Conservative microRNA studies and analyses in Scots pine (*Pinus sylvestris* L).

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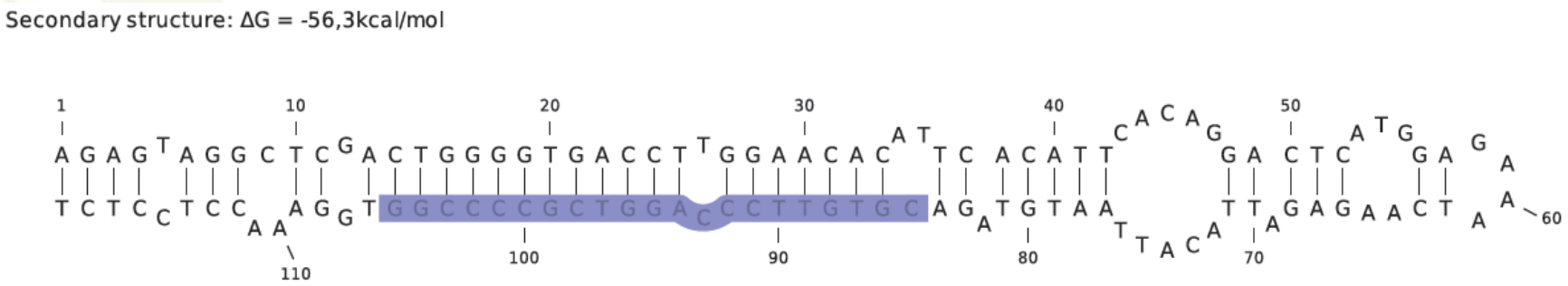
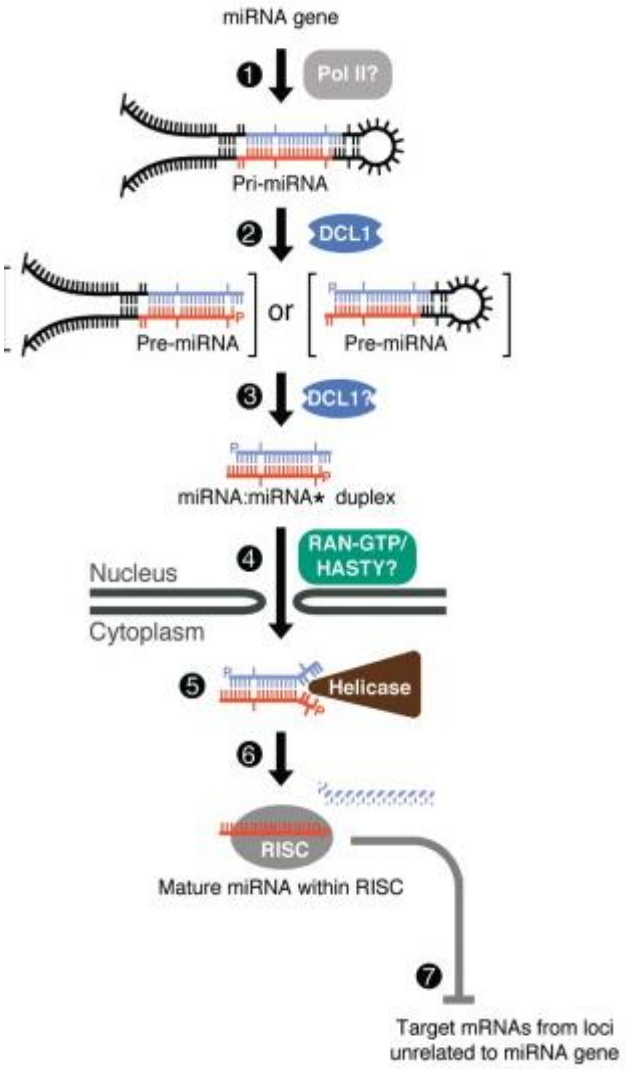
Introduction

- MicroRNAs (miRNAs) are non- protein coding RNAs ~19-25 nt in length that play an important role in many biological and metabolic processes, including regulation of gene expression, influencing plant growth and developmental processes, as well as responses to stress and pathogens.
- Methyl jasmonate (MJ) is plant hormone synthesized from linolenic acid and plays a role in plant responses to abiotic and biotic stresses, including wound response and plant defence.



miRNA biogenesis



Aims

- The aims of this study are to identify and characterise miRNAs in Scots pine, to elucidate the role of miRNAs in defence responses, to detect miRNA expression levels under stress conditions and to identify potential target genes.

Methods

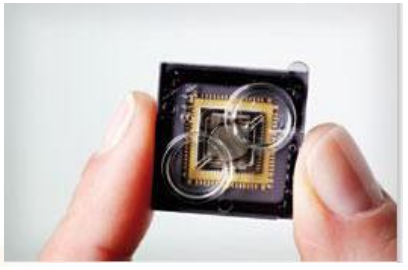
Plant material, growth conditions and sample collection

6 Scots pine ramets from 1 clone. 3 ramets were treated with 10 mM MJ and 3 untreated ramets were used as controls. Ramets were grown in growth chambers, at 17-22^{0}C under short day conditions (16 h light + 8 h dark). After 2 weeks growth, needles were collected and total RNA isolated.

DNA library construction and small RNA sequencing

Total RNA samples were enriched for small RNA and 6 small RNA non-barcoded libraries were prepared using Ion Total RNA-Seq Kit v2 (*Life Technologies*). Then template-positive Ion Sphere™ Particles (ISPs) containing clonally amplified DNA were prepared with the Ion OneTouch™ 2 and sequenced by Ion Personal Genome Machine® (PGM™) System in collaboration with the «Norwegian Forestry and Landscape Institute» (now NIBIO).



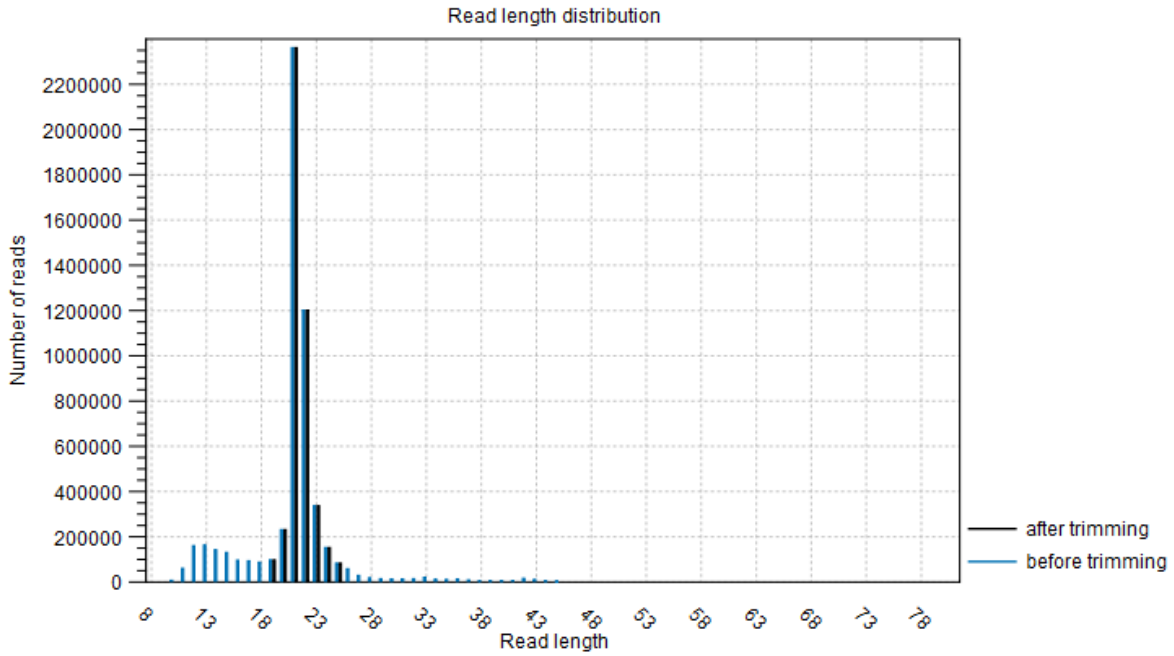
Methods

- Sequences were analysed using the CLC Genomics Workbench software version 7.5.1.
- We identified conserved miRNAs using previously reported miRNA sequences from various tree species as well as other plant species from miRBAse, the miRNA Registry Database (Release 20 and 21, http://www.mirbase.org).

Results

- Sequencing of the 6 small RNA libraries yielded approximately 4.5 million reads.
- 1021696 unique small RNA sequences were found.
- An average length - 21 nt (2,2 million reads).

2 Read length before / after trimming



Results

Resource	Sequences in resource	Sequences found	Percentage found
miRBase (Acacia auriculiformis)	7	4	57.1%
miRBase (Arabidopsis thaliana)	298	90	30.2%
miRBase (Oryza sativa)	592	82	13.9%
miRBase (Picea abies)	40	30	75.0%
miRBase (Pinus taeda)	36	33	91.7%
miRBase (Pinus densata)	30	21	70.0%
miRBase (Populus euphratica)	4	1	25.0%
miRBase (Populus trichocarpa)	352	73	20.7%
miRBase (Nicotiana tabacum)	162	30	18.5%
miRBase (Vitis vinifera)	163	27	16.6%
miRBase (Zea mays)	172	37	21.5%

Results

Annotation	Count	Percentage
Annotated	4,975	0.5%
with miRBase (Acacia auriculiformis)	41	0.8%
with miRBase (Arabidopsis thaliana)	457	9.2%
with miRBase (Oryza sativa)	307	6.2%
with miRBase (Picea abies)	1,676	33.7%
with miRBase (Pinus taeda)	1,459	29.3%
with miRBase (Pinus densata)	586	11.8%
with miRBase (Populus euphratica)	2	0.0%
with miRBase (Populus trichocarpa)	162	3.3%
with miRBase (Nicotiana tabacum)	82	1.6%
with miRBase (Vitis vinifera)	132	2.7%
with miRBase (Zea mays)	71	1.4%
Unannotated	1,016,721	99.5%
Total	1,021,696	100.0%

Results

Annotation	Count	%	Perfect matches	%	1 mismatch	%	2 mismatches	%
Annotated	317,195	7.1%	191,919	60.5%	29,258	9.2%	96,018	30.3%
with miRBase (Acacia auriculiformis)	773	0.2%	487	63.0%	272	35.2%	14	1.8%
with miRBase (Arabidopsis thaliana)	29,350	9.3%	27,086	92.3%	1,278	4.4%	986	3.4%
with miRBase (Oryza sativa)	13,021	4.1%	11,252	86.4%	1,331	10.2%	438	3.4%
with miRBase (Picea abies)	172,488	54.4%	111,95	64.9%	12,9	7.5%	47,638	27.6%
with miRBase (Pinus taeda)	83,194	26.2%	28,41	34.1%	10,781	13.0%	44,003	52.9%
with miRBase (Pinus densata)	12,392	3.9%	9,107	73.5%	1,497	12.1%	1,788	14.4%
with miRBase (Populus euphratica)	2	0.0%	0	0.0%	0	0.0%	2	100.0%
with miRBase (Populus trichocarpa)	1,412	0.4%	260	18.4%	567	40.2%	585	41.4%
with miRBase (Nicotiana tabacum)	1,621	0.5%	1,321	81.5%	139	8.6%	161	9.9%
with miRBase (Vitis vinifera)	2,126	0.7%	1,562	73.5%	424	19.9%	140	6.6%
with miRBase (Zea mays)	816	0.3%	484	59.3%	69	8.5%	263	32.2%
Unannotated	4,171,264	92.9%						
Total	4,488,459	100.0%						

Bioinformatic analysis of conserved miRNAs

- To identify potential precursor miRNAs specific to Scots pine, miRNA sequences were aligned to *Pinus* PGI_v9.0_032811 reference sequence.
- miRNA hairpin stem loop structures of these sequences were calculated using MFOLD (http://mfold.rna.albany.edu/?q=mfold) web server (Zuker, 2003) and CLC genomics workbench.
- Two mismatches were allowed between identified Scots pine miRNAs and currently known miRNAs in other plant species. Additional other parameters were set: additional downstream bases – 2, additional upstream bases – 2, missing downstream bases - 2, missing upstream bases- 2.
- MFE (minimal folding free energy), AMFE (adjusted MFE), MFEI (minimal folding free energy index), length of sequence, percent of nucleotide (A, U, G, and C), A + U content, G + C content, and number of base pairs were calculated (Zhang et al., 2006).

Bioinformatic analysis of conserved miRNAs

A small RNA was considered as a potential miRNA candidate only if it met the following criteria:

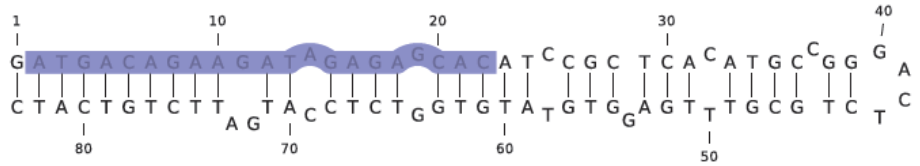
1. the predicted mature miRNA was allowed to have 0–2 nucleotide mismatches in sequence with the best matched known plant mature miRNA and sequence length was between 19 and 25 nucleotides;
2. a RNA sequence could fold into an appropriate stem–loop hairpin secondary structure;
3. the predicted mature miRNA is located in one arm of the hairpin structure;
4. less then 6 mismatches in the complementary site (the opposite miRNA* sequence in the other arm);
5. predicted secondary structures had folding free energy indexes (MFEIs) >= 0.85
6. 30–70% A + U contents (Zhang et al., 2006).

Potentional Scots pine pre-miRNAs sequences indentification

- Precursor microRNA sequences could be identified for 43 differentially expressed microRNAs.
- A total of 47 pre-miRNAs were found with MFEI 0.72-1.16 or 36 precursors with MFEI 0.85-1.16.
- For some miRNAs we found two precursors for 4 mature miRNAs – ath-miR157a-c, pta-miR946a-5p, vvi-miR319e, miR166a-g,j. It was difficult to detect wich precursor is true, because in some case miRNA was perfectly matched with miRNA site in precursor, but the MFEI was lower like it was with pta-miR946a-5p precursor from TC163753 with MFEI 0.81, but in case TC181939 MFEI was 1.01, but there were 2 mismatches. Using NCBI Blast, the identitu between precursors were not found in 1 case, but in 3 cases in vairet from 68%-92%.
- Precursors length variet from 80-245 nt.
- The (G+C)% content variet from 37.02-56.83%.

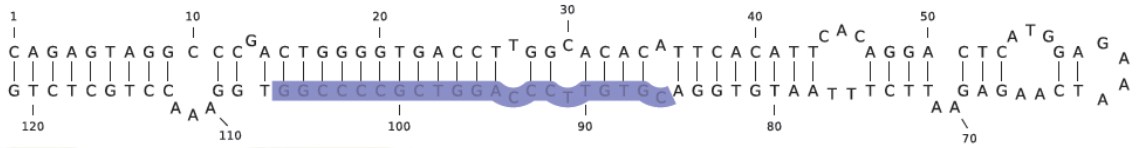
Potentional Scots pine pre-miRNAs sequences indentification

Secondary structure: ΔG = -36,2kcal/mol athMIR157abc

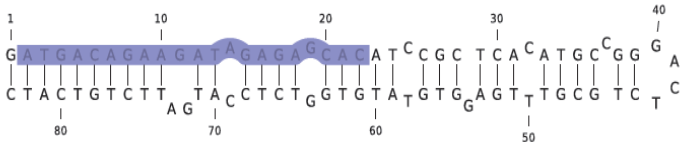


Secondary structure: ΔG = -46,1kcal/mol nta-miR408

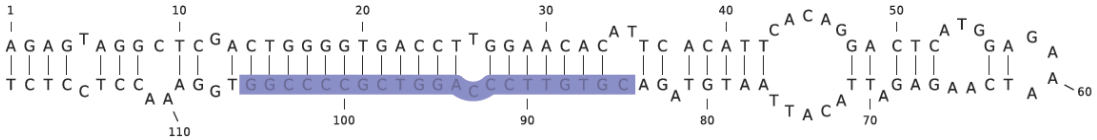
Secondary structure: ΔG = -64,8kcal/mol osa-miR398b/nta-miR398



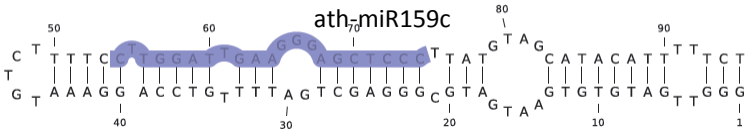
Secondary structure: ΔG = -36,2kcal/mol ath-miR157abc



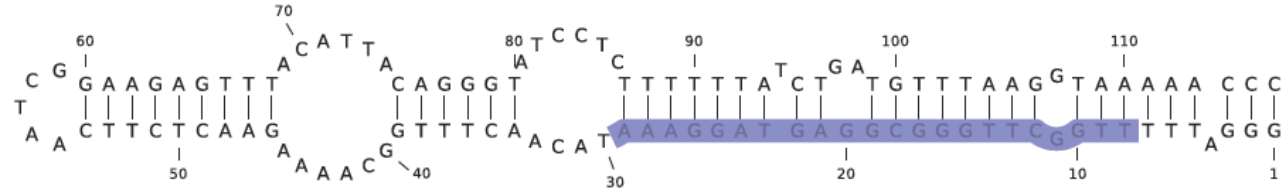
Secondary structure: ΔG = -56,3kcal/mol pta-miR398



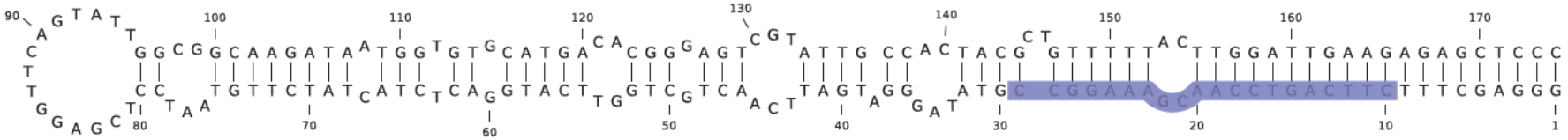
Secondary structure: ΔG = -37,6kcal/mol ath-miR159c



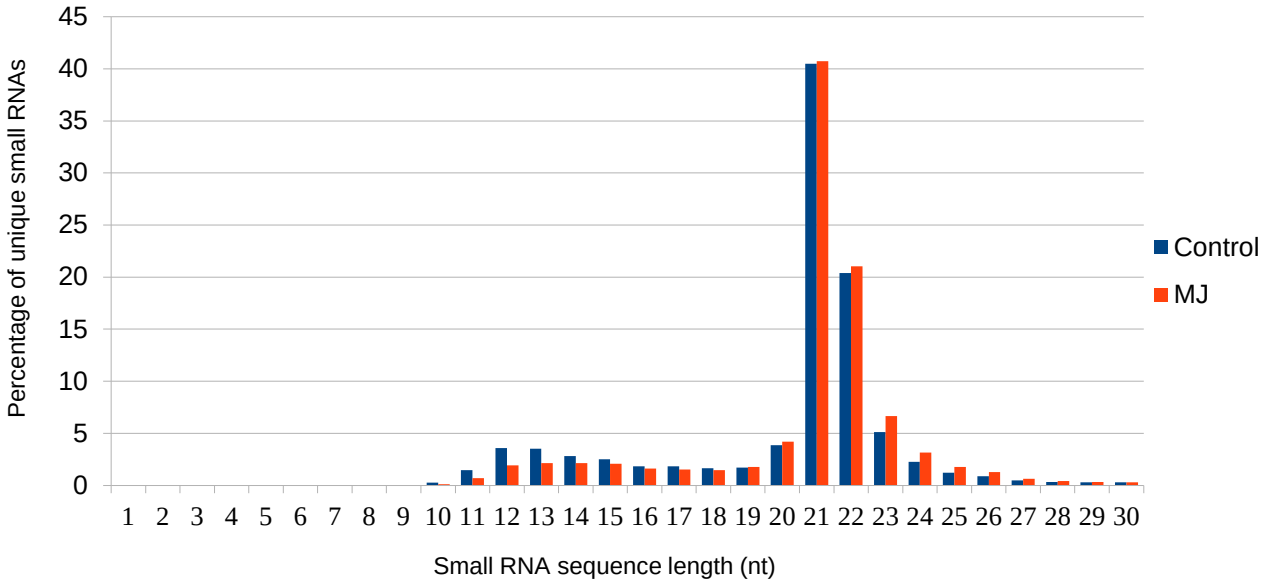
Secondary structure: ΔG = -33,6kcal/mol pab-miR482d



Secondary structure: ΔG = -74,2kcal/mol osa-171bcde



Differentially expressed conserved miRNAs



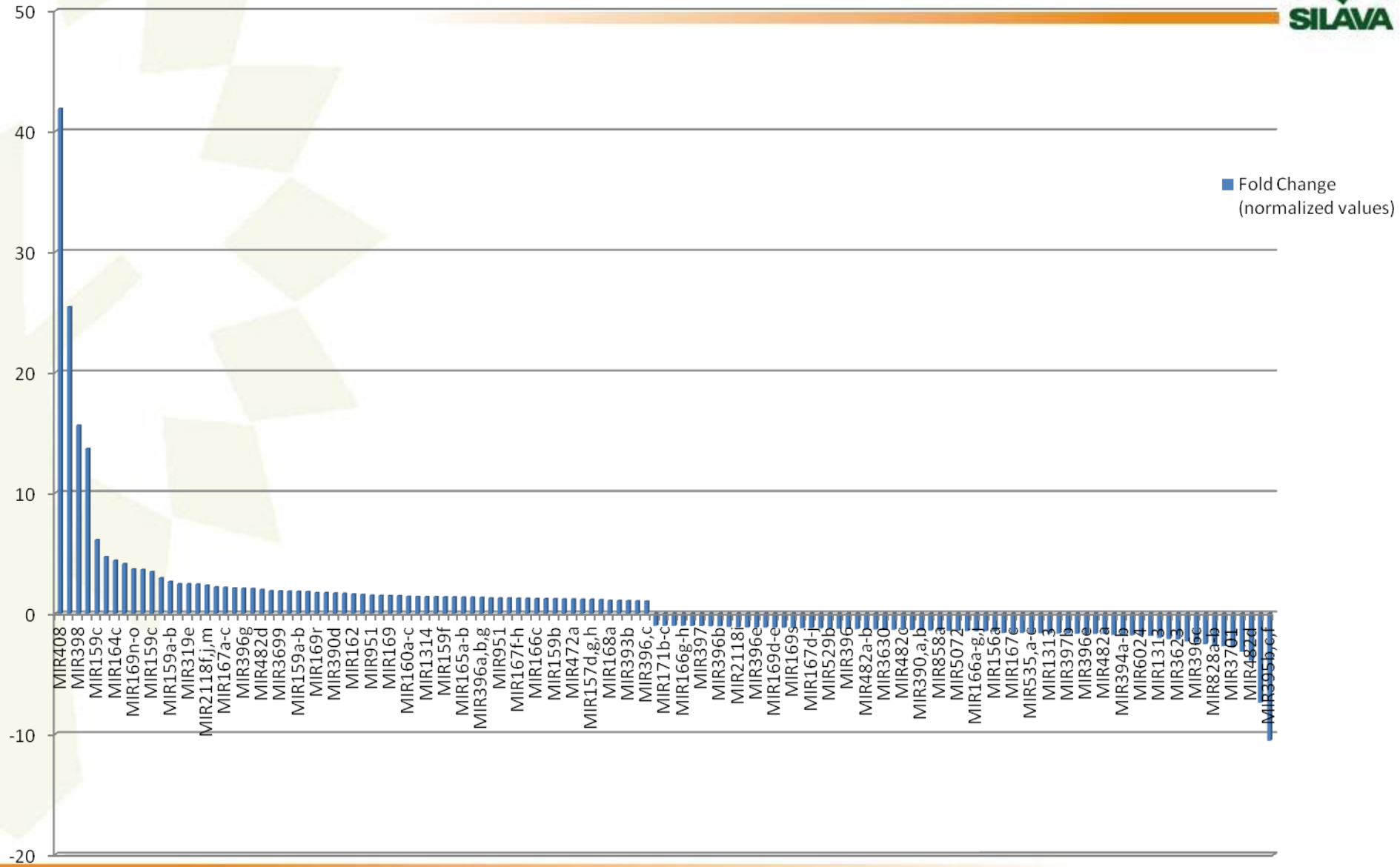
Size distribution of unique small RNA sequences from control and MJ-treated Scots Pine samples.

- The average nt size of small RNA population become smaller – from 21.57 (in controls) to 20.76 nt (in MJ treated).
- The population of small RNAs>21 nt in length decreased, whereas small RNAs 19-21 nt increased.
- After trimming average nt size was the same – 21.50 nt in controls and 21.46 nt in MJ treated samples.

Differentially expressed conserved miRNAs

- 38 families were differentially expressed, containing 75 differentially expressed miRNA groups (based on mature miRNAs from miRBase - each microRNA group contains multiple sequence variants).
- 20 miRNA families were not differentially expressed.
- MJ treatment significantly affected the expression of microRNAs: 14 microRNA families were up regulated, and 14 were down regulated. Additionally, 10 microRNA families contained members which were regulated in opposite directions.
- Based on fold change, the miR408 group is the most up regulated in Scots pine needles with fold change 41,88, followed by miR398 (two groups) with fold change 25,43 and 15,61. The most differently expressed group in up down regulated miRNAs is miR395 (mir395b/395c/395f) with fold change -10,54 followed by two miR482 groups (miR482a/482c and miR482d) with fold change -7,39 and -3,90.

Fold change


50
40
30
20
10
0
-10
-20
Fold Change (normalized values)
MIR408
MIR398
MIR159c
MIR164c
MIR169n-o
MIR159c
MIR159a-b
MIR319e
MIR2118f,j,m
MIR167a-c
MIR396g
MIR482d
MIR3699
MIR159a-b
MIR169r
MIR390d
MIR162
MIR951
MIR169
MIR160a-c
MIR1314
MIR159f
MIR165a-b
MIR396a,b,g
MIR951
MIR167f-h
MIR166c
MIR159b
MIR472a
MIR157d,g,h
MIR168a
MIR393b
MIR396,c
MIR171b-c
MIR166g-h
MIR397
MIR396b
MIR2118i
MIR396e
MIR169d-e
MIR169s
MIR167d-i
MIR529b
MIR396
MIR482a-b
MIR3630
MIR482c
MIR390,a,b
MIR858a
MIR5072
MIR166a-g,i
MIR156a
MIR167e
MIR535,a-e
MIR1313
MIR397b
MIR396e
MIR482a
MIR394a-b
MIR6024
MIR1313
MIR3623
MIR396c
MIR828a-b
MIR3701
MIR482d
MIR395b,c,l

Prediction of potentional target mRNAs for Scots Pine miRNAs

Potential mRNA targets for Scots Pine miRNAs were determined by searching for complementary hits using the psRNATarget program (http://plantgrn.noble.org/psRNATarget/) (Dai and Zhao, 2011) using the Pinus (Pine) DFCI Gene Index (PGI) version 9, released on 2011_03_26, as the source of sequence information.

Default settings were used, resulting in only mRNA target sequences with a score of <= 3 being considered as potential miRNA targets. This score insured only targets of high confidence were chosen.

Target genes with known function were identified for 46 mature miRNAs groups, from which 20 targets were found for up regulated miRNAs and 26 targets for down regulated miRNAs groups.

There were also 9 targets without known functions (7 targets for up regulated miRNAs, 2 targets for down regulated miRNAs).

Targets role for differentially expressed up regulated miRNAs

nta-miR408 - defense responses and/or in lignin formation (fold change – FC: 41,88)

osa-miR398b/nta-miR398 , ptc-miR396g - electron transport (FC: 25,43 and 2,70)

ath-miR157a-c- defense responses to abiotic stresses, tissue development, pathogen response (FC: 13,68)

ath-miR159c , pta-miR159a -metal handling (FC: 6,11 and 3,62)

zma-miR529 - plant tissue development, plant pathogen response, response to low-temperature exposure could, response to biotic or abiotic streses, cell death, growth of new organs, plant senescence, plant sexual reproduction,metabolism and lignin biosynthesis (FC: 4,70)

pta-miR947-development, disease resistance, stress responses (FC: 4,12)

osa-miR169n/o - role of some biosynthetic enzymes as targets for herbicides, stress responses (FC: 3,67)

pta-miR159c – mediating transcriptional repression, intra-cellular transport, mitosis, meiosis, development, metal handling immune response,disease resitance (FC: 3,46)

nta-miR171b, **osa-miR171b-f**- inhibition of photosynthesis,plant growth, development, cell death (FC:2,44 and 2,42)

vvi-miR319e - intracellular and extracellular signalling, abiotic stress signalling (FC: 2,44)

ath-miR167a//miR167b- stress response , response to plant diseases, may be involved in transcriptional regulation, vesicular transport and peroxisomal biogenesis (FC: 2,15)

pta-miR1312, pta-miR1315, **pab-miR951** - disease resistance (FC: 2,05, FC: 1,48, FC: 1,50)

nta/osa - miR172a//miR172f//miR172i - development ,signal transtruction, drought stress tolerance (FC 1,65)

pta-miR1314 - plant growth and development, plant maturation, protection from plant infection (FC: 1,56)

Targets function for differentially expressed down regulated miRNAs

ath- miR395b//miR395c//miR395f , **pde-miR3701**- biosynthetic pathway (FC: -10, 54, FC: -2,82)

ptc-miR172g - induction and inhibition growth, development, metabolism, flowering, germination (FC: -2,71)

ptc-miR828a/b, osa-miR396c, vvi-miR828b - responses to biotic and abiotic stresses, development, differentiation, cell shape, hormone responses , metabolism, defense etc (FC: -2,68, -2,42, -1,46)

vvi-miR171b - inhibition of photosynthesis,plant growth, development, cell death ()

ptc-miR482d - ripening process

vvi-miR3623, pde-miR3701, **pde-miR950** - disease resistance

pta-miR482d - abiotic stress responses, organelles synthesis

vvi-miR396a - plant development and abiotic stress responses

ptc-miR394a/b , **zma-miR166b-i** –development

ptc-miR482a - cell growth

pab-miR3701 - phenylpropanoid metabolic process, proteolysis , stress response, development

osa- miR397b - lignin biosynthesis

zma-miR159e - chemical defense and communication (mono-, sesqui- and diterpenes), photoprotection and energy transfer (carotenoids), growth regulation.

pta-miR1313 - cellular and morphogenic developmnet, growth responses, initiation of cellular signaling

ath-miR169l - role of some biosynthetic enzymes as targets for herbicides

ath - miR167c - transcriptase

pta - miR156a - plant defense signaling, regulation of a variety of plant developmental processes

ath/osa/ptc -miR166a-g, j – morphogenesis

ath-miR858a - secondary metabolism, cell shape development, cell division, signal transduction, and disease resistance

Summary

- Conservative miRNA sequences were identified
- Precursor miRNA sequences were identified
- MFEI were calculated to confirm that predicted are precursor miRNA hairpin stem-loop secondary structures
- Conservative miRNAs are differentially expressed after MJ treatment
- miRNA targets were identified

Thank you!