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The microRNA response associated with methyl jasmonate-induced resistance in Norway spruce bark

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ABSTRACT

Methyl jasmonate (MeJA) treatment elicits induced resistance (IR) against pests and diseases in Norway spruce (*Picea abies*). We recently demonstrated using mRNA-seq that this MeJA-IR is associated with both a prolonged upregulation of inducible defenses and defense priming. Gene expression can be regulated at both a transcriptional and post-transcriptional level by small RNAs, including microRNAs (miRNAs). Here we explore the effects of MeJA treatment and subsequent challenge by wounding on the Norway spruce miRNA transcriptome. We found clusters of prolonged down- or upregulated miRNAs as well as miRNAs whose expression was primed after MeJA treatment and subsequent wounding challenge. Differentially expressed miRNAs included miR160, miR167, miR172, miR319, and the miR482/2118 superfamily. The most prominent mRNA targets predicted to be differentially expressed by miRNA activity belonged to the nucleotide-binding site leucine-rich repeat (NBS-LRR) family. Among other predicted miRNA targets were genes regulating jasmonic acid biosynthesis. Our results indicate that miRNAs have an important role in the regulation of MeJA-IR in Norway spruce.

1. Introduction

Induced resistance (IR) is an important adaptation that allows plants to increase their resistance to herbivores and pathogens in response to a triggering stimulus (De Kesel et al., 2021). Two non-exclusive mechanisms underlie IR: prolonged upregulation of inducible defenses and defense priming (Wilkinson et al., 2019). Prolonged upregulated defenses remain activated for long periods following the triggering stimulus, while primed defenses remain at basal levels until a secondary challenge elicits an augmented defense response (Wilkinson et al., 2019; Mageroy et al., 2020a). These two IR mechanisms are associated with different costs and benefits and the optimal strategy is dependent on the environment (Wilkinson et al., 2019).

The plant hormone methyl jasmonate (MeJA) has for more than 20 years been used as a tool to study IR in conifers (Franceschi et al., 2002; Martin et al., 2002). Application of MeJA to the stem bark has been demonstrated to enhance Norway spruce (*Picea abies*) resistance to bark beetle attack and pathogen infection (Kozlowski et al., 1999; Erbilgin et al., 2006; Zhao et al., 2011). Recently, we showed that MeJA acts both as a direct inducer of defenses and as a stimulus of defense priming in Norway spruce (Mageroy et al., 2020a, 2020b). Using mRNA-seq analysis of bark tissues collected 4 weeks after MeJA treatment, we identified transcripts that showed prolonged upregulation, such as those coding for TIFY domain-containing proteins, and transcripts that were primed, such as those predicted to encode for pathogenesis-related (PR) proteins (Mageroy et al., 2020b).

Small RNAs (sRNAs) often have important and essential roles in regulating gene expression (Yu et al., 2017). MicroRNAs (miRNAs) are 20 to 22 nucleotide sRNAs that are generated from double stranded hairpin precursor RNAs and function as posttranscriptional regulators (Yu et al., 2017; Axtell and Meyers, 2018). miRNAs regulate gene expression post-transcriptionally by either guiding the cleavage of mRNAs or by inhibiting the translation of complimentary mRNAs (Song et al., 2019). miRNAs also trigger the generation of phased, secondary, small interfering RNAs (phasiRNAs) from target mRNAs (Fei et al., 2013). Some phasiRNAs act in *trans* (*trans* acting siRNAs; tasiRNAs) and directly silence mRNAs other than their source mRNA (Fei et al., 2013). Many phasiRNAs have been identified, however, their biological roles and targets remain poorly described (Liu et al., 2020). sRNAs are also known to affect gene expression by guiding RNA dependent DNA methylation (RdDM)(Tamiru et al., 2018). Although the role of sRNAs in plant growth and other biological processes has been extensively studied

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for more than 20 years, diverse new functions of sRNAs in plant immunity, defense priming, and epigenetic memory continue to be discovered (Soto-Suárez et al., 2017; Yakovlev and Fossdal, 2017; Sánchez-Sanuy et al., 2019; Wang and Galili, 2019).

We wished to understand how the differentially expressed mRNAs found in our previous study are regulated (Mageroy et al., 2020b). Given the importance of miRNAs in regulating gene expression, we use miRNA-seq analysis to explore the changes in miRNA expression following MeJA treatment and subsequent wounding challenge. By making use of the same bark tissues as those used for the mRNA-seq study, we found that as with the mRNA expression, MeJA treatment profoundly alters both the basal expression of miRNAs and the expression of miRNAs in response to a subsequent wounding challenge. Potential mRNA targets of the differentially expressed miRNAs were identified to allow for exploration of regulatory roles of miRNAs in Norway spruce MeJA-IR.

2. Materials and Methods

2.1. Plant Material and Experimental Setup

Plant material and experimental design was the same as previously described in Mageroy et al. (2020b). Briefly, six trees from a single clone (no. 137) were selected from a stand at the Hogsmark Experimental Farm in Ås, SE Norway (59°40′04.1″ N 10°42′46.2″ E). On April 30, 2013, three trees were sprayed with a solution of 100 mM methyl jasmonate (MeJA) and 0.1% Tween, and three trees were sprayed with a solution of only 0.1% Tween, as a control treatment (Fig. 1). Approximately 500 mL of solution was used per tree. Four weeks later, small areas of bark on all trees were mechanically wounded to elicit inducible defenses. Wounding was done by puncturing the bark to the cambium with a push-pin about 30 times inside four 10 × 10 mm areas evenly distributed around the stem circumference at about 1.75 m height from the base of the tree (Fig. 1). The rest of the bark area was left intact. Twenty-four hours after wounding, bark samples were collected for sRNA sequencing using a 10-mm cork borer. Bark plugs of wounded bark were collected at the four wounded areas around the stem (Fig. 1). Plugs of intact bark were collected at four sites situated about 18 cm below and 45 degrees to the left of each wounded area. This sampling technique has been used in a previous study that showed defense induction in spruce do not spread much in the tangential direction (Erbilgin et al., 2006). This gave four different bark treatment combinations: MeJA-treated and wounded (MW), MeJA-treated and intact (MI), control and wounded (CW), and control and intact (CI) (Fig. 1).

2.2. RNA Extraction

The cork bark was removed and the phloem was ground in liquid nitrogen. Total RNA was purified from phloem powder using Epicentre MasterPure™ Plant RNA Purification Kit (Epicentre, Madison, WI, USA; kit now replaced with MasterPure™ Complete DNA and RNA Purification Kit, Lucigene, Middleton, WI, USA) according to the manufacturer's instructions. Contaminating DNA was removed from the total RNA samples using the above-mentioned kit, according to the supplier's protocol. Total RNA preparations were then stored at −80 °C until sRNA library preparation.

2.3. sRNA Extraction, Library Preparation and Sequencing

Sequencing of sRNA libraries was performed as described by Yakovlev and co-workers (Yakovlev and Fossdal, 2017). These libraries were prepared from total RNA extracts using the Ion Total RNA-Seq Kit v2 for Small RNA Libraries (Cat. No. #4476289, ThermoFisher Scientific) with enrichment steps for sRNA as detailed in the Ion RNA-Seq Library Preparation guide (#4476286 revision E). The quality and quantity of the libraries were assessed at intermediate steps using Agilent Technologies 2100 Bioanalyzer Small RNA kit (#5067-1548, Agilent, Santa Clara, CA, USA). The 12 libraries (Fig. 1) were barcoded and pooled for sequencing, using a library concentration of 30 pM and an internal calibration standard (Cat. No. A27832). Libraries were templated on an automated Ion Chef (ThermoFisher; Chef Package Version IC.5.0.1), using the Ion PGM Hi-Q Chef Kits (Cat. No. A25948). Initial sequencing was performed with Hi-Q chemistry on an Ion PGM sequencer with a 316v2 chip (Cat. No. 4488145; Pub. No. MAN0010919

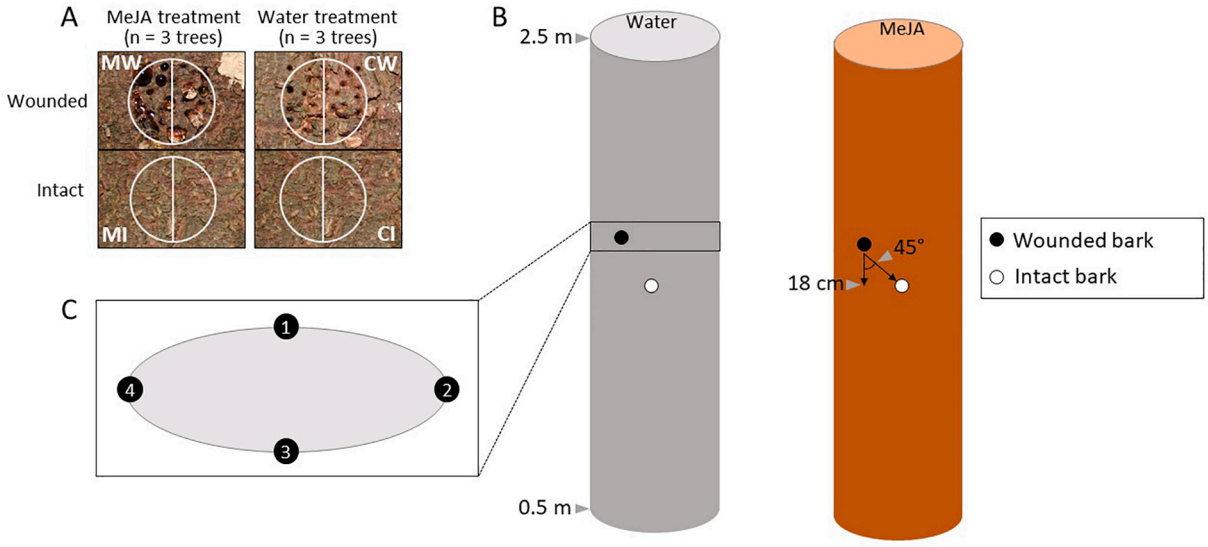

Fig. 1. Overview of experimental design and sampling. (A) Three Norway spruce trees were sprayed with methyl jasmonate (MeJA) and three trees were sprayed with water (control) on the lower stem between 0.5 m and 2.5 m from the ground. Four weeks later, small areas of bark on all trees were wounded while the rest of the bark area was left intact. This gave four treatment combinations: MeJA-treated and wounded bark (MW), MeJA-treated and intact bark (MI), control and wounded bark (CW), and control and intact bark (CI). (B) Twenty-four hours after wounding, bark plugs were sampled from all treatment combinations using a cork borer. (C) Each set of samples (wounded or intact) consisted of four sampling sites evenly distributed around the stem circumference. Each bark plug was split in two (A), and four half plugs from each treatment combination were pooled into one sample for miRNA analysis. This was the same bark tissue used in our previous study of the mRNA transcriptome (Mageroy et al., 2020b).

Rev. B.0). Further sequencing was done at the Uppsala Genome Center with two Ion Proton v3 chips (ThermoFisher Scientific; Cat. No. 4476610). The Ion Torrent Suite versions 5.0.4 and 5.2.1 were used, with internal calibration enabled, for both base-calling and de-multiplexing sequencing runs. The total numbers of unfiltered reads for each treatment, replicate, sequencing chip and instrument are provided in Supplementary Table S1. The three sequencing runs from the same barcoded sample we concatenated together to form one fastq file per replicated treatment (Supplementary Table S1). The BBtools Readlengths.sh script from BBMap was used to quantify raw read length (Bushnell, 2014).

2.4. Analysis

ShortStack version 3.8.3 was used to annotate and count miRNA reads from sRNA sequence data (Axtell, 2013). Parameters and options for ShortStack were: `–bowtie_cores 12 –bowtie_m 100 –ranmax 10 –mincov 0.2 rpm –dicermin 19 –foldsize 600`. The `–readfile` parameter used the input from the replicated treatments, where each sequence file comprised the fastq data concatenated from the libraries of two Ion Proton runs and the single Ion PGM sequencing run (12 files in total). A `–genomefile` containing only the *P. abies* Z4006 scaffolds from miRNA loci identified in (Xia et al., 2015) and (Yakovlev and Fossdal, 2017) was used for the alignment (Supplementary file S1). The `–locifile` option, which delimited the coordinates of annotated mature miRNAs, was also used (Supplementary file S2). Approximately 20 scaffolds were removed from (Xia et al., 2015) since they were labelled 'contaminants' or 'putative mitochondrial fragments' in that paper (Supplementary file S3). Two scaffolds (listed as 'putative contaminants'), were later returned since they were found to contain miR482g and one other miRNA loci as determined in (Xia et al., 2015). Read counts generated by ShortStack (Supplementary Table S2) and the DESeq2 R package version 3.5.0 (Love et al., 2014) were used to determine differential expression. Counts were first pre-filtered to exclude transcripts with less than 10 total counts over all libraries and variance stabilizing transformation (VST) was then performed using the DESeq2 vst function. Principal Component Analysis (PCA) plots were made using the DEseq2 plotPCA function and visualized using ggplot2 (Wickham, 2016). Pairwise comparisons were made between all libraries using the DESeq function of the DESeq2 package. A Benjamini and Hochberg, 1995 adjusted *p*-value of 0.05 was used as a cut-off to determine differentially expressed miRNAs. The R package NMF was used to generate a heatmap from VST counts of differentially expressed miRNA transcripts (Gaujoux and Seoighe, 2010). Clustering of miRNA transcripts by expression pattern was performed using VST counts and the Pearson method (parallelDist package version 0.2.4)(Eckert, 2018).

Predictions of mRNA targets of differently expressed miRNAs were inferred by referring to the miRNA targets already described in Yakovlev and Fossdal (2017) or Xia et al. (2015) and by using psRNATarget (Dai et al., 2018). The scoring schema V2 in psRNATarget was used to predict target sequences with a maximum expectation value of 3. The reference transcriptome used for mapping of mRNA-sequencing reads from the same bark samples (Mageroy et al., 2020b, see the Supplementary File S4), was used as the psRNATarget input file.

Protein family (Pfam) assignments of mRNA target sequences were taken from Mageroy et al. (2020b). Pfam enrichment analysis for each expression cluster identified in the heatmap was performed in R (Version 3.5.1) using bc3net (de Matos Simoes & Emmert-Streib, 2016). A Benjamini-Hochberg adjusted p-value of 0.05 was used as the cutoff for significance.

Network diagrams were produced in R using igraph (Csardi and Nepusz, 2006) and were visualized using the ggnet2 part of GGally, an extension of ggplot2 (Wickham, 2016; Schloerke et al., 2020). To aid readability, network diagrams were divided into three with miRNAs with shared mRNA targets appear in the same diagram.

3. Results

3.1. Effects of Methyl Jasmonate Treatment and Subsequent Wounding on the miRNA Transcriptome

In our previous work, we observed large-scale changes to the Norway spruce mRNA transcriptome in response to MeJA treatment and/or wounding challenge (Mageroy et al., 2020b). Small RNAs (sRNAs) can shape the mRNA transcriptome via both pre- and post-transcriptional mechanisms (Vaucheret, 2006). Thus, to explore the possible role of sRNAs in shaping the bark mRNA transcriptome following MeJA treatment and subsequent wounding challenge, we sequenced sRNAs from the same tissues that we used in our previous mRNA sequencing study (Mageroy et al., 2020b). In agreement with a previous study examining sRNA expression in Norway spruce (Nystedt et al., 2013), we found that the majority of sRNAs in Norway spruce bark were 21 nt miRNAs, with a very low frequency of 24 nt sRNAs (Fig. 2).

We focused only on the miRNA transcriptome, as miRNAs have previously been found to be abundant in Norway spruce and are well known regulators gene expression (Xia et al., 2015; Yakovlev and Fossdal, 2017). First, sRNA libraries were mapped to previously identified Norway spruce miRNA containing scaffolds (Xia et al., 2015; Yakovlev and Fossdal, 2017). Next, we used a principal component analysis (PCA) to assess global miRNA expression patterns and the consistency between biological replicates. The PCA demonstrated that replicates within the same treatment generally clustered together, except for replicate 1 (Fig. 3). This was probably due to the fact that the control replicate 1 had a lower number of reads than all other replicates (Supplementary Table S1). The PCA also suggested that treatment with MeJA had the greatest impact on the miRNA transcriptome, as the primary PCA axis (explaining 39% of the variance) correlated with treatment, whereas the secondary PCA axis (explaining 18% of the variance) correlated with challenge (Fig. 3).

Pairwise comparisons between all four treatment combinations (CI, CW, MI, MW; Fig. 1) identified 97 miRNA loci, belonging to 38 families, which were differentially expressed in at least one of the comparisons (Figs. 4 and 5). Comparisons to CI provide insight into how each treatments affects baseline miRNA expression. Comparisons to CW show how the short-term response to wounding in naïve bark differs from long-term MeJA-induced responses and the MeJA-primed response to wounding. Finally, the comparison between MI and MW also helps identify differences between prolonged response to MeJA and MeJA-primed response to wounding. The highest number of differentially expressed miRNAs were found for the comparisons between MeJA-treated and wounded versus the control and intact bark tissues (MW vs. CI) and for MeJA-treated and wounded versus the control and wounded bark tissues (MW vs. CW) (Fig. 4). The fewest number of differentially expressed miRNAs were found in the comparison between control wounded bark tissue and the untreated control bark (CW vs. CI) (Fig. 4).

Next, we explored the expression patterns of the differentially expressed miRNAs in more detail. Hierarchical clustering was used to group these miRNAs into three main clusters (Fig. 5; Supplementary Table S3). The expression patterns of these clusters matched expression patterns described in our mRNA transcriptomics study: miRNAs in cluster 1 exhibited "prolonged downregulation after MeJA treatment"; miRNAs in cluster 2 exhibited "primed upregulation following MeJA treatment and wounding"; and miRNAs in cluster 3 exhibited "prolonged upregulation after MeJA treatment" (Mageroy et al., 2020b). The highest number of differentially expressed miRNAs were found in clusters 3 and cluster 1. These had 41 and 33 different miRNA loci respectively, confirming that, as seen in the PCA analysis, the MeJA treatment had the largest effect on the miRNA transcriptome.

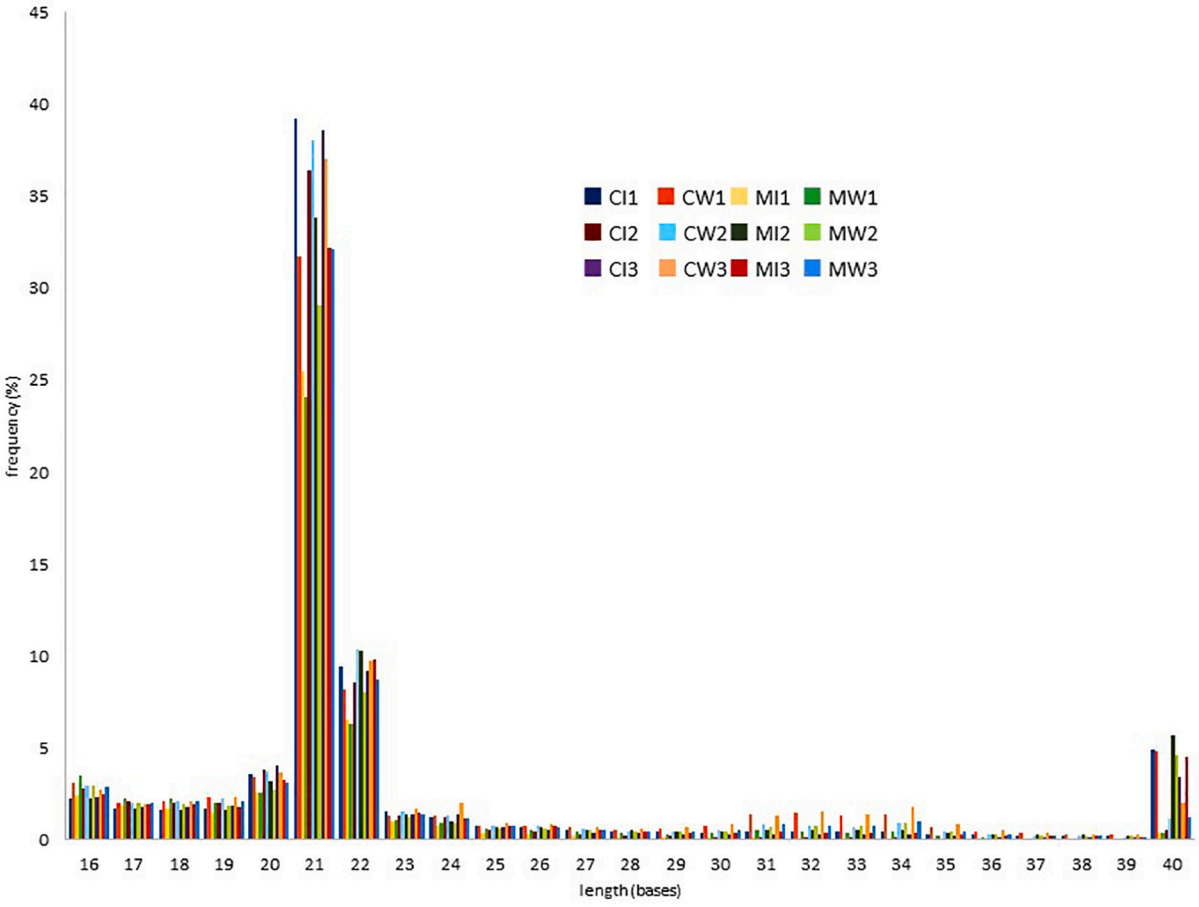

Fig. 2. Read length frequency for individual sRNA libraries from Norway spruce bark sprayed with methyl jasmonate or water and then wounded or left intact (legend; treatment combination and replicate). CI = control intact; MI = methyl jasmonate intact; CW = control wounded; MW = methyl jasmonate wounded. For an explanation of the experimental design and the different treatment combinations see Fig. 1.

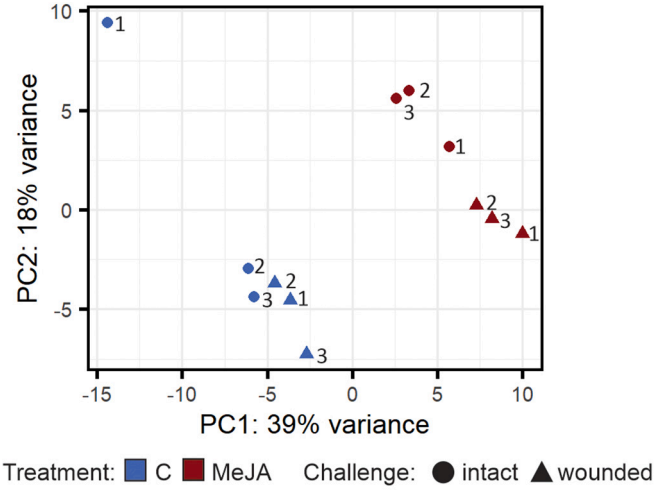

Fig. 3. Principle Component Analysis plot of Variance Stabilizing Transformation (VST) counts of sRNA transcripts. The stem bark of six Norway spruce trees that were sprayed with methyl jasmonate (MeJA) or water (n = 3). Four weeks later, the sprayed bark was then challenged with wounding or left intact. Bark was sampled 24 h after wounding. Numbers next to symbols indicate the treatment replicate number.

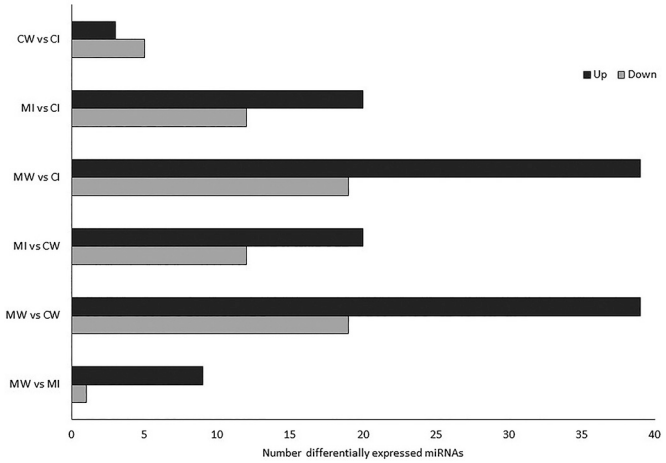

Fig. 4. Number of differentially expressed miRNAs in pairwise comparisons of treatment combinations. The stem bark of Norway spruce trees was first sprayed with methyl jasmonate (MeJA) or water (control), then wounded or left intact four weeks later, and finally sampled 24 h after wounding. The outcome was bark samples from four treatment combinations: MeJA-treated and wounded bark (MW), MeJA-treated and intact bark (MI), control and wounded bark (CW), and control and intact bark (CI).

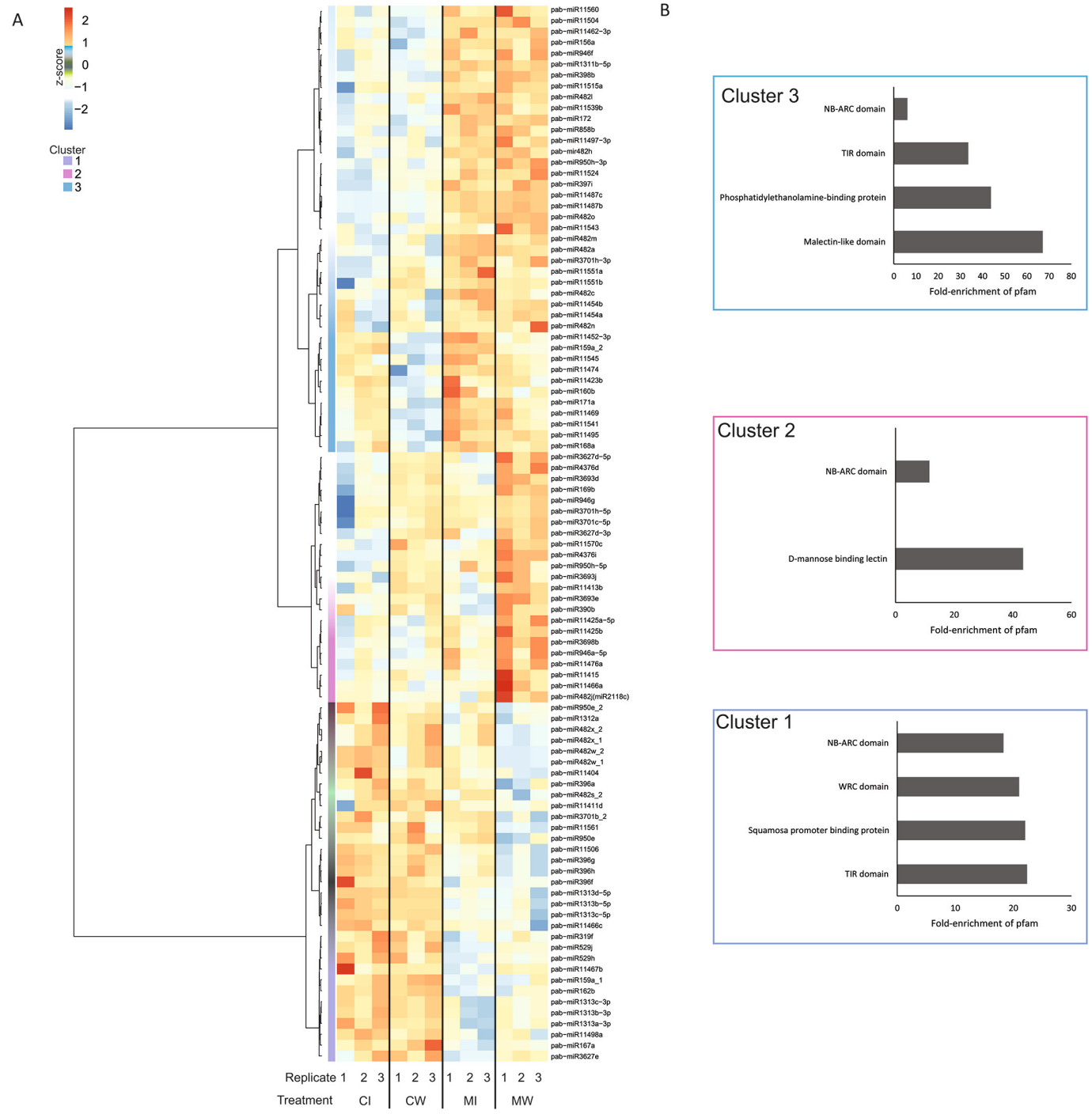

Fig. 5. Clustering of differentially expressed miRNAs and Pfam (protein family) enrichment analysis of their predicted mRNA targets in Norway spruce bark. (A) Heatmap of z-scores of VST counts computed for all miRNAs differentially expressed (p adjusted < 0.05) in at least one of the six pairwise comparisons between the four treatment combinations (see Fig. 1 for an explanation of the experimental design and treatment combinations). Differentially expressed miRNAs clustered into three main expression groups (Cluster 1–3). Cluster 1 = prolonged upregulated. Cluster 2 = primed upregulated. Cluster 3 = prolonged downregulated. (B) Pfam enrichment analysis of each expression cluster showing the top four enriched (or the two enriched in Cluster 2) Pfam categories with an adjusted p-value < 0.05.

3.2. mRNA Target Predictions

To explore how the miRNAs in the three clusters (Fig. 5) could have shaped the mRNA transcriptome we next identified potential miRNA targets using psRNATarget from the curated Norway spruce transcriptome, to which we mapped mRNA-seq libraries in our previous study (Mageroy et al., 2020b) (Supplementary Table S3 or Supplementary Figs. S1 Network diagrams 1–3). We also assessed the Pfam domain enrichment of the proteins encoded by the putative mRNA targets identified by psRNATarget for each expression cluster. While some Pfam accessions were enriched for all three clusters, such as the NB-ARC domain (PF00931.21), many were only overrepresented in specific clusters (Supplementary Table S4). Below we report the most notable miRNAs, enriched Pfams, and differentially expressed mRNA targets for each of the clusters, as well as those that were found in all three clusters.

3.2.1. Cluster 1: Prolonged Downregulation After MeJA Treatment

miRNA families found only in Cluster 1 included loci from miR162,

miR167, miR319, miR396, miR529, and miR1313. Families miR162, miR167, miR319, miR396, and miR529 are highly conserved among plants, while miR1313 seems to be unique for species in the pine family (Pinaceae) (Axtell et al., 2007; Morin et al., 2008; Liu et al., 2009; Li et al., 2011, 2019; Wong et al., 2011; Wan et al., 2012; Barik et al., 2015; Xia et al., 2015). miR162 is a conserved and known negative regulator of *DICER-LIKE1* (*DCL1*). It forms part of a self-regulatory loop between miR162 and *DCL1* and provides a key feedback component of miRNA biogenesis (Liu et al., 2009). In angiosperm plants, miR167 and miR319 target the *Auxin Response Factors 6/8* (*ARF6/8*) and the *TCP* (*TEOSINTE BRANCHED/CYCLOIDEA/PCF*) family of transcription factors, respectively (Nagpal et al., 2005; Schommer et al., 2008; Zhao et al., 2015). Family miR1313 has previously been identified as a trigger for phasiRNAs in Norway spruce (Xia et al., 2015).

3.2.2. Cluster 2: Primed Upregulation After MeJA Treatment and Wounding

miRNA families unique to Cluster 2 included miR169, miR390, miR3693, and miR4376. Families miR169 and miR390 are both widely conserved in plants (Axtell et al., 2007; Chen et al., 2017). Family miR4376 is conserved across most plants, but absent in monocots (Xia et al., 2015). As of yet, miR3693 has only been identified in *Picea* (Yakovlev and Fossdal, 2017). miR390 triggers the production of trans-acting siRNAs (tasiRNA) from *TRANS-ACTING SIRNA GENE 3* (*TAS3*). *TAS3* is the most studied *TAS* gene and the components of this pathway are present in all land plants (Xia et al., 2017). Targets of *TAS3* tasiRNAs include several class-B *ARF* genes (Xia et al., 2017). Members of the miR4376 family target *ACA10*, which encodes AUTOINHIBITED Ca^{2+}-ATPases and triggers formation of phasiRNAs (Xia et al., 2015).

3.2.3. Cluster 3: Prolonged Upregulation After MeJA Treatment

miRNA families that were differentially expressed and only found in Cluster 3 included loci from miR156, miR160, miR168, miR171, miR172, miR397, miR398, miR858, miR1311, and miR11487. Families miR156, miR160, miR168, miR171, miR172, miR397, and miR398 are highly conserved among plants (Chávez Montes et al., 2014). Family miR858 is found in gymnosperms and most eudicots, but is absent in most monocots (Chávez Montes et al., 2014). The miR1311 family is specific to Pinaceae and the miR11487 family has so far only been identified in *Picea*. In angiosperms, miR160 targets the class-C clade of ARF which include *ARF10* and *ARF16/17* (Mallory et al., 2005; Gutierrez et al., 2012). miR172 and the predicted mRNA target, *APETELA2-like* transcription factor *PaAP2L3* (Nilsson et al., 2007), were the only miRNA–mRNA target pair that had expression patterns expected of a transcript regulated by a miRNA (a concurrent upregulation of miR172 and the respective downregulation of *PaAP2L3*) (Supplementary Table S3). In angiosperms miR168 triggers phasiRNA production through *ARGONAUTE1* (*AGO1*) dependent activity (Gyula et al., 2018), but this has not yet been confirmed in spruce (Xia et al., 2015). Here we identified *NBS-LRRs* as potential targets of miR168 (Supplementary Figs. S1). Family miR1311 has been identified as a trigger of phasiRNA from *NBS-LRRs* in spruce (Xia et al., 2015). Interestingly, the miR11487b and miR11487c loci had the highest $\log_2$-fold changes in expression in MeJA treated bark tissues (MI and MW) compared to the control tissues (CW and CI) (Supplementary Table S5). Predicted miR11487 mRNA targets were annotated as possessing the epigenetic modification reader domain PWWP or the Putative S-adenosyl-L-methionine-dependent methyltransferase domain, which can be found in DNA methyltransferases.

3.2.4. miRNAs Found Across All Three Clusters

Families miR3701, miR482, and miR950 were found in all three clusters. Families miR3701 and miR950 are specific to conifers, while the miR482/miR2118 super-family is found in most plants excepts for ferns and monocots (Wan et al., 2012; Chávez Montes et al., 2014; Xia et al., 2015). The predicted targets of these miRNA families all comprise *NBS-LRRs* (Supplementary Table S3). Additionally, all of these miRNA families have been identified as triggers of phasiRNA production from *NBS-LRR* loci (Xia et al., 2015).

Lastly the miR393 family is a highly conserved miRNA involved in a variety of processes including host damage-associated molecular pattern mediated recognition (Huang et al., 2016). miR393 is commonly upregulated during pathogen and herbivore attack, or grafting (Bozorov et al., 2012; Pagliarani et al., 2017). This miRNA family is known to target transcripts of the auxin receptors TRANSPORT INHIBITOR RESISTANT1/AUXIN SIGNALING F-BOX (TIR1/AFBs; Si-Ammour et al., 2011; Bozorov et al., 2012), as well as to trigger secondary phasiRNA production in Norway spruce (Xia et al., 2015). Surprisingly we found the expression of all four members of the miR393 family to be absent in bark tissues (Supplementary Table S2).

4. Discussion

sRNAs play an essential role in controlling transcriptional and post-transcriptional silencing of genes affecting plant development, cell communication and signaling. In our analyses, we observed that bark treatment with MeJA altered the miRNA transcriptome both directly and by changing how the miRNA transcriptome responded to a subsequent wounding challenge. The most pronounced effect of MeJA treatment was a prolonged up- or downregulation of miRNAs expression. A subset of miRNAs were also identified as having the characteristics of a primed expression response after wounding in pretreated MeJA bark. However, we did not find an overall strong relationship between changes in the expression of specific miRNAs and that of their predicted mRNA targets. The general lack of correspondence could be due to the fact that while miRNAs can guide the cleavage of mRNAs they can also inhibit the translation of their mRNAs targets, meaning that a corresponding target mRNA reduction would not be observed in the transcriptome. Alternatively, miRNAs may fine tune the expression of mRNAs within specific MeJA-induced structures, like developing traumatic resin ducts in the cambium, which could form a small portion of the total bark sample from which we extracted RNA (Krokene et al., 2008; Celedon et al., 2017). Either way, further analysis of *AGO*-associated miRNAs and target protein expression, or an in situ hybridization of miRNA targets, would be required to confirm these hypotheses.

4.1. Defense Signaling

While there was minimal correlation between altered miRNA expression and that of their predicted mRNA targets, Pfam enrichment analysis of the predicted miRNA targets did show that the defense gene-associated NB-ARC domain was enriched among mRNA targets in all expression clusters (Fig. 5B). The NB-ARC domain, otherwise known as the nucleotide-binding site (NBS) domain, is one of the two core domains in NBS-LRR proteins which are well known to participate in pathogen sensing and plant defenses (McHale et al., 2006; Couto and Zipfel, 2016; Jones et al., 2016). Because overexpression of NBS-LRRs results in programmed cell death, it is costly and NBS-LRRs must therefore be tightly regulated (Wang et al., 2016; Zhang et al., 2016). Studies of the white spruce (*Picea glauca*) and Norway spruce genomes show that they contain over 500 highly diverse NBS-LRR genes representing 1.35% of all gene transcripts (Zhang et al., 2016; Van Ghelder et al., 2019). miRNAs can be effective regulators of NBS-LRR expression, as a small number of miRNAs can regulate substantial numbers of NBS-LRRs. Thus few evolutionary events are needed to produce an miRNA that can regulate many targets (Zhang et al., 2016).

Almost 800 phasiRNA-producing loci (PHAS loci) have been identified in spruce with sequence similarity to *NBS-LRR* genes (Xia et al., 2015). Complementary to these loci, spruce also has a large miR482/miR2118 superfamily with well-known triggers of *NBS-LRR* phasiRNAs, as well as 18 other miRNAs that target *NBS-LRRs* for phasiRNA production (Xia et al., 2015). In our study, we found miR482 loci plus other

miRNA triggers of *NBS-LRR* phasiRNA production in all three expression clusters. Given the diversity of NBS-LRR structure and function, finding *NBS-LRR* targeting miRNAs in all three expression clusters is perhaps not so unexpected (Lolle et al., 2020). Much more research is required to understand the diverse roles of NBS-LRRs in spruce defense and how *NBS-LRR*s are targeted and regulated by miRNAs.

4.2. Regulation of the Jasmonic Acid Pathway

The jasmonic acid (JA) pathway is an important signaling pathway that controls plant defenses which are effective against herbivores and necrotrophic pathogens (Wasternack and Song, 2016). Several miRNAs that are known to target regulators of the JA pathway were found to be differentially expressed in our analysis. miR167 and miR319, which target the JA biosynthesis regulators *ARF6/8* and *TCP2/4/10* respectively, were prolonged downregulated after MeJA treatment. In *Arabidopsis*, *arf6 arf8* double mutants produce only a third as much JA as wild type plants (Nagpal et al., 2005; Oh et al., 2008; Tabata et al., 2010). Similarly, the downregulation of *TCP* reduces JA levels in *Arabidopsis* and, in tomato, the miR319-TCP4/LA network appears to play a significant role in modulating JA biosynthesis during defense responses to root-knot nematode invasion (Schommer et al., 2008; Fujimoto et al., 2011; Zhao et al., 2015). We also found that miR160, which targets *ARF17* in *Arabidopsis*, a negative regulator of JA accumulation (Mallory et al., 2005; Gutierrez et al., 2012), was prolonged upregulated in bark tissue after MeJA treatment. If these regulators have similar functions in spruce as in *Arabidopsis* and tomato, JA levels should be increased after MeJA treatment. However, we did not find corresponding changes in expression levels of *ARF*s that are known targets of miR167, miR319 or miR160. Additionally, in our previous work on the same bark samples we found JA levels to be significantly increased in MeJA-treated bark after wounding, but not in intact MeJA-treated bark (Mageroy et al., 2020b). Thus, it remains unclear how miRNAs regulate JA biosynthesis in Norway spruce.

Another differentially expressed miRNA in our study was the highly conserved miR172, which is known to target *AP2-like* transcription factors in other plants (Nilsson et al., 2007; Chávez Montes et al., 2014; Chorostecki et al., 2017; Zhang and Chen, 2021). Our analysis also identified *PaAP2L3* as a co-regulated target of miR172. The AP2/ERF (ethylene response factor) superfamily has regulatory roles in many plant processes including growth and development, fruit ripening, hormone signaling, and defense responses (Gu et al., 2017). In *Arabidopsis*, miR172c forms an auto-regulatory loop with AP2, coordinated by a conserved heterodimeric protein module containing FRUITFUL (FUL) and ARF8 proteins (Gasser, 2015; José Ripoll et al., 2015). In our study, miR172 expression was prolonged upregulated after MeJA treatment, while its *AP2-like* target was downregulated only in MeJA-treated intact bark. In barley and *Arabidopsis*, miR172 modulates *AP2* expression by translational repression (Chen, 2004; Zhu and Helliwell, 2011; Anwar et al., 2018), and also could explain why an increase in miR172 does not always result in a synchronized decrease in the *AP2* mRNA target in our study. However, it is also likely that Norway spruce has other unknown miR172 loci that regulate *AP2-like* transcription factors as the current genomic reference is highly fragmented. Only one genomic miR172 locus is currently identified in Norway spruce (Xia et al., 2015), and based on comparison with other gymnosperms at least 2–3 miR172 family members are expected to be present (unpublished data). Recent publications support a role for miR172 in angiosperms plant defense (Gu et al., 2017; Wang and Galili, 2019). The role of miR172 and *AP2* in regulating reproductive development and meristem function in Norway spruce has already been described (Nilsson et al., 2007). However, the role of miR172 loci in spruce defenses is largely unexplored. To better understand if and how miR172, miR167, miR319, and miR160 regulate JA-mediated defense in Norway spruce, further analysis of the JA biosynthesis pathway, JA signaling and perception is required.

4.3. PhasiRNA Regulation of RNA-Dependent DNA Methylation

In this study, several phasiRNA trigger miRNAs were differentially expressed. The function of many phasiRNAs is still poorly understood in plants (Liu et al., 2020). Nevertheless, it is known that 21–22 nt secondary siRNAs, which may or may not be phased, can play a crucial role in non-canonical RdDM pathways (Cuerda-Gil and Slotkin, 2016). Xia et al. (2015) found that the phasiRNA network is greatly expanded in spruce. They suggest that the expanded miRNA and downstream phasiRNA pathways may be important in regulating transposable elements (TEs) in Norway spruce and thus compensate for the low abundance of 24-nt sRNAs in this species (Xia et al., 2015). Potentially 21 nt phasiRNAs could play a role in directing the activity of *de novo* DNA methyltransferases in spruce and thus in turn control transcriptional silencing of TEs. Given the increasing evidence for the regulation of DNA methylation at TEs which are linked to defense priming and IR (Wilkinson et al., 2019), this topic requires further research in spruce.

5. Conclusions

While we are beginning to understand the breadth of the mechanisms by which sRNAs, including miRNAs, regulate plant immunity in model angiosperms, our understanding in gymnosperms is very rudimentary. The data we present in this study indicate that several miRNAs, including members of miR167, miR172, miR319 and miR485/2118 families, have a role in the establishment and maintenance of MeJA-IR in Norway spruce. Further exploration of how miRNAs may regulate the defenses underpinning MeJA-IR in spruce could use single cell and/or tissue-specific transcriptomics (e.g. Celedon et al., 2017) as well as transgenic seedlings expressing short tandem target mimic (STTM) RNAs which inactivate specific miRNA families, as recently demonstrated in tomato (Canto-Pastor et al., 2019).

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Author contributions

M.H.M, A.V-S. and P.K. conceptualized the study. P.K. and M.H.M acquired the funding. M.H.M, A.V-S, and S.W.W analyzed the data. M.H.M wrote the original draft. M.H.M, P.K., A.V-S, and S.W.W reviewed and edited the manuscript.

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Data availability statement

Raw sRNA read data are found at NCBI with the BioProject PRJNA564212 and Biosamples: SAMN12687882, SAMN12687883, SAMN12687884, SAMN12687885.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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