Establishment of long-term methyl jasmonate-induced resistance in Norway spruce

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SWW, JT, PK and MHM proposed the original idea for the research. SWW conducted experiments and gathered data with assistance from MHM, TOS, and LSD. SWW analyzed the data with assistance from MHM. SWW and MHM wrote the manuscript with input from all authors. MHM, PK and JT provided funding for the research.

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Abstract

Norway spruce (*Picea abies*) is an economically and ecologically important tree species that grows across northern and central Europe. Treating Norway spruce with jasmonate has long-lasting beneficial effects on tree resistance to damaging pests, such as the European spruce bark beetle *Ips typographus* and its fungal associates. The (epi)genetic mechanisms involved in this long-lasting jasmonate-induced resistance (IR) have gained much recent interest but remain largely unknown. In this study, we treated 2-year-old spruce seedlings with methyl jasmonate (MeJA) and challenged them with the *I. typographus* vectored necrotrophic fungus *Grosmannia penicillata*. MeJA treatment reduced the extent of necrotic lesions in the bark and thus elicited IR against the fungus. The transcriptional response of spruce bark to MeJA treatment was analyzed over a 4-week time course using mRNA-seq. This analysis provided evidence that MeJA treatment induced a transient upregulation of jasmonic acid, salicylic acid and ethylene biosynthesis and downstream signaling genes. Additionally, genes encoding components of the RNA-directed DNA methylation pathway showed long-term repression, suggesting a possible role of DNA demethylation in the maintenance of MeJA-IR. These results provide new clues about the potential mechanisms underpinning long-term MeJA-IR in Norway spruce.

Introduction

Norway spruce (*Picea abies*) is an economically and ecologically important tree species that dominates Europe's boreal and sub-alpine coniferous forests (Caudullo et al., 2016). However, across much of its range, Norway spruce is threatened by the tree-killing European spruce bark beetle, *Ips typographus* (Biedermann et al., 2019). The exhaustion of tree defences, and ultimately the success of bark beetle attacks, is thought to be aided by infection of the tree with necrotrophic ophiostomatoid fungi carried by the beetle (Krokene, 2015; Zhao et al., 2019). Common fungal phytopathogens associated with *I. typographus* include *Grosmannia penicillata*, *Endoconidiophora polonica* and *Ophiostoma bicolor* (Linnakoski et al., 2010; Tanin et al., 2021).

Norway spruce and other members of the Pinaceae family have a comprehensive suite of defences to protect themselves against potentially deadly attackers. In addition to constitutive defences, such as lignified sclerenchyma cells and intracellular deposits of calcium oxalate crystals, these conifers have a range of inducible defences (Franceschi et al., 2005; Krokene, 2015). These include production of traumatic resin ducts (TRDs) filled with a terpene-rich oleoresin and activation of polyphenolic parenchyma cells rich in phenolic compounds such as stilbenes and flavonoids (Celedon and Bohlmann, 2019; Hammerbacher et al., 2020). Aside from enhanced production of terpenes and phenolics, induction of a hypersensitive cell death response and mobilization of pathogenesis-related (PR) proteins are also thought to provide resistance against biotic stress in conifers (Franceschi et al., 2005). Despite this extensive repertoire of inducible defences, *I. typographus* and its ophiostomatoid fungal associates continue to decimate Norway spruce plantations across central and eastern Europe (Hlásny et al., 2021). Thus, there is an urgent need to understand the defense mechanisms of Norway spruce in more detail and develop novel pest and disease strategies to protect this important tree species.

Exposure of plants to specific environmental stimuli, such as localized pathogen infection, beneficial microbes or specific chemicals, can make them more resistant to subsequent attack (Pieterse et al., 2014; Mauch-Mani et al., 2017). This phenomenon is known as induced resistance (IR) and provides a feasible route by which particularly young trees in a nursery setting could be protected against pests and diseases. Through direct induction of plant

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defences IR can provide immediate, and usually short-term, protection that lasts for days or a few weeks. More long-term plant protection by IR may occur through two non-mutually exclusive mechanisms: prolonged upregulation of inducible defences and priming of inducible defences (Conrath et al., 2015; Mauch-Mani et al., 2017; Wilkinson et al., 2019; De Kesel et al., 2021). Prolonged upregulation of inducible defences is where an IR eliciting stimulus (e.g. insect attack) induces defences and they remain upregulated for weeks or months. Whereas with priming of inducible defences, defences maybe transiently activated following an IR eliciting stimulus, but return to basal levels until a subsequent triggering stress upon which they activated faster and/or stronger. Although long-term IR may be underpinned by either prolonged upregulation or priming of inducible defences, enhanced plant resistance is often explained by a combination of the two mechanisms (Mageroy et al., 2020b). Considerable research on the mechanisms involved in IR has been conducted in model angiosperms. For example, increasing evidence suggests that epigenetic mechanisms are involved in the maintenance of particularly longer-lasting IR, which is often underpinned by a priming of inducible defences (Wilkinson et al., 2019; Parker et al., 2021). Despite the increasing knowledge about model angiosperms, our understanding of the mechanisms of IR in important tree species such as Norway spruce remains limited.

Application of jasmonic acid (JA), or its methyl ester methyl jasmonate (MeJA), can induce resistance against either *I. typographus* or its fungal associates in seedlings and mature Norway spruce (Kozlowski et al., 1999; Erbilgin et al., 2006; Zeneli et al., 2006; Krokene et al., 2008; Mageroy et al., 2020a). This MeJA-IR is linked to both direct and prolonged induction of defences, such as the formation of TRDs and new polyphenolic cells (Krokene et al., 2008; Celedon and Bohlmann, 2019). In addition, MeJA-IR in Norway spruce has also been associated with a priming of inducible defences. Zhao et al (2011a) demonstrated that MeJA treatment alone induces a minor increase in terpene levels in the bark compared to the massive terpene accumulation that occurred following wounding of bark treated with MeJA 4 weeks previously. This accumulation does not appear to be the result of *de novo* production, as enzymes involved in terpene biosynthesis are generally not primed at a transcriptional level (Mageroy et al., 2020b). Contrarily, MeJA treatment likely primed a swathe of *PR* genes to respond faster and stronger to wounding in mature spruce trees (Mageroy et al., 2020b).

Despite these recent findings, much is still unknown about the molecular mechanisms underpinning the establishment and maintenance of MeJA-IR in Norway spruce. In this study, we use a detailed mRNA-seq analysis with multiple time points to obtain a more complete understanding of the transcriptional changes following MeJA treatment and to identify changes in gene expression that contribute to long-term MeJA-IR. Additionally, we evaluate costs associated with MeJA-IR by quantifying growth and resistance of MeJA-treated spruce plants.

Methods

Plant Materials, Growth Conditions, and MeJA Treatment for Pathogen Bioassay and mRNA-seq

In May 2018, 2-year-old Norway spruce seedlings grown in multipot containers (50 cm^3 pots with 95 minipots per container, 791 seedlings m^{-2}) were purchased from the nursery Norgesplanter AS. The seedlings, which had overwintered at 7 °C with the root plugs wrapped in clingfilm, were transferred to 0.8 L pots (7.5 cm × 7.5 cm × 12 cm; Nelson Garden, product no. 5726) containing compost supplemented with mineral fertilizer (Plantasjen, EAN:7058782362802). Seedlings were grown outdoors with an irrigation system providing additional water when required. We performed all manipulations and experimentation from the end of July 2018 onwards, when yearly height and shoot growth had been completed.

On 31 July 2018, half of the spruce seedlings were sprayed with a 10 mM MeJA solution while the remaining half received a 0 mM MeJA control solution. The MeJA solution consisted of MeJA (Sigma-Aldrich, 392707) dissolved in tap water and supplemented with 0.1% Tween 20 (Sigma-Aldrich, P9416) to ensure even coating across all sprayed tissues. The control solution was identical except that it did not contain any MeJA. Using a 1.5 L pressurized spray bottle (Bürkle GmbH, 0309-0100), each plant was sprayed with a similar volume of solution, which was enough to saturate the entire stem surface. Following spraying, plants of the two pre-treatments were kept separate for at least 4 hours (hrs) to allow excess solution to evaporate before putting the plants back into close proximity to one another.

Necrotrophic Pathogen (Grosmannia penicillata) Bioassay

Grosmannia penicillata isolate 1980-91/54 (collected: 1980, Akershus (Ås), Norway) from the Norwegian Institute of Bioeconomy Research (NIBIO) fungal culture collection, was revived from a -150 °C culture stock and propagated on malt agar (2% malt extract and 1% agar). Seedlings were inoculated ~4 weeks (29 August 2018) after treatment with water or MeJA (Figure 1). 15 control and 15 MeJA treated plants were inoculated with *G. penicillata* and 15 plants were mock inoculated with sterile malt agar. The inoculation procedure was identical for all plants regardless of whether they were inoculated with a fungal or sterile agar inoculum. First, a wound, ~1 cm in length and half the circumference of the stem, was cut in the middle of the first internode by slicing open outer layers of bark with a scalpel. The bark tissue was not completely detached from the stem, and a ~5 mm^3 droplet of inoculum was placed behind the bark flap using a 5 ml needle-less syringe. To seal the inoculum in place behind the bark flap and to prevent contamination and/or drying out of the wound, Parafilm was wrapped around the stem at the inoculation site. Inoculum was created by homogenizing a batch of malt agar, with or without fungus. Inoculated plants were arranged into eight blocks. Seven blocks had two plants of each combination of treatment (water control or MeJA) and inoculation type (mock or fungal), while one block only had one plant of each combination. The position of plants was randomized in each block. Approximately 8 weeks after inoculation (25 October 2018), plant resistance was assessed by measuring the lesion length in the inner bark above and below each inoculation site. Parafilm and bark flap were removed, and the inner bark was exposed by removing the outer layers of bark using a scalpel. In both mock and fungal inoculated plants, a distinct area of darkened, necrotic tissue was visible around the wound site (Figure 2a). The axial length of each lesion was measured using calipers (Digital Vernier Caliper, Cocraft).

Pathogen Bioassay Statistical Analysis

A three-factor ANOVA was performed to assess the effect of treatment and inoculation on lesion length, using R v3.6.1 (R Core Team, 2019). To ensure the data conformed to ANOVA's normality assumption, lesion lengths were log-transformed. The ANOVA included treatment, inoculation, and block as fixed effects as well as the interaction between treatment and inoculation. Due to an imbalance in the number of replicates per block and a significant effect of the treatment × inoculation interaction, type III sum of squares were used. A Tukey post-

hoc test with a statistical significance threshold of $p < 0.05$ was used to evaluate whether mean lesion lengths differed significantly between treatment and inoculation combinations.

Bark Harvesting and RNA Extraction

Bark was harvested 3 hrs, 6 hrs, 24 hrs, 72 hrs, 1 wk and 4 wks after treatment from the first stem internode of four seedlings per treatment (water and MeJA). Each seedling was treated as a separate sample, therefore each treatment (e.g. MeJA treated) had four replicates for each harvest time. Immediately following harvesting the bark samples were flash frozen in liquid nitrogen and then stored at -80 °C. Using a pestle and mortar, all bark samples were later ground to a powder. For the samples collected 4 wks post-treatment only half of the harvested bark was ground to a powder.

Total RNA was extracted from 30-50 mg of bark powder per sample using the MasterPure Complete DNA and RNA Purification Kit (Lucigen, MC85200). To denature ribonucleases and reduce polyphenolic contamination of extracted nucleic acids, 0.5% β-mercaptoethanol (Sigma-Aldrich, M3148) and 1% polyvinylpyrrolidone (PVP; Sigma-Aldrich, P-5288) were added to the extraction buffer. To reduce carbohydrate contamination, nucleic acids were precipitated using 0.5 volumes of 7.5 M lithium chloride precipitation solution (ThermoFisher Scientific, AM9480), an incubation step at -20 °C for 2 hrs, and centrifugation for 30 minutes at $16500 \times g$ and 4 °C. Nucleic acids were resuspended in 60 µl of nuclease-free water.

mRNA Library Preparation and Sequencing

Preliminary RT-qPCR analysis (Supplemental Figure 1) suggested that the 3 hrs, 24 hrs, 1 wk and 4 wks time points were enough to capture the key trends in the transcriptome data. Therefore, the RNA samples from these time points were selected for global transcriptome analysis. Prior to sequencing, quantity and quality of RNA was assessed using a Nanodrop and Agilent 2100 Bioanalyzer. All samples had an RNA integrity number (RIN) value ≥ 8.5. Library preparation and sequencing of all RNA samples was performed by BGI Tech Solutions (Tai Po, Hong Kong). Across the 32 samples, 1.2 billion 150 bp paired-end (PE) clean reads were generated in total, with the minimum, maximum and mean number of read pairs per sample being 27.9, 38.1 and 36.3 million, respectively (Supplemental Data Set 1). BGI removed adapter sequences, contamination and low-quality reads. They reported that for all samples, ≥ 96% of nucleotides had a Phred quality score of > 20. To confirm that BGI's read filtering had

been comprehensive and that the sequencing quality was sufficient for mRNA-seq analysis, FastQC v0.11.8 (Andrews, 2010) was used to analyze the FASTQ files provided by BGI. Per-sample FastQC reports were grouped into a single report using MultiQC v1.7 (Ewels et al., 2016). The only issues flagged by FASTQC were a higher than expected level of read duplication and the first 10-15 positions of each read having an uneven proportion of the four bases and showing a K-mer bias. Both issues are common for mRNA-seq datasets and are simply artefacts of highly expressed transcripts and the use of random hexamer primers for cDNA synthesis, respectively.

Read Alignment and Counting

The Bowtie 2 package v2.3.1 (Langmead and Salzberg, 2012) was used to align reads to the curated Norway spruce reference transcriptome described in (Mageroy et al., 2020b). First, the transcriptome was indexed using the bowtie2-build function, and then reads were aligned using the bowtie2 function run with the parameter settings: '--very-sensitive' '-q' '-k 10'. With option k being set to 10, up to 10 valid alignments were reported per read pair rather than the default single best alignment. This approach allowed multi-mapping reads to be recognized and removed. On average, 22.5% (8.2 million) of the raw read pairs in each sample remained following alignment and subsequent multi-mapper removal (Supplemental Data Set 1). The number of uniquely aligned fragments mapping to each gene were counted using the featureCounts function from the Rsubread R package v2.0.1 (Liao et al., 2014, 2019). The following options were specified in the featureCounts function: 'isGTFAnnotationFile = TRUE', 'isPairedEnd = TRUE'. The Gene Transfer Format (GTF) reference transcriptome annotation file was created using AUGUSTUS run with the parameter settings: '--strand=both', '--genemodel=partial', '- -species=arabidopsis' (Stanke et al., 2004).

Global Pattern Assessment and Differential Expression Analysis

To assess the global patterns in the data, count tables created by featureCounts were loaded into R and all genes with < 100 total read counts across all samples were removed. Remaining read counts were transformed with a variance stabilizing transformation (vst) (Anders and Huber, 2010) using the DESeq2 vst function run with the following option settings: 'blind=TRUE', 'nsub=1000', 'fitType = "parametric"'. Transformed count data was used for a principle component analysis (PCA), a hierarchical clustering analysis (HCA) and heatmap

analysis of sample-to-sample distances. The PCA was performed using the DESeq2 plotPCA function run with the following options: 'intgroup=c("Treatment","Time")', 'ntop = all genes ≥ 100 read counts', 'returnData = TRUE'. The outcome of the PCA was displayed using the R package ggplot2 v3.2.1. The R package pheatmap v1.0.12 (Kolde, 2019) was used to cluster samples and create heatmaps displaying sample-to-sample distances. Samples were clustered using the complete-linkage method and the euclidean distances between samples.

Differential expression analysis was conducted in R using the package DESeq2 v1.24.0 (Love et al., 2014). Genes showing a significantly altered expression profile across time as a result of MeJA treatment were identified using the DEseq function run with the following parameter settings: 'test="LRT"', 'full = ~ time + treatment:time', 'reduced = ~ time'. Genes with an adjusted p-value < 0.001 were selected from the results table created by the DESeq2 results function run with the parameter settings: 'alpha = 0.001', 'cooksCutoff = T', 'lfcThreshold = 0' (Benjamini and Hochberg, 1995).

Hierarchical Clustering and Expression Pattern Visualization

Genes were grouped by expression pattern using agglomerative hierarchical clustering. A correlation-based distance measure was used to calculate between-gene dissimilarity based on counts transformed using the DESeq2 vst function run with the argument values: 'blind=FALSE', 'nsub=1000', 'fitType = "parametric"'. A dendrogram was created from the dissimilarity data using Ward's minimum variance clustering method. For visualization, the dendrogram was displayed together with the heatmap, with each row in the heatmap representing a separate gene with transformed counts displayed as Z-scores. All aforementioned steps were conducted using the aheatmap function from the NMF R package v0.21.0 (Gaujoux and Seoighe, 2010). All bar charts and line plots showing numbers of differentially expressed genes and gene expression profiles, respectively, were created using the R package ggplot2. Expression profile plots used the same transformed counts that were used for the hierarchical clustering.

Protein Signature Enrichment Analysis

Predicted protein sequences for all genes with ≥ 100 total read counts across all samples were annotated with protein signatures (e.g. protein domain) using the Pfam database v32.0 (El-Gebali et al., 2019) and hmmscan from the HMMER package v3.2.1 (Wheeler and Eddy, 2013).

These annotated genes provided the background distribution of Pfam protein signatures for enrichment analysis, which was conducted using the enrichment function from the bc3net R package v1.0 (de Matos Simoes and Emmert-Streib, 2016). For a Pfam protein signature to be classified as enriched in a candidate protein list, it had to have an adjusted p-value ≤ 0.05 (Benjamini and Hochberg, 1995). Fold-enrichment plots displaying significantly enriched protein signatures were created using ggplot2.

Gene Ontology (GO) Enrichment Analysis

Enrichment analysis of biological function GO (Gene Ontology) terms was conducted using the enrichment tool of ConGenIE (http://congenie.org/), the online host of information and data relating to the Norway spruce genome project (Nystedt et al., 2013; Sundell et al., 2015). The background for the analysis consisted of all genes in the spruce genome assigned at least one biological function GO term. For a GO term to be reported it had to be assigned to one or more transcript in a cluster and it had to have a false discovery rate (FDR) adjusted p-value < 0.05.

Identification of Differentially Expressed Genes Related to Defence or Epigenetics

Differentially expressed genes were annotated using predicted amino acid sequences, blastp v2.8.1, (Altschul et al., 1990, 1997; Camacho et al., 2009) and the Swiss-Prot database (Bateman, 2019). Genes predicted to encode for hormone biosynthesis enzymes, defence regulators, defence metabolite biosynthesis enzymes or pathogenesis-related (PR) proteins were identified by searching the blastp outputs for key terms (e.g. "WRKY", " Pathogenesis-related" and "anthocyanidin reductase"). Differentially expressed genes predicted to encode for epigenetic regulators were identified using the lists of epigenetic regulator genes provided in Mageroy et al (2020b).

Plant Materials, Growth Conditions, and Treatments for Chlorophyll Fluorescence Measurements

Thirty containerized 2-year-old Norway spruce seedlings from a single full-sib family were potted in 8 cm pots on 5-6 June 2001 and placed in a greenhouse. When all plants had broken bud and started shoot elongation (21 June), they were treated with 0, 5, 25, 50 or 100 mM MeJA in water (n = 6 plants per treatment). All solutions were supplemented with 0.1% Tween 20. Chlorophyll fluorescence was measured on three occasions (1 day, 6 days, 8 wks after MeJA treatment) using a PAM-2000 fluorometer (Walz, Effeltrich, Germany) employing the

saturation pulse method. Needles were dark-adapted for at least 45 min prior to fluorescence measurements and the actinic photosynthetic photon flux density used was 250 μmol m^{-2} s^{-1}. Maximal PSII fluorescence (Fv/Fm) was measured after 3.5 hrs in darkness using a white saturating pulse (750 μmol m^{-2} s^{-1}) lasting 0.8 seconds. The distance to needles was adjusted and fixed before fluorescence measurements were made to avoid either signal overload or weak signals during data collection. Chlorophyll fluorescence data was analyzed with a one-way ANOVA and a Tukey's HSD test. Statistics were performed in R v3.5.1 (R Core Team, 2019).

Plant Materials, Growth Conditions, and Treatments for Growth Measurements and Anatomical Analysis

In May 2017, 2-year-old Norway spruce seedlings grown in multipot containers (75 cm^3 pots with 60 minipots per container, 500 seedlings m^{-2}) were purchased from the nursery Telemark Skogplanter AS. Seedlings had previously been treated with the insecticide Merit Forest (active ingredient: imidacloprid) in the nursery. The seedlings, which had overwintered at 7 °C with the root plugs wrapped in clingfilm, were transferred to single pots (9 cm × 9 cm × 10 cm; Nelson Garden, Product No. 5726) containing compost supplemented with mineral fertilizer (Hagebutikken, Product: 30632748). Seedlings were placed on 12 watering trays with 15 pots per tray. The trays were placed in a growth room with constant temperature and humidity (20 °C, 60% relative humidity) and 20 hours light. Each plant was watered with 100-200 mL of water three times per week and trays were rotated within the growth room to ensure equal lighting for the whole research period. On 31 May 2017, the seedlings were sprayed with ~30 mL of 0 or 10 mM MeJA solutions. The 10 mM MeJA solution consisted of MeJA dissolved in tap water and supplemented with 0.1% Tween 20 to ensure even coating across all sprayed tissues. The control solution was identical except that it did not contain any MeJA.

Growth Measurements

Seedling growth was assessed by measuring plant height twice: on the day of MeJA treatment (31 May 2017) and 5 ½ wks later (9 July 2017). Plants were harvested for shoot, root, and stem analyses ~10 wks after MeJA treatment (10 August 2017). The 10 uppermost current-year lateral shoots were cut off, placed in paper bags, and stored at room temperature for up to 5 months. Dead shoots without needles were not harvested. Washed roots were placed in a water bath and scanned using an Epson Expression 11000XL (Epson America Inc.). Root

lengths and diameters were analyzed from root images using WinRhizo v2013a (Régent Instruments Inc., Canada). Root lengths were binned into the following diameter classes: <0.39 mm; 0.4-0.79 mm; 0.8-1.19 mm; 1.2-1.59 mm; 1.6-1.99 mm; ≥2 mm. Roots were then placed in paper bags. To determine tissue dry weight, lateral shoots and roots in paper bags, dried in a drying cabinet at 65 °C for 24 hrs and weighed with a Mettler-Toledo PB602 balance (accuracy to 0.01 g; Mettler-Toledo GmbH, Switzerland). For growth measurements, two-sided t-tests were performed. Root length data was normalized by log transformation and p-values were adjusted to account for multiple testing using the Benjamini and Hochberg correction. Statistics were performed in R v3.5.1 (R Core Team, 2019).

Anatomical analysis of stems

A stem section was taken from the lowest 1 cm of the stem of each seedling for microscopy. Stem cross-sections were made using razor blades to cut the thinnest possible section. Sections were placed on glass slides and analyzed using a light microscope (Leica 020-525.732, Leica Microsystems Wetzlar GmbH, Germany) equipped with the Leica Application Suite software v4.0 (Leica Microsystems Limited, Switzerland). A 90 degree sector of each cross-section was magnified 2.5× and inside this sector the following anatomical parameters were measured: the proportion of TRDs across the tangential width of the 2017 annual sapwood growth, the radial thickness of the 2017 annual sapwood growth, and the radial thickness of the secondary phloem (the organized part of the inner bark). These anatomical parameters are illustrated in Supplemental Figure 2. Two-sided t-tests were performed to assess the effect of MeJA treatment on anatomical parameters. Statistics were performed in R v3.5.1 (R Core Team, 2019).

Results

MeJA Elicits IR to a Necrotrophic Pathogen in Spruce Seedlings

To confirm the protective effect of the MeJA treatment, control and 10 mM MeJA-treated spruce plants were challenged with the necrotrophic fungal pathogen *Grosmannia penicillata* or a mock agar control (Figure 1). *Grosmannia penicillata* was chosen for the experiment as it causes substantial necrosis when inoculated in the phloem of unprotected spruce trees (Zhao et al., 2019). Plants treated with MeJA generally exhibited shorter necrotic lesions in the phloem than the water controls 8 weeks after inoculation (Figure 2). In fact, lesion lengths in

plants treated with MeJA before fungal inoculation were similar to those of the mock inoculated control plants (Figure 2). These results provide evidence of MeJA-IR and confirms the suitability of the experimental setup we used to investigate the mechanisms behind MeJA-IR in spruce.

Profiling the Transcriptional Response to MeJA treatment

To assess the transcriptional response to the resistance-inducing MeJA treatment, mRNA-seq was performed on bark tissue harvested 3 hrs, 24 hrs, 1 wk and 4 wks after treatment with water or MeJA (Figure 1). Both a principal component analysis (PCA) and a hierarchical clustering analysis (HCA) were performed to confirm that replicates behaved consistently and to check the global patterns in the data. Both analysis methods suggested that MeJA treatment induced a transient shift in the bark transcriptome (Figure 3). Although the 3 hrs samples were somewhat similar between treatments, by 24 hrs after treatment plants treated with MeJA were distinct from the water controls. The separation between the two treatments was reduced at 1 wk, and 4 wks after treatment the MeJA samples were very similar to the water controls (Figure 3). This was evidenced by the 4 wks samples not clustering perfectly by treatment (Figure 3b). Apart from the 4 wks time-point, individual replicates of all other treatment groups clustered together, further confirming the quality of the dataset.

Having found that MeJA induced a transient shift in the global transcriptome (Figure 3), next we identified individual genes that showed a significantly (FDR-adjusted p-value (p.adj) < 0.001) altered expression profile across time after MeJA treatment. In total, 6330 differentially expressed genes were identified (Supplemental Data Set 2). Based on expression pattern, these genes grouped into thirteen clusters (Figure 4a). While each cluster had a subtly different expression profile, there were broadly four main patterns (Figure 4; Supplemental Data Set 2):

Pattern 1: genes transiently upregulated in response to MeJA treatment, before returning to a basal expression level (i.e., the expression level in the water controls; cluster 2, dark pink; cluster 5, purple), or a near basal expression level (cluster 1, yellow; cluster 4, orange; cluster 13, sandy brown).

Pattern 2: genes initially upregulated in response to MeJA treatment, then returning to a basal level before exhibiting slightly repressed expression long-term (cluster 3, dark green; cluster 9, turquoise).

Pattern 3: genes repressed in response to MeJA treatment, before returning to a basal (cluster 8, pale pink; cluster 12, brown) or near basal expression level (cluster 6, dark grey; cluster 7, fluorescent green; cluster 11, light blue) 4 wks after treatment.

Pattern 4: genes first repressed in response to MeJA treatment, before climbing back to and above basal expression levels (cluster 10, red).

To assess whether there were particular functional annotations associated with each of the expression patterns, we performed Protein family (Pfam) domain and Gene Ontology (GO) term enrichment analysis (Figure 4c; Supplemental Data Sets 3 and 4). The enriched Pfam domains and GO terms associated with the clusters displaying MeJA-induced repression (pattern 3) were generally related to processes involved in primary cell functioning and metabolism, such as cell division, the Krebs cycle, photosynthesis, and transcription. For example, cluster 7 was enriched for terms such as "DNA polymerase family B" (PF00136.20), "regulation of cell cycle phase transition" (GO:1901987), "DNA replication" (GO:0006260) and "gene silencing" (GO:0016458; Supplemental Data Sets 3 and 4). Furthermore, "Phosphoenolpyruvate carboxylase" (PF00311.16) and "photosystem II assembly" (GO:0010207) were enriched among the genes of cluster 8. Photosynthesis-related terms such as "photosynthesis, light harvesting" (GO:0009765) and "chlorophyll biosynthetic process" (GO:0015995) were also enriched for cluster 10 which long-term displayed MeJA-induced expression (pattern 4; Figure 4c; Supplemental Data Sets 3 and 4). These responses suggest that while photosynthesis maintenance and functioning may initially be repressed by MeJA, longer term it may be upregulated to higher than basal levels in compensation.

Another commonality between patterns 3 and 4 was that they both had an overrepresentation of genes involved in terpenoid biosynthesis (Supplemental Data Set 4). Interestingly, also the clusters 1 and 4, which were transiently upregulated after MeJA treatment (pattern 1), had enriched terms related to terpenoid production, such as "Terpene synthase family, metal binding domain" (PF03936.15) and "terpenoid biosynthetic process"

(GO:0016114), respectively (Supplemental Data Sets 3 and 4). Thus, the composition or quantity of terpenoids in bark tissue was likely modulated in response to MeJA treatment.

In addition to terpene biosynthesis, numerous other annotations related to 'response to biotic stress' were overrepresented in the pattern 1-clusters (Figure 4c; Supplemental Data Set 3 and 4). This was exemplified by clusters 1 and 2. Enriched GO terms associated with one or both clusters included "regulation of innate immune response" (GO:0045088), "regulation of defense response to fungus" (GO:1900150), "response to chitin" (GO:0010200), "regulation of plant-type hypersensitive response" (GO:0010363) and "response to wounding" (GO:0009611; Supplemental Data Set 4). Other enriched GO terms associated with the pattern 1-clusters included "salicylic acid-mediated signaling pathway" (GO:0009863), "jasmonic acid-mediated signaling pathway" (GO:0009867) and "jasmonic acid biosynthetic process" (GO:0009695; Supplemental Data Set 4). Additionally, clusters 1 and 2 contain genes encoding for JAZMONATE ZIM DOMAIN (JAZ) proteins, which are key regulators of the response to JA (Chini et al., 2016). This was evidenced by the "tify domain" (PF06200.13) and "Divergent CCT motif" (PF09425.9; renamed "Jas motif"), which were enriched in both clusters and are characteristic of JAZ proteins (Howe et al., 2018) (Figure 4c; Supplemental Data Set 3). Notably, these two pattern 1-clusters also contained ethylene (ET) and abscisic acid (ABA) responsive genes, as evidence by the enriched GO terms "response to ethylene stimulus" (GO:0009723) and "response to abscisic acid stimulus" (GO:0009737; Supplemental Data Set 4).

Biosynthesis of Defence Hormones in Response to MeJA treatment

Based on the results of the protein signature and GO term enrichment analysis and the known role of JA and SA in the regulation of defences against biotic stress, we performed a more detailed analysis of the response to MeJA of genes involved in JA and SA biosynthesis and downstream signaling genes. We searched the list of 6330 genes that were differentially expressed after MeJA treatment for genes annotated as encoding JA or SA biosynthesis enzymes, receptors or related transcriptional regulators. In other species JA is known to positively regulate the expression of its own biosynthesis genes (Wasternack and Song, 2016). It was therefore unsurprising that genes predicted to encode enzymes involved in all steps of the conversion of galactolipids to the bioactive form of JA, (+)-7-iso-jasmonoyl-L-isoleucine,

were generally upregulated following MeJA treatment (pattern 1-clusters; Figures 4 and 5; Supplemental Data Set 5).

Interestingly, considering the antagonism between JA and SA in some angiosperms (Thaler et al., 2012), genes predicted to encode enzymes involved in the biosynthesis of the precursors of SA, such a phenylalanine ammonia-lyase (PAL), were also predominantly found in the pattern 1-clusters (Figures 4 and 6; Supplemental Data Set 5). The same was true for the downstream signaling pathways. Homologs of master regulators that control SA- and JA-dependent defence responses in Arabidopsis were, generally, also found in the pattern 1-clusters (Figures 4 and 7; Supplemental Data Set 5).

Previous work in conifers has demonstrated that ET accumulates in response to MeJA treatment and in turn is an important signaling compound for the induction of defences by MeJA (Hudgins and Franceschi, 2004). Thus, we also explored in more detail the expression of ET biosynthesis genes. ET is synthesised from the amino acid methionine in a three-step process involving the enzymes S-adenosyl-L-methionine (SAM) synthetase, 1-aminocyclo-propane-1-carboxylic acid (ACC) synthase (ACS), and ACC oxidase (ACO) (Lin et al., 2009). All differentially expressed ET biosynthesis genes were in pattern 1-clusters (Figure 4; Supplemental Data Set 4). Two genes predicted to encode for SAM synthases were in cluster 1 and one ACO gene was in cluster 5 (Figure 4; Supplemental Data Set 5). Notably, no ACS genes were differentially expressed in response to MeJA treatment. Nevertheless, all together these results suggest that MeJA induces the biosynthesis of the defence hormones JA, SA and ET in spruce bark.

Spruce Defence Genes are Differentially Expressed in Response to MeJA treatment
The upregulation of genes involved in defence hormone biosynthesis and downstream signaling genes suggested that MeJA treatment likely directly induced spruce defences. Evidence in support of this conclusion is that genes involved in the biosynthesis of terpenes, the major component of oleoresin, were differentially expressed in response to MeJA treatment (Figure 4; Supplemental Data Sets 3 and 4).

MeJA can also induce an accumulation of phenolic compounds (Krokene et al., 2008). The general phenylpropanoid pathway may form a huge range of phenolic metabolites with

diverse functions (Deng and Lu, 2017). As mentioned above, MeJA treatment was found to transiently upregulate genes annotated to encode for PAL, which catalyzes the first step of the general phenylpropanoid pathway and contributes to biosynthesis of SA and defensive phenolics (Figure 6; Supplemental Data Set 5). To focus on phenylpropanoid pathway genes associated with MeJA-IR, we searched the list of differentially expressed genes for genes predicted to encode biosynthesis enzymes for defensive phenolics involved in spruce defence against ophiostomatoid fungal pathogens (Hammerbacher et al., 2011, 2013, 2018, 2019). Among the genes differentially expressed by MeJA treatment we identified genes predicted to encode chalcone synthase, chalcone isomerase, flavanone-3-hydroxylase, flavonol-3'-hydroxylase, flavonol-3'5'-hydroxylase, dihydroflavonol 4-reductase, anthocyanidin synthase, anthocyanidin reductase and leucoanthocyanidin reductase (Supplemental Data Set 2). Generally, these biosynthesis genes were upregulated in response to MeJA treatment (pattern 1-clusters; Figure 4; Supplemental Data Set 2). Notably, these genes are all involved in the production of flavonoids and none were annotated as encoding for stilbene synthases. Overall, the post treatment transcriptome analysis suggests that the biosynthesis of defence-related phenylpropanoids was upregulated by MeJA.

In addition to terpene- and phenolic-based defences, PR proteins are also thought to be an important component of spruce defence against biotic stress (Franceschi et al., 2005). Hypothetically, MeJA treatment could trigger an upregulation of *PR* genes and a long-term accumulation of PR proteins, which could provide enhanced resistance to subsequent attack. To assess this, we searched the list of differentially expressed genes for genes annotated as PR proteins. Interestingly, there was not a consensus pattern (Supplemental Figure 3) and genes predicted to encode for PR proteins were found in clusters belonging to all four expression patterns (Figure 4; Supplemental Data Set 5). Thus, although some PR proteins may accumulate in response to MeJA treatment, based on gene expression data alone, this is not the general pattern.

MeJA Treatment Induces Differential Expression of Epigenetic Regulators
Increasing evidence suggests that epigenetic mechanisms are involved in the immunological memory of plants and in maintenance of IR (Wilkinson et al., 2019; Parker et al., 2021). Interestingly, the GO term enrichment analysis suggested that many genes coding for

epigenetic regulators were differentially expressed following MeJA treatment. This was exemplified by the cluster 7 MeJA-repressed genes (Figure 4). Overrepresented GO terms in this cluster included "DNA methylation or demethylation" (GO:0044728), "chromatin silencing" (GO:0006342) and "histone modification" (GO:0016570; Supplemental Data Set 4).

To further investigate the specific epigenetic regulators that were differentially expressed following MeJA treatment we checked our list of 6330 differentially expressed genes against a list of epigenetic regulators in Norway spruce compiled by (Mageroy et al., 2020b). Multiple epigenetic regulators were differentially expressed in response to MeJA treatment (Figure 8a). These epigenetic regulator genes displayed several expression patterns and fell into all the thirteen clusters shown in Figure 4 (Supplemental Data Sets 2 and 6). MeJA induced a transient upregulation of most histone acetyltransferase genes. In contrast, regulators of DNA methylation were repressed following MeJA treatment (Figure 8b), in particular genes related to RNA-directed DNA methylation (RdDM; Figure 8b; Supplemental Data Set 6). These genes included DNA-directed RNA polymerase V subunit 1 (NRPE1), the largest subunit of Pol IV NUCLEAR RNA POLYMERASE D 1 (NRPD1), and RNA-DEPENDENT RNA POLYMERASE 2 (RDR2). Additionally, two of the differentially expressed DNA methyltransferases were predicted to encode for DNA (cytosine-5)-methyltransferase (DRMs; Figure 8b; Supplemental Data Set 6). In Arabidopsis, DRMs are guided by small RNAs (sRNAs), derived by Pol IV (or Pol II), to specific genome locations where they establish DNA methylation (Zhang et al., 2018). Over 60% of the differentially expressed RdDM-related genes we detected were in pattern 3-clusters, with the mean pattern suggesting that RdDM began to be repressed between 3 and 24 hrs after MeJA treatment (Figure 8b; Supplemental Data Set 6). One differentially expressed DNA demethylase (MA_111413g0010) was identified. This transcript was initially marginally upregulated before being downregulated to below basal expression levels (Figure 8b).

Evaluating the cost of methyl jasmonate treatment
Our transcriptome analysis showed that genes involved in primary cell functioning and metabolism were transiently downregulated after MeJA treatment. Thus, while MeJA treatment promotes resistance against necrotrophic pathogens, it may also incur some costs. To further evaluate potential costs associated with MeJA-IR, we determined the effects of MeJA treatment on above- and below-ground growth. MeJA treatment reduced apical leader

growth resulting in a reduction in shoot dry weight (Figure 9 a-b). Annual sapwood growth was also reduced by MeJA treatment, whereas the secondary phloem was enlarged, perhaps due to the swelling of polyphenolic cells (Figure 9 c-e)(Franceschi et al., 2002). In contrast, MeJA treatment had no effect below ground, with the weight and length of roots being no different between water and MeJA treated plants (Figure 9 f-g). We also measured the effects of a range of MeJA concentrations on chlorophyll fluorescence 1 day, 6 days, and 8 weeks after treatment. Only a high concentration of 100 mM MeJA induced a transient reduction in chlorophyll fluorescence (Supplemental Figure 4).

Discussion

The plant hormone MeJA has previously been demonstrated to elicit IR against pests and pathogens in both young and mature Norway spruce trees (Kozlowski et al., 1999; Erbilgin et al., 2006; Zeneli et al., 2006; Zhao et al., 2011b; Mageroy et al., 2020a; Puentes et al., 2021). In this study, we confirmed that MeJA elicits IR by demonstrating that MeJA treatment of 2-year-old seedlings increases resistance to infection by the bark beetle-vectored fungal pathogen *G. penicillata* four weeks later (Figure 2). Subsequently, we explored the molecular mechanisms behind the establishment and maintenance of MeJA-IR. This analysis provided an understanding of the general transcriptional response to MeJA in the bark tissue of Norway spruce seedlings. Furthermore, it suggested that there are costs of MeJA-IR which we evaluated by measuring photosynthetic capacity as well as above- and below-ground growth.

Transcriptional Response of Norway Spruce Bark to MeJA
The transcriptional response to jasmonate treatment has been explored in multiple previous studies on several plant species (e.g. Men et al., 2013; Shi et al., 2015; Hickman et al., 2017; Liu et al., 2017; Benevenuto et al., 2019). Although these previous analyses were generally conducted over much shorter timeframes (hours and days rather than hours, days, and weeks) and used leaf rather than woody stem tissues, their findings had many similarities to those of our study. In Arabidopsis, the transcriptional response to MeJA has been explored in detail. For instance, Hickman et al. (2017) performed mRNA-seq on samples harvested at 14 time points over a 16-hour period after MeJA treatment. This high-resolution time course analysis suggested that in Arabidopsis there is a rapid pulse of differential expression, with many genes showing peak change within 3 hrs after treatment. While we similarly observed a pulse of

change, the peak up- or downregulation of most genes appeared to occur many hours later in Norway spruce than in Arabidopsis (Figure 4). One possible explanation is that it may take longer for MeJA to enter and move through bark than leaf tissue. If so, there would be a longer delay between treatment and MeJA entering spruce cells, where MeJA is converted to the bioactive form of JA, JA-isoleucine, which then stimulates changes in transcription. This hypothesis could be tested using a cell type-specific metabolomics and transcriptomics approach, the latter as demonstrated by Celedon et al. (2017).

Another similar study in whitebark pine (*Pinus albicaulis*) seedlings demonstrated that MeJA treatment of needles resulted in a transient upregulation of numerous genes related to hormone signaling, including hormone biosynthesis genes, hormone-related transcriptional regulators (e.g. homologs of MYC2 and ethylene responsive TFs), and phenylpropanoid and terpenoid biosynthesis genes (Liu et al., 2017). We found similar genes upregulated in our study. However, unlike whitebark pine needles, we did not observe upregulation of ethylene biosynthesis gene ACC synthase in spruce bark (Supplemental Data Set 5). Another similar MeJA response in whitebark pine needles and Norway spruce bark was the downregulation of multiple genes involved in photosynthesis (Liu et al., 2017). At least in the first 24 hrs after MeJA treatment, we found genes involved in photosynthesis to be repressed (Figure 4; Supplemental Data Sets 2, 3 and 4). Although bark tissue, particularly of mature trees, appears brown and lacking in chlorophyll, stem photosynthesis do occur in conifers including Norway spruce (Berveiller et al., 2007; Saveyn et al., 2010; Tarvainen et al., 2018). Thus, some transcriptional responses to MeJA seem to be conserved across angiosperms and gymnosperms and across multiple tissue types.

Direct Induction of Spruce Defences May be Key to MeJA-IR
IR can be underpinned by a direct and prolonged upregulation of defences induced by the IR-eliciting stimuli (Wilkinson et al., 2019). In spruce, MeJA treatment has previously been shown to directly induce a range of defences. For example, Zulak et al. (2009) demonstrated that in 2-year-old Norway spruce seedlings MeJA treatment induced terpene biosynthesis and the subsequent accumulation of terpenes in bark tissue. Similarly, in our study we also found an upregulation of certain terpene biosynthesis genes, such as pinene and carene synthases (Figure 4, pattern 1-clusters; Supplemental Data Set 2). However, not all terpene biosynthesis

genes were upregulated. For example, three of the four differentially expressed isoprenyl diphosphate synthases (IDS), which produce the precursors of all monoterpenes, sesquiterpenes and diterpenes, were found among repressed clusters (Supplemental Data Set 2). This finding appears at odds with the accumulation of terpenes in spruce bark following MeJA treatment that is often reported (e.g., Zulak et al., 2009). However, some IDS genes in Norway spruce are not induced by MeJA but may instead be mildly (Schmidt and Gershenzon, 2007, 2008; Schmidt et al., 2011). IDS genes that are unresponsive to or repressed by MeJA could be involved in basal terpene production or the production of other metabolites derived from the general terpene precursors.

It is unclear how a bark-specific increase in terpene levels contributes to IR lasting longer than a few weeks. Previous studies have shown that increased terpene levels in the bark of spruce seedlings after MeJA treatment often are transient and that only a few specific terpenes remain at high levels 1-2 months after treatment (Martin et al., 2002; Zulak et al., 2009). This is in line with our finding that by 4 weeks after MeJA treatment genes associated with terpene biosynthesis were nearly back to basal expression levels (pattern 1-clusters in Figure 4). However, Zhao et al. (2011) demonstrated that if bark treated with MeJA 4 wks before was wounded this resulted in much higher terpene levels than if the bark was left intact. The increase after wounding is unlikely to be explained by a primed gene induction, as terpene biosynthesis genes are not induced by wounding MeJA treated bark (Mageroy et al., 2020b). Nevertheless, MeJA treatment does induce the formation of TRDs in the new sapwood (Martin et al., 2002; Figure 9 and Supplemental Figure 2). Although they take at least 2-4 weeks to form (Celedon and Bohlmann, 2019), fully developed TRDs provide a large reservoir of defensive, terpene-rich oleoresin (Krokene, 2015). This resin can inhibit the growth of ophiostomatoid fungi and may have contributed to the MeJA-IR we observed against *G. penicillata* (Figure 2)(Solheim, 1991). Furthermore, with the inoculation method we used bark was wounded prior to deposition of the fungal inoculum. This wounding may have released local resin pools or induced a flow of resin from surrounding bark tissue though axial and radial resin ducts (Nagy et al., 2000; Krokene, 2015). More detailed, cell-specific metabolome and transcriptome time course data for both the bark and sapwood, similar to that of Celedon et al. (2017), is required to clarify how terpenes contribute to long-lasting MeJA-IR.

Multiple studies have shown that MeJA treatment also induces swelling of polyphenolic cells and accumulation of phenolic compounds in Norway spruce bark (Franceschi et al., 2002; Krokene et al., 2008; Li et al., 2012). Over the past decade, Hammerbacher, Gershenzon and colleagues have provided an increased understanding of the biosynthesis of specific polyphenolic compounds in spruce bark (e.g. stilbenes and flavonoids) that provide resistance to ophiostomatoid fungi (Hammerbacher et al., 2011, 2018, 2019). In this study, we found that genes predicted to encode for enzymes involved in flavonoid biosynthesis were upregulated following MeJA treatment (Supplemental Data Set 2). The upstream general phenylpropanoid pathway, which provides the precursor metabolites for flavonoid biosynthesis, was also transiently upregulated. Thus, although previous studies have mostly worked with *Endoconidiophora polonica*, the model ophiostomatoid species, it is likely that an accumulation of phenolic compounds derived from the phenylpropanoid pathway is key to the MeJA-induced resistance against *G. penicillata* we observed in this study. A holistic study of phenolic biosynthesis that profiles metabolites, protein levels and enzyme activities should be conducted, such as was performed by Zulak et al. (2009) for terpenes.

PR proteins are another important pathogen defence in plants. Numerous genes predicted to encode for PR proteins were differentially expressed in Norway spruce bark following MeJA treatment. While we did not find a consensus expression pattern exhibited by *PR* genes (Supplemental Figure 3), there were some interesting results. For instance, some *PR* genes were transiently induced following MeJA treatment (upregulated the first 4 wks after treatment) while others were mildly prolonged upregulated (upregulated for more than 4 wks; Supplemental Figure 3; Supplemental Data Set 5). This inducibility of *PR* genes in response to jasmonate is consistent with previous work in conifers (Davis et al., 2002; Pervieux et al., 2004). Additionally, we have previously demonstrated that there was a stronger upregulation of numerous *PR* genes in the bark of mature spruce trees that had been treated with MeJA and then wounded, compared to trees which had been treated with water before wounding (Mageroy et al., 2020b). The current study suggests that MeJA-IR could be underpinned by a prolonged accumulation of PR proteins in addition to a primed induction.

Hormonal Regulation of MeJA Induced Defences

Many genes that were upregulated after MeJA treatment were annotated as being involved in defence regulation (Figure 4; Supplemental Data Sets 2-4). Furthermore, many of the same clusters of upregulated genes were also involved in hormone biosynthesis and signalling (Figures 4-7; Supplemental Data Sets 2-5). More specifically, there was a strong association between upregulated genes and the major defence regulatory hormones JA and SA, along with the supporting hormone ET. MeJA treatment has previously been demonstrated to induce an accumulation of JA and SA in the bark of 6-7 year old Norway spruce saplings (Schmidt et al., 2011). Furthermore, a study in 4-year-old saplings of *Pseudotsuga menziesii*, another Pinaceae species, demonstrated that MeJA induced ET accumulation (Hudgins and Franceschi, 2004). Thus, it is unsurprising that genes encoding many of the enzymes required to produce JA, SA and ET hormones were transiently upregulated following MeJA treatment in our study (Figures 5-7). However, there were some interesting exceptions. With regards to ET biosynthesis, genes encoding ACS, thought to be a rate limiting step in ET biosynthesis, were notably absent from the list of upregulated genes. Ralph et al. (2006) reported that mechanical wounding and herbivory upregulated multiple ACS genes in 2-year-old Sitka spruce (*Picea sitchensis*) and concluded that the ACS enzyme was the rate limiting step in ET biosynthesis in spruce, in line with many studies in angiosperms. However, it has recently been suggested that ACC oxidase can be the rate limiting enzyme in ET biosynthesis under some conditions (Houben and Van de Poel, 2019). Thus, despite the unresponsiveness of ACS genes to MeJA, it is still plausible that ET levels increase in Norway spruce bark in response to MeJA treatment. For SA, two biosynthesis pathways have been proposed: the ISOCHORISMATE SYNTHASE (ICS) pathway and the PAL pathways (Lefevere et al., 2020). No ICS genes have been reported in spruce (Schmidt et al., 2011) and the only enzymes in the PAL pathway that were differentially expressed in our study were PAL and aldehyde oxidase. Since both of these enzymes are involved in the synthesis of many metabolites it is difficult to know how much the upregulation of these genes contributes to SA accumulation in Norway spruce.

In addition to hormone biosynthesis genes, we observed upregulation of homologs of *MYC*, *EIN3/EIL1* and *NPR1*, major regulators of JA, JA/ET and SA dependent signalling pathways and defence responses. Studies in Arabidopsis and tomato have demonstrated that JA activates MYC and EIN3/EIL1 by targeting JAZ repressors for degradation (Lorenzo et al., 2004; Fernández-Calvo et al., 2011; Chang et al., 2013). When concentrations of the bioactive JA-Ile

are low, JAZ repressors inhibit the activity of the master regulatory TFs. However, when JA-Ile accumulates, the co-receptor complex of JA-Ile, JAZ and the F-box protein CORONATINE INSENSITIVE1 (COI1) is formed, resulting in JAZ proteins being ubiquinated and thus targeted for degradation (Howe et al., 2018). As reported in other species (e.g. Hoo et al., 2008; Hickman et al., 2017; Sun et al., 2017; Wang et al., 2020), we found that numerous genes encoding for JAZ proteins were upregulated by MeJA treatment. It is likely that this upregulation acts to prevent over-activation of JA-dependent responses.

Other TF families that were upregulated in response to MeJA treatment included the NAC (CUP-SHAPED COTYLEDON/NO APICAL MERISTEM/ATAF), myb domain protein (MYB), ethylene response factor (ERF), and WRKY families. In Arabidopsis and other well studied angiosperm species, members of these TF families can play an intermediate role in JA, JA/ET and SA signalling pathways and are important regulators of defence responses (Huang et al., 2016; Chen et al., 2017; Erb and Reymond, 2019; Yuan et al., 2019). For instance, MYB TFs are known to be key regulators of phenylpropanoid biosynthesis in many species (Liu et al., 2015). We also found NACs, MYBs, ERFs and WRKYs that were downregulated by MeJA treatment. This could be because they are negative regulators of defences or, alternatively, they regulate other pathways and processes which are repressed by MeJA treatment (e.g. growth and development). Additionally, these TFs do not always act below the master regulators (e.g. MYC2) in hormone signalling pathways. For example, there is evidence that specific MYBs can interact directly with JAZ repressors (Qi et al., 2011; Zhou et al., 2017). Future studies using RNA interference (RNAi) to repress MeJA-inducible TFs would be helpful for assessing the role of TFs in the regulation of defence responses in Norway spruce (e.g. *PR* gene expression, terpene and phenolic accumulation).

Role of Epigenetic Mechanisms in Maintenance of MeJA-IR
Our data showed that MeJA treatment induced an upregulation of defence regulators (e.g. MYC) and downstream defence genes (e.g. *PR* genes and genes involved in defence metabolite biosynthesis) which lasted for at least 4 weeks. Moreover, our previous work provides strong evidence of an augmented induction of *PR* genes after wounding of MeJA-treated trees (Mageroy et al., 2020b). Together this suggests that MeJA-IR in spruce is underpinned by both a prolonged upregulation and priming of genes. Long-term changes in basal gene expression

and/or gene responsiveness are probably underpinned by epigenetic mechanisms (Wilkinson et al., 2019). For instance, histone tail modifications leading to chromatin decompaction and enhanced TF binding to promoter regions has been linked with defence gene priming in Arabidopsis (Jaskiewicz et al., 2011; Schillheim et al., 2018; Baum et al., 2019). Furthermore, changes in DNA methylation both in gene promoters and elsewhere in the genome has been linked to both a prolonged upregulation and priming of genes (López Sánchez et al., 2016; Cambiagno et al., 2018; Furci et al., 2019). Many different proteins are required to implement histone tail modifications, changes in DNA methylation and chromatin remodelling (Pikaard and Scheid, 2014; Van Oosten et al., 2014). Interestingly, we found that in response to MeJA treatment, many genes predicted to encode for these epigenetic regulators were differentially expressed. Further study is required to determine what changes MeJA induces to the epigenetic landscape in spruce bark and how these changes contribute to shifts in gene expression associated with MeJA-IR.

We found that many of the Norway spruce RdDM genes were repressed by MeJA treatment (e.g. DRM2, NRPE1, RDR6). In Arabidopsis, RdDM pathways establish new DNA methylation and maintain DNA methylation at CHH context sites (Zhang et al., 2018). Arabidopsis and rice (*Oryza sativa*) mutants with defects in RdDM genes are, as expected, hypomethylated, particularly in CHH context (Stroud et al., 2013; Tan et al., 2016; Tang et al., 2016). The canonical RdDM pathway involves 24 nucleotide (nt) small RNAs (sRNAs) and long noncoding scaffold RNAs that help guide the methylation machinery, such as Argonaute 4 (AGO4), to specific loci (Matzke et al., 2014). Interestingly, aside from in reproductive tissue, the expression of 24 nt sRNAs is very low in Norway spruce (Nystedt et al., 2013; Chávez Montes et al., 2014; Nakamura et al., 2019; Wilkinson et al., 2021). However, it is has been reported in Arabidopsis that 21/22 nt sRNAs can also guide DNA methylation via a non-canonical RdDM pathway which involves AGO6 (Cuerda-Gil and Slotkin, 2016). As 21/22 nt sRNAs are common in spruce, perhaps the non-canonical RdDM pathway with 21/22 nt guide sRNAs is actually the canonical pathway in vegetative tissue of Norway spruce (Wilkinson et al., 2021). Nevertheless, regardless of the canonical pathway, the observed repression of Norway spruce RdDM genes indicates that DNA hypomethylation is induced by MeJA treatment, which in turn could contribute to long-term changes expression and/or responsiveness of spruce defence genes.

In addition to RdDM genes, we also found that MeJA treatment repressed genes encoding for DNA glycosylases, enzymes which catalyse the first step in active DNA methylation removal (Zhang et al., 2018). However, this repression occurred later than the RdDM pathway repression, further supporting our hypothesis that MeJA triggers a loss of DNA methylation. The later repression of the DNA glycosylases also provides evidence for an RdDM-DNA glycosylase feedback phenomenon previously observed in Arabidopsis. Two studies in 2015 provided evidence that the expression of ROS1, the major vegetative DNA glycosylase in Arabidopsis, is regulated by the RdDM pathway (Lei et al., 2015; Williams et al., 2015). Decreased or increased methylation in a specific region of *ROS1* promoter leads to reduced or enhanced gene expression, respectively. Future studies should aim to explore the methylation in DNA glycosylase promoters in spruce in response to changes in RdDM expression triggered by MeJA treatment. Taken together with the existence of many predicted DNA methylation pathway genes in Norway spruce, our data suggests that RdDM and other DNA methylation pathways described in angiosperms also function in conifers and that MeJA treatment may induce DNA hypomethylation.

Cost of long-term MeJA-IR

Although we observed a transient downregulation of gene expression related to photo-synthesis in bark tissue after treatment with 10 mM MeJA, this did not seem to influence the photosynthetic ability of needles (Supplemental Figure 4). We did, though, observe clear negative effects of MeJA treatment on shoot and sapwood growth (Figure 9). This growth reduction could be due to the redirection of carbon resources into the formation of MeJA-induced TRDs (Figure 9e), which are filled with metabolically costly defence-related terpenes (Gershenzon, 1994), or the swelling of phenolic rich polyphenolic cells, which may explain the secondary phloem enlargement in MeJA treated trees (Figure 9d). TRDs and enlarged polyphenolic cells are likely important for long-term MeJA-IR and therefore in Norway spruce enhanced resistance to biotic stress induced by MeJA may come at a direct cost to growth. Whether this has an impact on long-term fitness and reproduction of MeJA-treated trees is difficult to assess in a tree species that does not reproduce until it is 25 years old. But, as previously discussed by Mageroy et al. (2020a), when the alternative is death from bark beetle attack, investment in defense may be more strategic for a long-lived plant species.

Conclusions

This detailed study of transcriptional changes in Norway spruce bark after MeJA treatment showed that changes in gene expression can be detected as early as 3 hrs after treatment. Expression of some transcripts returned to basal levels by 1 wk, while induction of others lasted until 4 wks after treatment. MeJA-induced transcript clusters were strongly associated with defence responses to biotic stress, whereas many MeJA-repressed gene clusters were linked with primary cell functioning and metabolism. Additionally, we found that genes encoding machinery related to the RdDM pathway were repressed, suggesting that epigenetic changes and DNA hypomethylation are important in the establishment and maintenance of MeJA-IR in Norway spruce.

Supplemental Information

Supplemental_Data_Set_1 - mRNA-seq raw read data and alignment statistics

Supplemental_Data_Set_2 - Differentially expressed genes

Supplemental_Data_Set_3 - Overrepresented protein signatures

Supplemental_Data_Set_4 - Enriched GO terms

Supplemental_Data_Set_5 - Hormone biosynthesis and defence genes

Supplemental_Data_Set_6 - Epigenetic regulators

Supplemental_Figure_1 - RT-qPCR

Supplemental_Figure_2 - Microscopy measurements of stem cross sections

Supplemental_Figure_3 - *PR* gene expression

Supplemental_Figure_4 - Chlorophyll fluorescence

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Conflict of Interest Statement

The authors declare no competing interests.

Data Availability Statement

Raw reads from mRNA sequencing have been submitted to NCBI. BioProject accession:
PRJNA768356.

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Figure legends

Figure 1. Experimental setup to study establishment and maintenance of methyl jasmonate (MeJA)-induced resistance in Norway spruce seedlings. Two-year-old seedlings were treated with either water (blue lines), 10 mM MeJA (dark red lines) or other concentrations of MeJA (orange lines; details below). For the resistance assay, plants were inoculated with a

necrotrophic fungal pathogen or mock 4 weeks (wks) after treatment. Eight weeks later, necrotic lesions were measured. mRNA-seq was conducted on mRNA extracted from bark tissue harvested from plants at 3 hours (hrs), 24 hrs, 1 wk and 4 wks after treatment. Chlorophyll fluorescence was measured 1 day, 6 days and 8 wks after treatment with 0, 5, 25, 50, or 100 mM MeJA. Plant height growth was measured twice over a period of 5.5 wks. Shoots, roots and stems were then harvested at 10 wks to explore the impact of MeJA on plant growth and morphology.

Figure 2. MeJA elicits induced resistance against *Grosmannia penicillata*. Two-year-old Norway spruce seedlings were treated with either water (blue) or 10 mM MeJA (red) four weeks prior to inoculation with either a *G. penicillata* or sterile malt agar inoculum. Symptoms were assessed 8 weeks post inoculation. (a) The first stem internode, with wound and/or fungal induced cell death, from all plants of one representative experimental block. (b) The full axial length of wounds or necrotic lesions (indicated in (a)) were measured on all stems. Results are presented as mean ± 95% confidence interval of the mean. Points show individual replicates (n = 15). Bars with different letters are significantly different (ANOVA followed by Tukey post hoc test, p < 0.05).

Figure 3. Treatment with methyl jasmonate (MeJA) induces a transient shift in the transcriptome of Norway spruce bark. Principle component analysis (PCA) (a) and hierarchical clustering analysis (HCA) (b) plots displaying how treatment of 2-year-old spruce seedlings with water (blue) or 10 mM MeJA (red) impacts on the bark transcriptome over the subsequent 4 weeks. All genes with a total mRNA-seq read count of ≥ 100 across the 32 samples (n = 4 per treatment and time point) were included in the analyses. Both the PCA and the HCA utilised counts normalised with a variance-stabilizing transformation. Samples in the HCA were clustered using the Euclidean distances between samples (darker blue for lower distances) and the complete-linkage method.

Figure 4. Response of the Norway spruce bark transcriptome to methyl jasmonate (MeJA) treatment. Expression profiles and functional characterisation of 6330 genes showing a significantly (adjusted p-value < 0.001) altered expression pattern, over a 28-day period, between bark tissues of 2-year-old spruce seedlings treated with water (control, blue) or 10 mM MeJA (red). (a) Differentially expressed genes were grouped into 13 clusters (indicated

by the coloured boxes with associated gene numbers (N)) using Spearman distances and Ward's method. The 13 clusters were assigned to one of four patterns (P) based on their general expression profile in MeJA relative to water treated plants: 1 – Upregulated; 2 – Upregulated, then downregulated; 3 – Downregulated; 4 – Downregulated, then upregulated. Read counts are displayed as per gene z-scores normalised for library size and transformed using the DESeq2 function vst. (b) Per-cluster mean expression profiles with 95% confidence intervals. Variance-stabilizing transformation (VST) transformed counts are approximately on a $\log_2$ scale. (c) Significantly overrepresented protein signatures (adjusted p-value ≤ 0.05). If a cluster had more than five significantly enriched protein signatures, only the five with the highest fold enrichment are displayed. See Supplemental Data Set 3 for the full list of enriched protein signatures.

Figure 5. Jasmonic acid (JA) biosynthesis genes are upregulated, either transiently or for at least four weeks, in Norway spruce bark in response to methyl jasmonate (MeJA) treatment. Expression profiles of genes displaying a significantly (adjusted p-value < 0.001) altered expression pattern across time as a result of MeJA treatment and which were annotated as encoding for enzymes (yellow boxes) involved in the (+)-7-iso-JA-Ile biosynthesis pathway. In the plots, faint lines indicate the mean expression profiles, with 95% confidence intervals, of individual transcripts and the thicker lines depict the mean per enzyme category profile, for water controls (blue) and MeJA treatments (red). Read counts were normalised using the variance stabilizing transformation (vst) in DEseq2. The JA biosynthesis pathway is based on knowledge from angiosperms such as *Arabidopsis thaliana* and *Solanum lycopersicum* and was adapted from Wasternack and Hause (2013) and Wasternack and Song (2017). Compound abbreviations: α-LeA, α-linolenic acid; 13-HPOT, (13S)-hydroperoxyoctadecatrienoic acid; cis-(+)-OPDA, cis-(+)-12-oxophytodienoic acid; OPC-8, 3-oxo-2-(2-pentenyl)-cyclopentane-1-octanoic acid; (+)-7-iso-JA, (+)-7-iso-jasmonic acid; (+)-7-iso-JA-Ile, (+)-7-iso-jasmonoyl-L-isoleucine. Enzyme abbreviations: PLA1, phospholipase A1; 13S-LOX, 13S-lipoxygen-ase; AOS, allene oxide synthase; AOC, allene oxide cyclase; OPR, OPDA reductase; JAR1, JA-amino acid synthetase.

Figure 6. Genes involved in the biosynthesis of salicylic acid (SA) are transiently upregulated in Norway spruce bark in response to methyl jasmonate (MeJA) treatment. Expression

profiles of genes displaying a significantly (adjusted p-value < 0.001) altered expression pattern across time as a result of MeJA treatment and which were annotated as encoding for enzymes and transporters involved in the biosynthesis of SA (yellow boxes). In the plots, faint lines indicate the mean expression profiles, with 95% confidence intervals, of individual transcripts and the thicker lines depict the mean per protein category profile, for water (blue) and MeJA (red) treatments. Read counts were normalised using the variance stabilizing transformation (vst) in DEseq2. The pathway is based on what is known about the biosynthesis of SA in angiosperms. Enzymes in grey are involved in the biosynthesis pathway but no differentially expressed genes were annotated to encode for them. Due to space limitations not all enzymes have associated expression profiles, see Supplemental Data Set 5 for the full list of differentially expressed biosynthesis genes. Compound abbreviations: DQ, 3-dehydroquinate; DHS, 3-dehydroshikimate; Shikimate-3-P, shikimate 3-phosphate; EPSP; 5-enolpyruvoyl-shikimate 3-phosphate; t-CA, trans-cinnamic acid. Enzyme/transporter abbreviations: DHQ-SDH, dehydroquinate-shikimate dehydrogenase; SK, shikimate kinase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; CS, chorismate synthase; CM1, chorismate mutase 1; PPA, prephenate aminotransferase; ADT/PDT, arogenate dehydratase/prephenate dehydratase (annotated as being encoded for by the same genes); CM2, chorismate mutase 2; PPY, phenylpyruvate aminotransferase; TAT, tyrosine aminotransferase; pCAT, plastidial cationic amino-acid transporter; PAL, phenylalanine ammonia-lyase; AO, aldehyde oxidase; BA2H, benzoic acid 2-hydroxylase; ICS, isochorismate synthase; IGS, isochorismoyl-glutamate synthase; IPGL, IC-9-Glu pyruvoyl-glutamate lyase; EDS5, ENHANCED DISEASE SUSCEPTIBILITY 5.

Figure 7. Methyl jasmonate (MeJA) treatment induces a rapid and transient upregulation of regulators of jasmonic and salicylic acid (JA and SA) dependent defences in Norway spruce bark. Expression profiles of genes displaying a significantly (adjusted p-value < 0.001) altered expression pattern across time as a result of MeJA treatment and which were annotated as encoding for proteins involved in regulation of JA (a) and SA (b) dependent defences. In the plots, faint lines indicate the mean expression profiles, with 95% confidence intervals, of individual transcripts and the thicker lines depict the mean per protein category profile, for water (blue) and MeJA (red) treatments. Read counts were normalised using the variance stabilizing transformation (vst) in DEseq2. The signalling pathways are based on what is known

in *Arabidopsis thaliana*. Compound abbreviations: (+)-7-iso-JA-Ile, (+)-7-iso-jasmonoyl-L-isoleucine. Protein abbreviations: COI1, CORONATINE INSENSITIVE1; JAZ, JASMONATE-ZIM-DOMAIN PROTEIN; NAC, Petunia NAM and Arabidopsis ATAF1, ATAF2, and CUC2; EIN3, ETHYLENE INSENSITIVE 3; ERF, ETHYLENE RESPONSIVE FACTOR; NPR1, NONEXPRESSER OF PR GENES 1; TGA, TGACG motif-binding.

Figure 8. Methyl jasmonate (MeJA) treatment alters the expression of regulators of multiple epigenetic modifications in Norway spruce bark, including DNA methylation. (a) Bars show the number of annotated genes in different epigenetic regulator categories. Genes showing a significantly (adjusted p-value < 0.001) altered expression pattern across time as a result of treatment are indicated in grey. (b) The individual transcripts (faint lines with 95% confidence intervals) and category means (solid lines) for differentially expressed regulators of DNA methylation homeostasis in water (blue) and MeJA (red) treated bark (See Supplemental Data Set 6). Read counts were normalised using the variance stabilizing transformation (VST) in DEseq2. Epigenetic regulator categories are taken from Mageroy et al (2020b).

Figure 9. Effect of methyl jasmonate (MeJA) treatment on growth and traumatic resin duct development. 2-year-old Norway spruce plants were sprayed with water (blue) or 10 mM MeJA (red). (a) Plant height was measured twice over a period of 5½ weeks after MeJA treatment. Shoots, roots and stem cross-sections for (b)–(g) were harvested 10 weeks after treatment. (b) Dry weight of the 10 uppermost current-year lateral shoots on each plant. (c, d) Radial thickness (i.e. width) of the current-year annual sapwood growth (c) and secondary phloem (d) on stem cross-sections. (e) Proportion of traumatic resin ducts (TRD) across a quarter of the tangential width of the current-year annual sapwood growth. (f) Root dry weight. (g) Total root length for different root diameter classes. All results are presented as mean ± 95% confidence interval of the mean. Points show individual data points (n = 15). Asterisks indicate significant differences (Two-sided t-test, n.s = not significant; *** = p < 0.001, ** = p < 0.01).