Differentially expressed conserved microRNAs in mature and rejuvenated silver birch *in vitro* propagated tissues.

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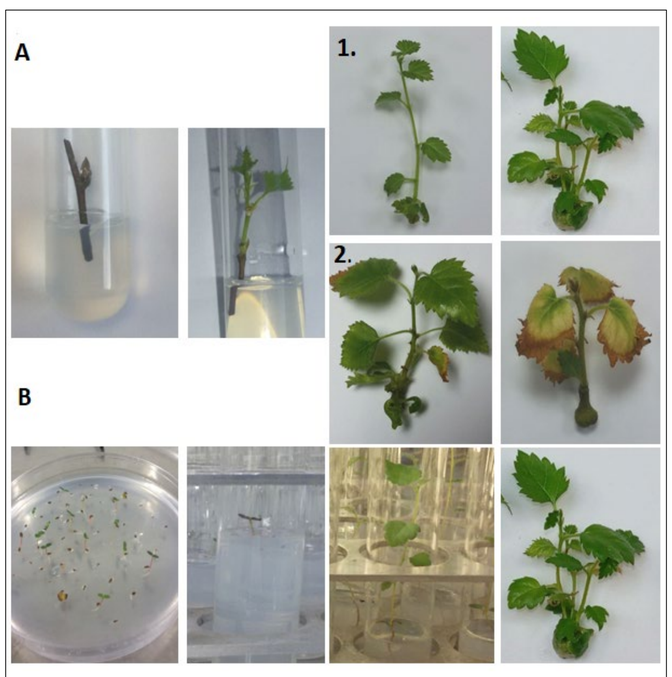
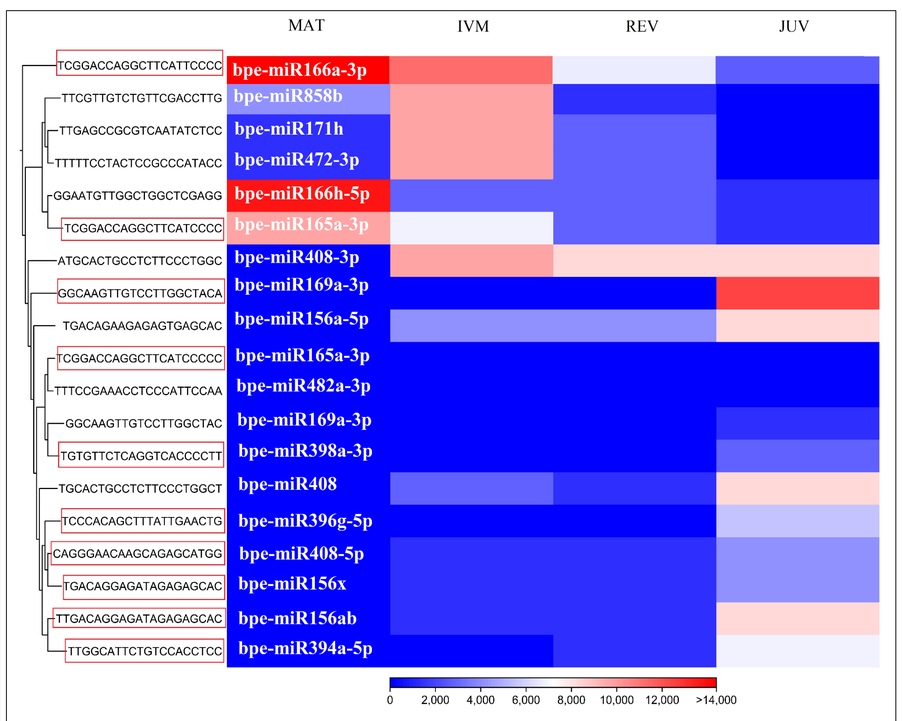
The aim of this study was to analyse expression of juvenility-associated miRNAs during silver birch *in vitro* propagation. The rejuvenated and mature *in vitro* samples were selected according to their propagation ability and morphological properties. Total RNA was extracted from leaves of four rejuvenated (REV samples) and four mature (IVM samples) *in vitro* shoots – two different clones were analyzed (clones VKA and 54-257) in two biological replicates. IVM cultures from the clone VKA exhibited typical signs of maturity (thick stems, large and thick leaves, inability to proliferate), and IVM cultures from clone 54-257 also had partially yellow leaves. All *in vitro* samples had been maintained in *in vitro* culture for approximately 10 months when collected for analysis. Leaves from a mature (approximately 20 years old) silver birch (3 samples, clone VKA) were used as a mature control (MAT), and 3 week old seedlings (4 samples, seeds collected from clone VKA) were used as a juvenile control (JUV). Samples for MAT control were collected in three different months - on 21st May, 10th July and 3rd September 2018, but seeds for the JUV control seedlings were collected in November 2019 and grown in turf substrate for 3 weeks. The obtained results were verified on eight different REV and IVM genotypes (clones) by RT-PCR.

RESULTS

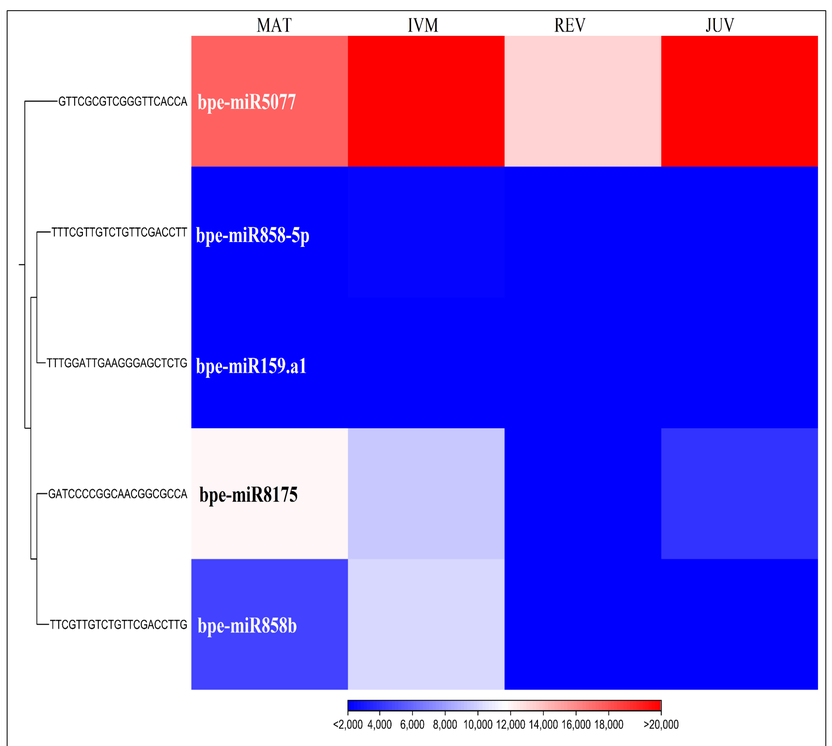
A total of approximately 37.9 million reads were obtained from the 15 libraries. After trimming of sequences by length, 14.87 million reads remained, which were clustered into 2776807 unique sequences with a minimum read count of five over all libraries. Comparison of these unique sequences with the miRBase v22 database identified 2600 conserved miRNA isomiR sequences, which were assigned to 291 miRNA groups, belonging to 50 different miRNA families. The largest number of different miRNA families were found in IVM (46 families) and in REV samples (45 families), and the smallest number were found in MAT samples (33 families). Expression of some miR156 isoforms was high in juvenile tissues and has been previously reported to regulate phase transitions in a range of species. The miR156 family was found in all sample types, with the highest read count (696) in JUV samples and lowest (28) in MAT samples. Additional miRNAs, such as miR394 and miR396, that were previously reported to be highly expressed in juvenile woody plant tissues were also identified in this study. The miR172 family was found in all sample types, but with very low expression levels - in JUV samples the expression value was 1, 7 in MAT samples, 1 in REV samples and 2 in IVM samples. Only 9 isomiRs were found for the miR172 family. Comparing the MAT and JUV samples, 19 isomiR groups (8 down regulated and 11 up regulated in JUV samples) were differentially expressed (FC ≥1.5 and an FDR corrected p-value of ≤0.05). A total of 41 differentially expressed miRNA isomiR groups were identified between the sample types. Five isomiR groups were differentially expressed between IVM and REV samples, all of which were more highly expressed in IVM samples. Of these, four were more highly expressed in MAT and IVM samples, but had lower expression in JUV and REV samples. Based on the obtained results and the identified miRNA precursor sequences, 19 pre-miRNA markers for RT-PCR were created for 9 miRNA families and verified on eight different REV and IVM genotypes. Two markers (miR156_789 and miR172_19231) were developed in a previous study also were verified. In the case of miR172_789, 2x higher RT-PCR expression values were observed in MAT-1 samples (collected in June) compared to MAT-2 samples (collected in September), which could be due to leaf senescence and a subsequent decrease in miR172 expression.



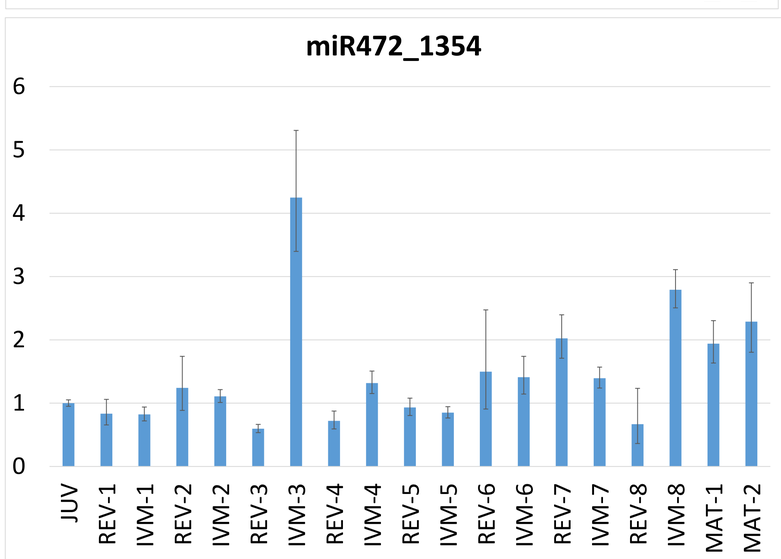
In vitro initiation of silver birch shoots and rejuvenation. A. Rejuvenated shoots that proliferate (1.) and mature shoots that eventually die (2.). B. Juvenile shoots (from seeds) that proliferate.

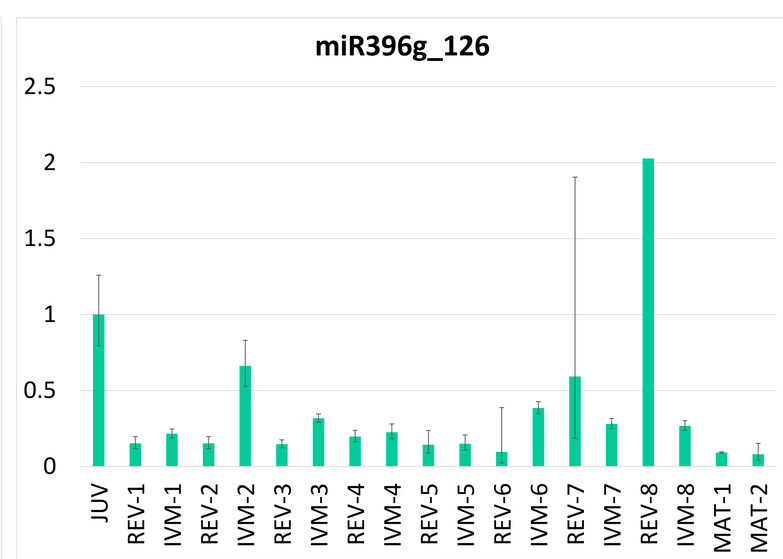


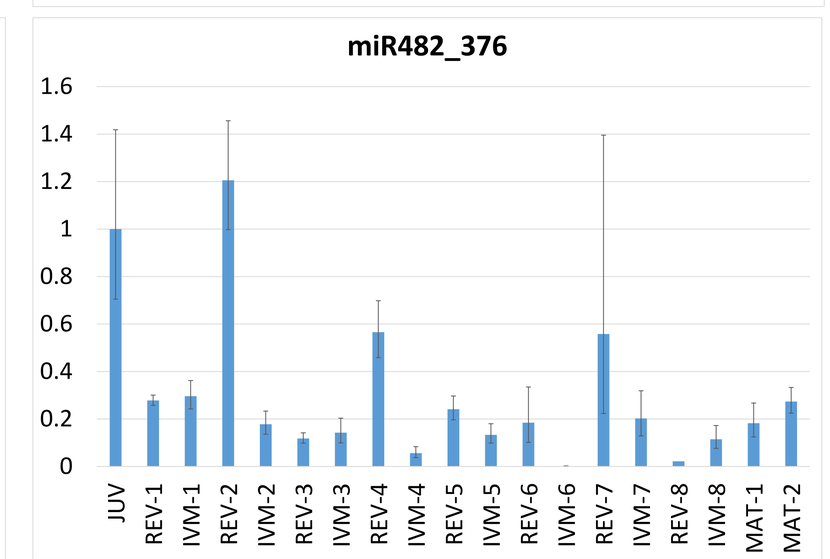
Differentially expressed miRNA between MAT-JUV. Heat map of differentially expressed miRNAs; the colour bars represents normalised expression values. Sequences in the red boxes indicate isomiRs that have a similar expression pattern in IVM-REV compared to MAT-JUV.



Differentially expressed miRNA between IVM-REV. Heat map of differentially expressed miRNAs; the colour bars represents normalised expression values.







Relative expression (RQ) of the miRNA precursors using RT-PCR. REV - rejuvenated *in vitro* shoots, IVM - typical mature *in vitro* shoots from 8 different genotypes (clones), JUV - juvenile control, MAT-1 - mature control, leaves collected at end of June, MAT-2 - mature control, collected in September.

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