05$).

sequence homology. All miR172 family members had very low expression in all analysed libraries, with only seven sequence reads in the MAT sample, one read in the JUV sample, two reads in IVM1 and IVM2 samples, and one read in the REV1 and REV2 samples.

Discussion

Both methods, real-time PCR and high-throughput sequencing of small RNAs identified members of the miR156 and miR172 microRNA families that were differentially expressed. The real-time PCR approach determined the expression of precursor miRNAs, while the high-throughput sequencing provided information about the expression levels of mature miRNAs. Precursor miRNAs with differing sequences can produce identical mature miRNAs, and as these precursor miRNAs are found in distinct genomic loci, they may have differing expression regulatory mechanisms, and therefore precursor miRNAs could be expressed at different developmental stages or tissues, which produce identical or highly similar mature miRNAs (Bielewicz et al. 2012). Conversely, a single precursor miRNA can produce a range of miRNA variants (isomiRs), either by imprecise cleavage (templated isomiRs), or by post-transcriptional addition or removal of specific nucleotides to miRNA ends (non-templated isomiRs) (Zhai et al. 2013). Therefore, real-time PCR and high-throughput sequencing approaches are complementary.

Both real-time PCR and high-throughput sequencing identified differentially expressed miR156 family members. This is in accordance with the central role that the miR156 family plays in the transition from the juvenile phase to maturity in a number of plant species (Wu et al. 2009; Wang et al. 2011; Xing et al. 2014; Jia et al. 2017; Ahsan et al. 2019a; Ahsan et al. 2019b). Interestingly, real-time PCR analysis identified two miR172 family members that were highly expressed in mature control samples (Krivmane et al. 2022). However, high-throughput sequencing did not identify any differentially expressed mature miR172 miRNAs between the mature and juvenile controls, and expression of miR172 miRNAs was very low in all samples analysed. MiR172 miRNAs target AP2-like genes, but the majority of studies indicating the involvement of miR172 and AP2-like genes in transition to maturity have been done in annual model plant species. In miRNA and gene expression studies of avocado, macadamia and mango, expression of AP2-like genes did not correspond to miR172 abundance or tree maturity (Ahsan et al. 2019b). Therefore, further studies in long-lived perennial species are needed to fully elucidate the miRNAs involved in developmental phase transition processes.

The relatively low expression in one *in vitro* rejuvenated clone (REV-1) of the four miRNAs that were highly expressed in the juvenile control, may indicate that this sample was not fully rejuvenated, or that it reverted to a mature state during maintenance under *in vitro* conditions. Alternatively, this could reflect differing expression patterns due to the differing genotypes and their differing genetic background. Further analysis of the small RNA high-throughput sequence data may be able to identify additional differentially expressed mature miRNAs, and provide a more complete overview of differences in expression of mature microRNAs in *in vitro* samples with similar propagation properties, but with differing genetic backgrounds. In addition, transcriptome data obtained from the same samples, can provide an opportunity to

analyse expression changes in protein-coding genes, and to correlate the relative expression levels of mature miRNAs and their potential target genes.

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