Article

Differentially expressed conserved plant vegetative phase-change related micro RNAs as markers of juvenility for silver birch *in vitro* propagation

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Abstract: In plants, phase change from the juvenile stage to maturity is a tightly controlled process at the physiological and genetic level, which is controlled by evolutionary highly conserved microRNAs. These phase changes are more pronounced in woody plant species, but the majority of molecular genetic studies on the regulation of this transition has been done in annual model or crop species. This process is of particular significance for the *in vitro* propagation of woody plant species, as individuals or tissues that have undergone the transition to vegetative maturity are recalcitrant to propagation. Development of effective methodologies for silver birch vegetative propagation are required to increase the efficiency of breeding programs. Conserved miRNAs that were differentially expressed between juvenile and mature silver birch tissues were identified using high-throughput sequencing of small RNA libraries. These differentially expressed miRNAs could potentially be utilized to develop markers indicating the juvenility or maturity of silver birch explants and *in vitro* cultures. In addition, the obtained results will provide an insight into the molecular mechanisms regulating vegetative phase change in silver birch and other perennial woody plant species.

Keywords: miRNA156, micropropagation, tree breeding, recalcitrant, woody plants, perennial plants.

1. Introduction

In plants, phase change from the juvenile stage to maturity is a tightly regulated process at the physiological and genetic level [1]. This transition occurs in all plants, but while the particular phases are more pronounced in woody plant species, the majority of molecular genetic studies on the regulation of this transition has been done in annual model or crop species [2]. This process is of particular significance for the *in vitro* propagation of woody plant species, as individuals or tissues that have undergone the transition to vegetative maturity are recalcitrant to propagation. Mature plant tissues can be rejuvenated by a number of methods, including *in vitro* culturing [3]. Variations in growth media composition or other *in vitro* conditions can be used to successfully rejuvenate mature tissues. Some birch genotypes can be rejuvenated and regenerated *in vitro*, while others persist in a mature state and progressively senesce and cannot be successfully propagated [4,5].

Silver birch (*Betula pendula* Roth.) is one of the most ecologically and economically significant tree species in northern European boreal forests. Forest tree breeding programs, producing highly productive and qualitative improved silver birch reproductive material have been developed in many northern European countries [6,7]. Vegetative reproduction of selected germplasm for use in controlled crosses can shorten the breeding cycle by 10 – 15 years [4]. Vegetatively propagated material can be used for further breeding and establishment of seed orchards, from which genetically improved nursery stock is obtained. Clonal propagation of high quality, selected silver birch germplasm can also

 doi:10.20944/preprints202212.0584.v1

increase the productivity of birch stands, compared to conventional plantations [8]. Often, plus-trees and other valuable individuals are only identified after they have reach maturity, and to obtain the most accurate assessment of tree productivity and quality, phenotypic evaluation of silver birch is often undertaken when trees have reached their mature phase.

Therefore, development of effective methodologies for silver birch vegetative propagation are required to increase the efficiency of breeding programs. The use of improved reproductive material for forest stand establishment and renewal can increase the value of the obtained timber and contribute to the sustainable development of the forest industry. Additionally, fast-growing forest stands provide an active carbon dioxide capture sink, which is important for meeting climate change targets. Accurate assessment of vegetative phase status can assist in the development of *in vitro* protocols and in selecting the optimum explants and growth conditions. Morphological, anatomical and physiological traits have been used as vegetative phase-change markers in a number of species, such as differences in leaf or stem morphology, cellular and subcellular anatomy, and variations in plant hormones and concentrations. However, often variations in traits associated with the vegetative phase change are subtle and difficult to unambiguously classify [9–11]. Genetic markers, based on the functional and regulatory genes involved in vegetative phase change can provide a more accurate assessment of the physiological status of plant tissues and organs.

Studies with model and annual plant species indicate that the main endogenous regulators of juvenility are the evolutionary highly conserved microRNAs (miRNAs) miR156, miR172 and their target genes [12,13]. Similar regulatory pathways and genes have also been associated with vegetative phase change in a number of woody plant species [9,14–17]. MiRNAs are small, non-protein coding RNAs usually between 20–24 nucleotides in length, and they regulate a range of cellular processes, principally by post-transcriptional control of gene expression, either by cleavage or translational repression via complementary binding to messenger RNA (mRNA) [18]. Primary microRNA transcripts (pri-RNAs) are one to several hundred nucleotides in length, and contain at least one hairpin-stem loop. Enzymes such as Dicer-like enzyme 1, Hasty and others are cleave these primary transcripts into approximately 70 nt long precursor miRNAs (pre-miRNAs), which are subsequently cleaved into the mature ~20-24 nt long miRNA molecules [19]. Based on the sequence homology of mature miRNA molecules, miRNAs are categorized into families, which have the same or similar target genes. However, the primary and precursor sequences that encode identical or highly similar mature miRNAs can have differing sequences surrounding the mature miRNA site, and the precursors may have differing expression patterns at different developmental stages or tissues, or in response to different abiotic or biotic conditions [20]. These miRNA isoforms (isomiRs) can vary in sequence, length, or both. Due to their short length, one miRNA could interact with a number of mRNAs [21].

MiR156 targets *SQUAMOSA* promoter binding protein-like (SBP/SPL) transcription factor genes, repressing the expression of SPL genes, which promote the transition to maturity by up-regulating key MADS-box genes, such as *APETALA (AP1), LEAFY (LFY) and FRUITFULL (FUL)*, which induce flowering [22]. Additionally, via the genes *SPL9* and *SPL10*, miR156 suppresses expression of miR172 which leads to elevated levels of miR172 target genes belonging to the *AP2*-like transcription factor family genes: *APETALA2* (*AP2*), *TARGET OF EAT1* (*TOE1*), *TOE2* and *TOE3*, *SCHLAFMUTZE* (*SMZ*), and *SCHNARCHZAPFEN* (*SNZ*) [3].

In this study, conserved miRNAs that were differentially expressed between juvenile and mature silver birch tissues were identified using high-throughput sequencing of small RNA libraries. These differentially expressed miRNAs could potentially be utilized to develop markers indicating the juvenility or maturity of silver birch explants and *in vitro* cultures. In addition, the obtained results will provide an insight into the molecular

 doi:10.20944/preprints202212.0584.v1

mechanisms regulating vegetative phase change in silver birch and other perennial woody plant species.

2. Results

2.1. Identification of Conserved miRNAs in Silver Birch

Sequencing of the 15 small RNA libraries yielded a total of approximately 37.9 million reads. After trimming of sequences by length (minimum length 18 nt and maximum length 25 nt), 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. These 291 one miRNA groups belonged to 51 different miRNA families. Of the 2600 conserved miRNA sequences, 1932 were ambiguously annotated, i.e., a small RNA sequence was similar to the mature regions of two different miRBase sequences (from the same miRNA family). Of the conserved miRNA sequences identified in our data set, 43.27% were identified from *Glycine max*, 16.55% from *Populus euphratica* and 7.92% from *Oryza sativa* (Table 1). Only 0.01% were identified from *Acacia magnum* and 0.23% from *Pinus densata*.

Table 1. Conserved small RNAs identified from miRBase.

Annotation	Read count	Percentage
Total matches with mature miRNA sequences in miRBase	8664	0.31
Acacia auriculiformis	110	1.27
Acacia mangium	1	0.01
Glycine max	3749	43.27
Oryza sativa	686	7.92
Zea mays	161	1.86
Vitis vinifera	393	4.54
Prunus persica	381	4.40
Populus euphratica	1434	16.55
Populus trichocarpa	422	4.87

 doi:10.20944/preprints202212.0584.v1

Pinus densata	20	0.23
Pinus taeda	69	0.80
Picea abies	298	3.44
Arabidopsis lyrata	431	4.97
Arabidopsis thaliana	368	4.25
Nicotiana tabacum	114	1.32
Brassica rapa	27	0.31
Unannotated	2768143	99.69
Total	2776807	100.00

The largest number of different miRNA families were found in IVM samples (46 families), then in REV samples (45 families), and the smallest number were found in MAT samples (33 families). The most isomiRs were found in the miR166 family with 470 isomiRs, as well as the miR159 family with 376 isomiRS, and the miR156 family with 314 isomiRs. Of the 50 conserved miRNA families identified, only one family (miR395), was not found in IVM samples and three families (miR2950, miR9726 and miR1507) were not found in REV samples (Supplementary file 1). The most highly expressed isomiRs in all sample types were from the miR166 (TCGGACCAGGCTTCATTCCCC and TCTCGGACCAGGCTTCATTCC) and miR156 (TTGACAGAAGATAGAGAGCAC) families.

The miR156 family was found in all sample types, with the highest read count (696) in JUV samples and lowest (28) in MAT samples. 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. The highest number of isomiRs were found in IVM samples (1291) followed by JUV samples (1183), and the smallest number were found in MAT samples (645).

Interestingly, four miRNA families (miR947, miR950, miR951 and miR3711), which previously were reported as conifer specific miRNAs [23], were also found in this study, but with low expression values (read counts) – 9, 15, 31 and 45. Of these, miR947 was not found in JUV samples. miR950 was found in all sample types, but miR951 were found only in REV and IVM samples. miR3711 was not found in MAT samples.

2.2. Differentially expressed miRNAs

The identified conserved miRNAs were analysed to identify significantly differentially expressed miRNAs between the four sample types (REV, IVM, JUV, MAT). The threshold for differential expression was an absolute fold-change FC ≥1.5 and an FDR corrected p-value of ≤0.05.

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Comparing the MAT and JUV samples, 19 isomiR groups (8 down regulated and 11 up regulated in JUV samples) were differentially expressed miRNAs (DE miRNAs): three isomiR groups from each of the families miR156 and miR408, two isomiR groups from each of the miR165, miR166, miR169 families, and one isomiR group from each of the miR171, miR394, miR396, miR398, miR472, miR482, miR858 families (Figure 1). IsomiR groups from the miR166 and miR165 families were highly expressed in MAT samples, intermediately expressed in IVM and REV, (with lower expression in REV samples) and had low expression in JUV samples. Opposite expression patterns were observed for miR169 isomiR groups, which were highly expressed in JUV samples, intermediately expressed in REV and IVM samples (with lower expression in IVM samples) and had low expression in MAT samples (Figure 1; Supplementary file). Two miR156 isomiR groups had higher expression in JUV samples and lower in MAT samples, with similar expression patterns between the REV and IVM samples i.e. higher expression in REV samples than in IVM samples. The third differentially expressed miR156 isomiR group was also highly expressed in JUV samples and lowly expressed in MAT samples, but higher expression was found in IVM samples compared to REV samples.

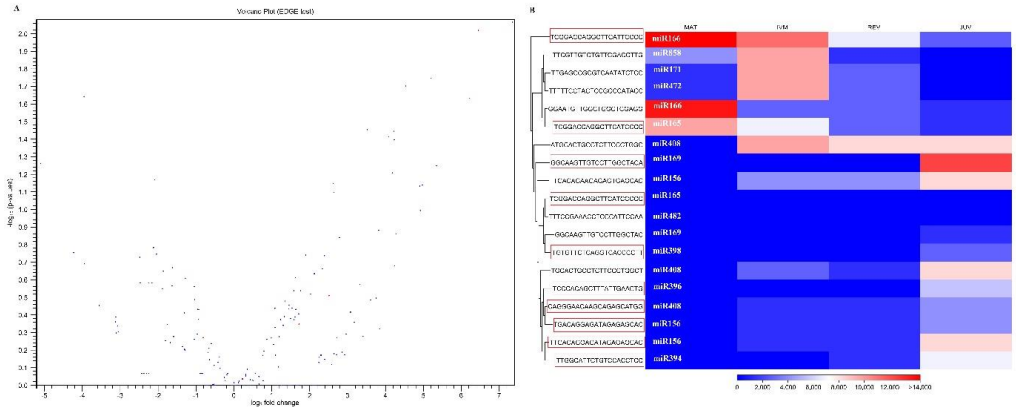

Figure 1. DE miRNA expression (MAT-JUV) profile in all sample types. (A) Volcano plot of 19 DE miRNAs. The X-axis is the fold change (log2 scale) of up and down regulated miRNAs. The Y-axis represents the p-value (log10) for the statistical test of differences between the samples. The dots represent miRNA isomiR groups with an absolute fold change > 1.5-fold change. Blue dots represent the miRNAs that were differentially expressed with a P value ≤ 0.05. (B). Heat map of DE miRNAs; the colour bars represents normalised expression values. Sequences in the red boxes indicate isomiRs that have a similar expression pattern in IVM-REV campared to MAT-JUV.

Five isomiR groups were differentially expressed between IVM and REV samples, all of which were more highly expressed in IVM samples (Figure 2). Of these, four were more highly expressed in MAT and IVM samples, but had lower expression in JUV and IVM samples (two miR858 isomiR groups, one miR159 isomiR group and one miR8175 isomiR group),. Both of the miR858 isomiR groups were also statistically significantly differentially expressed between MAT and JUV samples, as well as IVM and JUV samples, and the miR8175 isomiR group was differentially expressed between REV and MAT samples.

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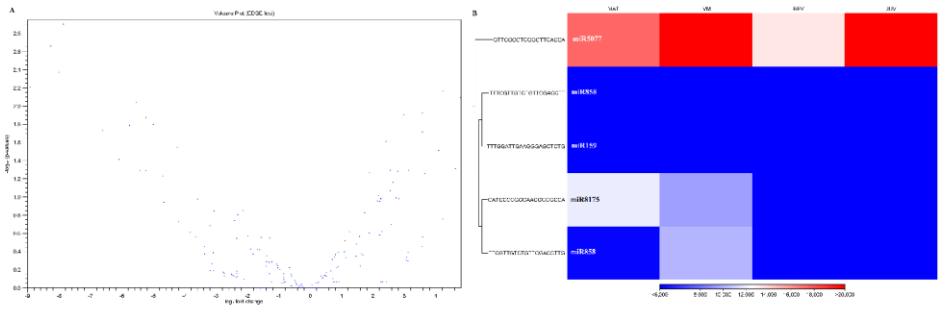

Figure 2. DE miRNA expression (IVM-REV) profile in all sample types. (A) Volcano plot of 5 DE miRNAs for IVM and REV samples. The X-axis is the fold change (log2 scale) of up and down regulated miRNAs. The Y-axis represents the p-value (log10) for the statistical test of differences between the samples. The dots represent miRNA isomiR groups with an absolute fold change > 1.5-fold change. Blue dots represent the miRNAs that were differentially expressed with a P value ≤ 0.05. (B). Heat map of DE miRNAs; the colour bars represents normalised expression values.

The comparisons most likely to identify miRNAs associated with vegetative phase change in silver birch were the MAT-JUV and IVM-REV comparisons described above. However, differentially expressed miRNS were identified in all pairwise comparisons between sample types. A total of 41 differentially expressed miRNA isomiR groups were differentially expresssed between the sample types (Supplementary file 2).

Fourteen isomiR groups were differentially expressed between REV and JUV samples. Two isomiR groups each from the miR169 and miR403 families, and one isomiR group from each of the miR396, miR5368, miR5077 families had higher expression in the JUV samples and seven – miR157, miR166, two of miR171 isomiR group, miR472, miR482, miR2118 were more highly expressed in REV samples

Seventeen miRNA isomiR groups were differentially expressed between IVM and JUV samples, 12 more highly expressed in IVM samples (two miR171 groups, two miR858 isomiR groups, and one isomiR group from each of the miR157, miR166, miR167, miR319, miR472, miR482, miR2118, miR3627 families), and 5 (two miR169 isomiR groups, one isomiR group from each of the miR394, miR395, miR396 families) were more highly expressed in JUV samples. Of these, two miRNA isomiR groups – miR169 and miR394 were also highly expressed in IVM samples, but the miR482 isomiR group, was highly expressed in JUV samples (Figure 3). Only three isomiR groups (miR169, miR394 and miR396) had the expected expression pattern - higher expression in JUV, lower in MAT and IVM samples.

Eleven miRNA isomiR groups were differentially expressed between REV and MAT samples, 10 more highly expressed in REV samples, and 1 more highly expressed in MAT samples. From these families fourmiR156 isomiR groups, two 408 isomiR groups,one miR157 isomiR group, one miR403 isomiR group one miR8175 isomiR group, one miR2118 isomiR group, were down regulated, and one miR169 isomiR group was up regulated in MAT samples.

Ten miRNA isomiR groups were differentially expressed between the IVM and MAT samples, nine more highly expressed in IVM samples (three miR156 and miR408 isomiR groups, one isomiR group from each of miR157, miR171, miR3711), and one more highly expressed in MAT samples (one miR169 isomiR group).

2.3. Identification of Potential miRNA Precursors for DE miRNAs

Mapping of the mature miRNA sequences to the *Betula pendula* scaffold assembly, vv1.2 scaffolds (University of Helsinki id 35079) identified 55 potential precursor sequences

 doi:10.20944/preprints202212.0584.v1

for 35 isomiR groups (20 miRNA families - 623 isomiRs) from a total number of 41 differentially expressed isomiR groups (708 isomiRs). (Supplementary File). The most precursors (20) were found for the miR156 family, and only one potential precursor sequence was found for the miR171, miR319, miR408, miR2118, miR3627, miR5368 families. No potential precursors were found for the differentially expressed miR159, miR858, miR3711, miR5077, miR8175 family isomiR groups. Homologous sequences in the *Betula pendula* scaffold assembly were found for some (miR156, miR166, miR167, miR169, miR171, miR319, miR403, miR5368) isomiR groups but these had an MFEI of less than 0.85 and therefore were not considered as precursors. A number of sequences were identified as potential precursors for multiple miRNA isoform groups from the same family. This is due to the parameters utilized, which allows a maximum of two mismatches between a miRNA and a potential precursor miRNA sequence.

Different precursors on the same silver birch contig were found for miR403, miR408 (Contig129) and for miR156, miR165 (Contig2096). For both miR3627 and miR395, three and four repeated precursor sequences were found on one contig, respectively. This suggests the clustering of the miRNA precursor sequences in the silver birch genome.

Potential precursor sequences for conserved miRNAs were analysed for the ability to form the required stem-loop structure, and the minimal folding free energy indexes were calculated. Predicted pre-miRNA sequences were trimmed in the primary miRNA sequence region until the next bulge or loop after the miRNA* region. Minimum folding free energy indexes ranged from 0.48–1.32, with most being > 0.85 (45 precursor sequences (82%)) (Supplementary file 3).

2.4. miRNA target gene identification

Analysis of conserved miRNA putative target genes identified 757 genes targeted by 3740 isomiRs from 19 miRNA groups that were differentially expressed between the MAT and JUV samples (Supplementary file 4). The predicted target genes included stress-related gene families such as Leucine Rich Repeat (LRR) protein genes (miR156, miR398), protein kinase domain (miR408), TIR-NBS or TIR-NBS-LRR type disease resistance proteins (miR472 and miR482), heat shock proteins (miR156 and miR482), and Blue copper-like proteins (miR408). Target transcription factors included genes such as SBP (miR156), MYB (miR858, miR166), HD ZIP III (miR165 and miR166), zinc finger protein genes, Superoxide dismutase [Cu-Zn] (miR156 and miR398) and others. Target genes also included transferases like Glutathione S-transferase (miR169), Acetyltransferase, GNAT family protein (miR408), DNA methyltransferase (miR166).

The most target genes (22) of the identified putative target genes (targeted by one miRNA group—miR166-3p) were associated with malate dehydrogenase or NAD-dependent malate dehydrogenase. Twelve of the identified putative target genes (targeted by three miRNA groups—miR482-3p, miR472-3p, miR166-3p) were associated with disease resistance proteins - NBS-LRR, TIR-NBS-LRR and TIR-NBS type disease resistance proteins. Plant NBS-LRR proteins are involved in the detection of pathogen-associated proteins, most often the effector molecules of pathogens, which are responsible for virulence. Eight targets for three miRNA groups of miR156 with 29 isomiRs were associated with squamosa promoter binding like-proteins. One gene targeted by bpe-miR166a-3p, was maturase, which are prokaryotic enzymes that aid self-excision of introns in precursor RNAs and have evolutionary ties to the nuclear spliceosome. Five miR169 isomiRs targeted extracellular ligand-binding receptor precursors. The miRNA group bpe-miR858btargeted four different MYBPA1 protein genes.

Analysis of conserved miRNA putative target genes of the five isomiR groups differentially expressed between IVM and REV samples, identified 195 genes (Supplementary file 4). The predicted target genes included MYB and MYBPA1 transcription factors, which were targeted by miR858b and miR858-5p isomiR groups.

 doi:10.20944/preprints202212.0584.v1

Bpe- miR159a targeted a number of genes, including AUX/IAA protein gene; DNA-directed RNA polymerase genes (two target genes), NBS-LRR type or TIR-NBS disease resistance protein genes (two target genes).

3. Discussion

Higher expression of members of the miR156 family was observed in juvenile samples, confirming the role of this miRNA family in juvenility [13]. Interestingly, the expression of the miR172 family was extremely low in all sample types, and none of the identified miR172 transcripts had significantly higher expression in mature samples. Previous studies of the expression of the miR172 family in other woody tree species and correlation with plant maturity have reported differing results. Expression of miR172 was not significantly different between juvenile and mature tissues in macadamia and mango, but was higher in mature avocado tissues [16]. Higher expression of miR172 in mature tissues has been reported in other woody plant species as well [9,24]. However, most studies of miR172 expression in phase transition have been done in annual species, where vegetative phase change is often difficult to separate from the transition to flowering [2], and many studies in woody tree species were investigating transition to flowering. In studies of perennial species investigating traits other than flowering, the expression of miR172 was not as well correlated with other developmental traits, e.g. rooting in *Eucalyptus* [25], and seed germination in *Nelumbo nucifera* Gaertn. [26]. In this study, the explants taken for establishment of *in vitro* cultures were closed buds, and were not obviously transitioning to flowering. This could explain why the expression of miR172 was low and not significantly different between juvenile and mature sample types.

Other significantly differentially expressed conserved miRNAs identified in this study included miR394 and miR396, both of which were also differentially regulated between juvenile and mature tissue types in avocado, mango and macadamia [16]. MiR394 regulates processes in the shoot apical meristem (SAM) [27] and regulates flowering time in *Arabidopsis thaliana* [28]. The miR396 family repress expression of the transcription factor class GROWTH REGULATING FACTOR (GRF), and GRF-INTERACTING FACTOR (GIF) transcriptional co-regulators, and this complex is crucial in many aspects of plant growth and development in angiosperms [29]. A number of the differentially expressed miRNAs are ancient and indicate their central role in plant development. MiR398 is extremely ancient, and is found in both gymnosperms and angiosperms. This miRNA has been shown to be involved in growth and development processes in a number of plant species, as well as regulating abiotic and biotic stress responses [30]. Other ancient and highly conserved microRNAs include miR165/miR166, which regulate HD-ZIP III (homeodomain-leucine zipper) transcriptional factors and are involved in a range of plant development processes [31,32]. MiR171 is highly conserved in land plants, found in many lineages, from bryophytes to angiosperms, and regulates shoot meristem activity and phase transition [33]. MiR408 plays significant roles in regulating plant growth, development and stress response in a range of plant species [34]. Many of these highly conserved miRNA families have been shown to play a role in very diverse processes, as they interact with transcription factor gene families. The miR858 family targets the large MYB families that regulate numerous biological processes from plant development to stress responses and secondary metabolism pathways [35].

Other differentially expressed miRNAs are less well studied, and so currently have been shown to be involved in specific processes, however, further studies may show that they are also involved in other plant developmental aspects. MiR482 targets *NBS-LRR* genes as well as other plant resistance mechanisms [36], and miR8175 is upregulated in aluminium tolerance responses in plants [37]. MiR5077 is reported to be involved in sugar metabolism in pear fruit development [38], which could play a role in vegetative phase transition, as sugars have been shown to be a cue for this transition [39].

Further analysis of the sequencing data will potentially enable identification of novel miRNAs that are differentially expressed between juvenile and mature silver birch tissues.

 doi:10.20944/preprints202212.0584.v1

Identification of precursor sequences and *in silico* analysis of the folding structures can indicate if the expected stem-loop hairpin structures can be formed. Further research in this direction is needed to confirm the expression of the precursor miRNAs using real-time PCR. This will also provide information on the expression of precursor sequences that encode the same or highly similar mature miRNAs. Transcriptome analysis will provide an opportunity to compare expression of miRNAs with their potential target gene expression levels. The expression of the miRNAs identified in this study need to be examined in a wider range of germplasm, to determine the effect of genetic background on miRNA expression, and to examine variation of miRNA expression of different clones with similar *in vitro* propagation properties.

4. Materials and Methods

4.1 Plant material and extraction of RNA

Plant material for miRNA expression analysis was collected and stored at–80 °C until RNA extraction. Total RNA was extracted from leaves of four rejuvenated (REV samples) *in vitro* shoots – two different clones in two biological replicates (clones VKA and 54-257) and of four mature *in vitro* shoots (IVM samples) - two different clones in two biological replicates (clones VKA and 54-257) were analyzed. IVM cultures from the clone VKA exhibited typical signs of maturity (thick stems, large and thick leaves, inability to proliferate). IVM cultures from clone 54-257 exhibited the previously mentioned typical signs of maturity, as well as having 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 collected in three different months - on 21st May, 10th July and 3rd September 2018, but seeds for JUV control seedlings were collected in November 2019 and grown in turf substrate for 3 weeks. RNA was extracted using a standard phenol/chloroform/isoamyl alcohol protocol [40]. Total RNA, extracted from all samples, was treated with the Turbo DNA-free kit (ThermoFisher Scientifc, Cat. No. AM1907) following the manufacturer's instructions.

4.2 RNA quality control

RNA concentration measured with a Qubit and QuantiT™ RNA BR Assay Kit (ThermoFisher, Cat. No. Q10210). RNA purity (DNA contamination) 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 [41]. Each forward primer 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 1×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 reactions 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 detected, RNA samples considered 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 reanalyzed prior to reverse transcription and real time PCR analysis. RNA purity was measured with a Qubit and QuantiT™ dsDNA HS Assay Kit (ThermoFisher, Cat. No. Q33120). Total RNA and small RNA quality, quantity and integrity number (for total RNA) was also verified using the Agilent Technologies 2100 Bioanalyzer with RNA Agilent RNA 6000

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Nano Kit and Agilent Small RNA kit. Total RNA preparations were stored at −80 °C until further analysis.

4.3 Small RNA enrichment, library preparation and sequencing analysis

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. Each amplified sRNA library was quantified and the quality analyzed using the Agilent Technologies 2100 Bioanalyzer with a High Sensitivity DNA Kit. Template-positive Ion Sphere™ Particles (ISPs) were prepared and enriched using the ExT Kit (Cat. No. A30670 revision E.0) on the IonChef Instrument following the manufacturer's protocol. The enrichment was then assessed using the Qubit® 2.0 Fluorometer with a dsDNA BR Assay kit. The prepared libraries were sequenced using two Ion 530 chips (Cat. No. A27764 on an Ion GeneStudio™ S5 System. The sRNA sequences were analyzed using the CLC Genomics Workbench software version 21.0.5 (QIAGEN). Low quality reads and adapter sequences were removed and sequences were filtered by length for miRNA identification: minimum length 19 nt and maximum length 25 nt.

4.4 Conserved miRNA identification

To identify conserved miRNAs expressed in silver birch, all unique small RNA sequences were compared to annotated mature plant miRNAs in miRBase (v22). Sequences from 16 species in miRBase were utilised, of which eight were tree species, including seven woody species: three conifer species - *Pinus taeda*, *Pinus densata*, *Picea abies*, four broadleaf tree species - *Populus trichocarpa*, *Populus euphratica, Acacia auriculiformis, Acacia mangium* and one fruit tree - *Prunus persica*, as well as other plant species - *Arabidopsis thaliana*, *Arabidopsis lyrata*, *Glycine max*, *Oryza sativa*, *Nicotiana tabacum*, *Vitis vinifera*, *Zea mays*, *Brassica rapa*. Mature miRNA and pre-miRNA sequences of these species were obtained from miRBase (v22). Two mismatches were allowed between silver birch miRNA sequences and miRNAs obtained from miRBase. Using the CLC genomics Workbench software, sequences were counted and assigned to families by comparison with mature miRNA sequences from miRBase. The parameters for sequence comparison were: two additional upstream and downstream bases, two missing upstream and downstream.

Potential precursor and target gene sequences were identified for the differentially expressed conserved mature miRNA sequences. To identify potential silver birch precursor miRNA sequences, miRNA sequences were aligned to the *Betula pendula* scaffold assembly, vv1.2. (id 35079) (available from Genome evolution database at https://genomevolution.org). Small RNA sequences were mapped to silver birch sequences (contigs) using the Multi Blast tool (allowing two nucleotide mismatches, gaps – one nt mismatch, extension – two nucleotides) using the CLC genomics workbench software. Previously described criteria were used with some modifications: minimum length (in nt) of the double stranded segment within the folded sequence - 19; minimum length (inclusive, in nt) of mature/star miRNA - 19; maximum length (exclusive, in nt) of mature/star miRNA - 25.

In addition, the minimum free-folding energy index (MFEI) was calculated to confirm that the precursor sequences conformed to the requirements for forming the miRNA precursor structures. Sequences with a maximum of two mismatches with the miRNA sequences were identified and taking into account reports that plant pre-miRNAs vary from approximately 80–200 nt in length, regions flanking the mapped mature miRNAs (approximately 100 nt downstream and 100 nt upstream) were used to predict folding structures using the UnaFold program (http://www.unafold.org/mfold/applications/rna-folding-form.php) web server and the CLC genomics workbench software. If the length of a sequence was less than 200 nt, the entire available sequence was used as a miRNA precursor sequence. MFE (minimal negative folding free energy, ΔG), AMFE (adjusted

MFE), MFEI (minimal folding free energy index), length of sequence, nucleotide percentage (A, U, G, and C), A + U content, G + C content, and number of base pairs were calculated. The MFEI was calculated using the formula:

[(MFE/length of the RNA sequence) *100]/(G+C) %

Predicted secondary structures of precursor miRNAs have folding free energy indexes (MFEIs) >= 0.85, distinguishing them from other RNAs such as tRNAs, and rRNAs whose MFEI are between 0.59 and 0.66.

4.4 Target gene identification

Identification of potential miRNA target genes was done by searching for complementary regions between the identified miRNAs in this study and by using all the *Populus trichocarpa* unigene DFCI Gene index (PPLGI), version 5, released on 2010.04.16 sequence input using the psRNATarget-Plant Small RNA Target Analysis Server [42].

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1: Supplementary file 1. miRNA families identified in all sample types and sequencing libraries ; Supplementary file 2. Differentially expressed miRNA isomiR groups; Supplementary file 3. Potential precursor miRNA sequences for differentially expressed miRNAs; Supplementary file 4. Target genes of differentially expressed miRNAs).

Author Contributions: Conceptualization and methodology, B.K, I.S., D.R.; formal analysis, B.K..; investigation, B.K, I.S.; resources, I.S., D.R..; writing—original draft preparation, B.K..; writing—review and editing, B.K., I.S., D.R..; supervision, project administration, funding acquisition, D.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Latvian Council of Science grant No. lzp-2019/1-0387 "Development of molecular markers for assessment of juvenility during micropropagation of silver birch (*Betula pendula* Roth)".

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The raw sequence reads were deposited in the NCBI database (BioProject ID: PRJNA916462).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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