Maternal environment modulates the defence response in *P. pinaster* progenies

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Abstract

Conifers are woody, long-lived, stationary organisms, with rather limited ranges of seed dispersal that could benefit from a previous adaptation of the progeny to the prevailing environmental conditions. The adaptational process is unlikely to be the result of genetic changes (chromosomal rearrangements, mutations, deletions, or insertions) across one generation, since mutation frequencies are very low. A more likely mechanism to enhance the progenies' fitness is the so called epigenetic memory or maternal effect. In this study, we used qRT-PCR analysis of gene expression patterns of selected candidate genes to provide evidence of a maternal effect at a basal level and upon induction with methyl jasmonate (MeJA) in *Pinus pinaster* progenies. The maternal trees of three independent genotypes were left untreated as a control (CNT) or subjected to either mechanical wounding (MW) or methyl jasmonate (MJ) during seed development. The observed maternal effects in the progeny operate on different levels in various pathways and biological processes. Additionally, the maternal environment seems to qualitatively influence and/or regulate defence-related and unrelated responses across at least one generation. Progenies originating from trees treated with MeJA seemed to exhibit an inherited induced resistance. In contrast, progenies derived from trees exposed to mechanical wounding displayed a priming response in comparison to progenies of the CNT maternal environment. We also observed that the genotype seems to predetermine the degree of maternal effects, especially in genes involved in epigenetics. To our knowledge, this study is the first of its kind and provides valuable evidence of maternal effects in *P. pinaster* progenies.

Keywords: Pinus pinaster, maternal effects, epigenetic memory, defence, methyl jasmonate, mechanical wounding, shikimate pathway, phenylpropanoid pathway, methionine metabolism.

Table of Contents

Abbreviations

cDNA	complementary deoxyribonucleic acid
CTAB	hexadecyltrimethylammonium bromide
EDTA	ethylenediaminetetraacetic acid
H_2O	water
LiCl	lithium chloride
MeJA	methyl jasmonate
mRNA	messenger ribonucleic acid
NaAc	sodium acetate
NaCl	sodium chloride
PVP	polyvinylpyrrolidone
RT	room temperature
Tris-HCl	tris(hydroxymethyl)aminomethane-hydrochloride
PCR	polymerase chain reaction
qRT-PCR	quantitative reverse transcribed-polymerase chain reaction

1 Introduction

The gymnosperm *Pinus pinaster*, or maritime pine, belongs to the *Pinus* genus in the *Pinaceae* family. The species is divided into two subspecies, *P. pinaster* subsp. *pinaster* and *P. pinaster* subsp. *atlantica* which naturally thrive in warm temperate climates, but are very tolerant to different climate conditions. While the natural distribution of subsp. *pinaster* is limited to the western Mediterranean (France, Spain, Italy, Corsica, Sardinia, Morocco, and Tunisia), subsp. *atlantica* is restricted to the costal Atlantic areas (Portugal, southwest France, and north Spain (Galicia)) (Alía & Martín, 2009; Forestry Compendium, 2002). Nevertheless, *P. pinaster* has been widespread and cultivated throughout Europe, South Africa, South America, Oceania, and Asia. In Europe, as well as in North Africa, *P. pinaster* has been historically used for dune consolidation and conservation, stabilisation of coastal areas, soil conservation, and afforestation of low fertility soils. However, its commercial value lies in timber, resin, and pulp production, as well as in Chrismas tree cultivation (Forestry Compendium, 2002). Furthermore, the pine's bark is processed to extract polyphenolic compounds which are used in medicine as an antioxidant in disease treatment (Iravani & Zolfaghari, 2011). *P. pinaster* belongs to the commercially and economically most valuable tree species in Spain, Portugal and France (Alía & Martín, 2009). Like many trees, *P. pinaster* is susceptible to a variety of pathogens and insect pests that damage various parts of the plant, such as cones, buds, shoots, twigs, leaves, stem, and roots (Forestry Compendium, 2002). The resulting economic losses can be very high when outbreaks occur. Therefore, it is important to further study known defence signalling pathways and illuminate novel molecular mechanisms which might contribute to an improved resistance and/or tolerance in *P. pinaster*. This knowledge can potentially be transferred to other economically important plant species.

1.1 Conifer Defence

Conifers combine two defensive strategies, the constitutive (continuous) defence and the induced defence. Both interact on a spatial and temporal level and can be distinguished in chemical and mechanical defensive aspects.

The constitutive defence is ever present, non-specific, determined by genetics and previous exposure to attack. It comprises of distinct structures and cell types located in different plant tissues. The periderm and outer bark are a physical barrier, consisting of highly lignified and suberized cells that act as the first line of defence. When this mechanical barrier is overcome, the attacker faces different defence related cell types inhabited by the inner bark and secondary phloem (Hudgins *et al.*, 2004; Franceschi *et al.*, 2005). Some of these cell types store and produce toxic chemicals, such as polyphenolic parenchyma (PP) cells, which are involved in the production and storage of phenolic compounds (Franceschi *et al.*, 1998), as well as resin ducts, resin blisters, and resin cells that produce and store terpenoids (Franceschi *et al.*, 2005; Bohlmann, 2008). Other cell types are more involved in structural defence, such as the thick and heavily lignified sclereids and sclerenchyma cells as well as cell types containing calcium oxalate crystals (Hudgins *et al*, 2004; Franceschi *et al.*, 2005; Bohlmann, 2008). Resinous terpenoids and phenolic compounds are also produced in the secondary xylem which is adjacent to the heartwood. The tree's heartwood is protected via lignification and impregnation with phenolic compounds (Franceschi *et al.*, 2005).

Every defence in plants is costly, reallocating carbon resources from defence unrelated physiological processes to defence related physiological processes and thereby, resulting in reduced growth or yield. Also, accessing carbon can be difficult when under conditions in which carbon becomes limiting, such as under drought stress (Oliva *et al.*, 2014). However, induced defences are of lower cost to the plant in relation to constitutive defences, hence they are only switched on upon attack (Bolton, 2009; Oliva *et al.*, 2010). Induced defences are rather specific and include different signalling pathways which are probably adapted to the type of trophic interaction with different biotic agents' feeding habits (herbivoric, biotrophic, necrotrophic). Insects usually elicit a defence response by feeding on the plant which leads to the expression of defence related genes and the production of toxic compounds (Lippert *et al.*, 2007), whereas pathogens have to be recognized on the molecular level prior to defence induction.

In conifers, mechanical induced defences include i) programmed cell death, leading to the withdrawal of nutrients and the isolation of a pathogen (only effective against biotrophs), ii) the formation of wound callus which might subsequently be lignified, suberized, or impregnated with phenolic compounds to stop further invasion, iii) the formation of wound periderm to contain attackers, and iv) the lignification of fibres (Franceschi *et al.*, 2005). Inducible chemical defences are rather specific to different natural enemies and include i) the *de novo* production of traumatic resin ducts (Franceschi *et al.*, 2005; Bohlmann, 2008), ii) the synthesis of toxic substances such as resinous terpenoids (isoprenoid pathway), alkaloids, and phenolic compounds (phenylpropanoid pathway) (Franceschi *et al.*, 2000; Franceschi *et al.*, 2005), and iii) the synthesis of jasmonate (JA) and other phytohormones, such as salicylic acid (SA) and ethylene (ET) to activate hormonal signalling pathways. These hormone mediated signalling pathways lead to the expression of pathogenesis-related (PR) and antimicrobial proteins and further, to systemic acquired resistance (SAR) (SA mediated signalling) or induced systemic resistance (ISR) (JA/ET mediated signalling) (Pieterse *et al.*, 2009).

1.1.1 The Shikimate and Phenylpropanoid Pathway

The shikimate and phenylpropanoid pathway are essential to plant defence against pathogen and insect attack. Therefore, several studies have focused on studying the general gene expression and specific transcriptional changes of the pathways' key enzymes. Furthermore, many studies were able to verify that the shikimate and phenylpropanoid pathway are induced upon mechanical wounding and treatment with MeJA in conifers (Franceschi *et al.*, 2002; Hudgins *et al.*, 2003; Hudgins *et al.*, 2004; Lippert *et al.*, 2007; Arnerup *et al.*, 2011; Moreira *et al.*, 2012; Yaqoob *et al.*, 2012; Hammerbacher *et al.*, 2014).

The shikimate pathway is one entry point to the secondary metabolism and precedes the aromatic compound biosynthesis. It is initiated by 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHP) that catalyses the conversion of the primary metabolism substrates erythrose-4-pyrophosphate (E4P) and phosphoenolpyrovate (PEP) to 3-deoxy-D-arabino-heptulosunate 7-phosphate (Figure 1) (Herrmann, 1995; Toghe *et al.*, 2013). There are three DAHP synthase paralogues in Norway spruce (DAHP1, DAHP2, and DAHP3). Arnerup *et al.* (2011) suggested that a shift in transcript abundance between DAHP1 and DAHP2 seems to mark the entry point to the secondary metabolism in Norway spruce (*Picea abies*), resulting in the production of chorismate, the common precursor to the amino acids *L*-phenylalanine, *L*-tyrosine, and *L*-tryptophan (Hermann, 1995; Toghe *et al.*, 2013). The conversion of *L*-phenylalanine to cinnamic acid by the rate limiting enzyme phenylalanine ammonia-lyase (PAL) marks the first step in the general phenylpropanoid pathway. Cinnamic acid is then further transformed to *p*-coumaric acid by the enzyme cinnamic

acid 4-hydroxylase (C4H). The activation of *p*-coumaric acid to *p*-coumaroyl-CoA marks the central branching point of the general phenylpropanoid pathway and provides the precursor for a wide array of different defence compounds, such as lignin polymers, lignans, flavonoids, stilbenes, and several more (La Camera *et al.*, 2004; Vogt, 2010).

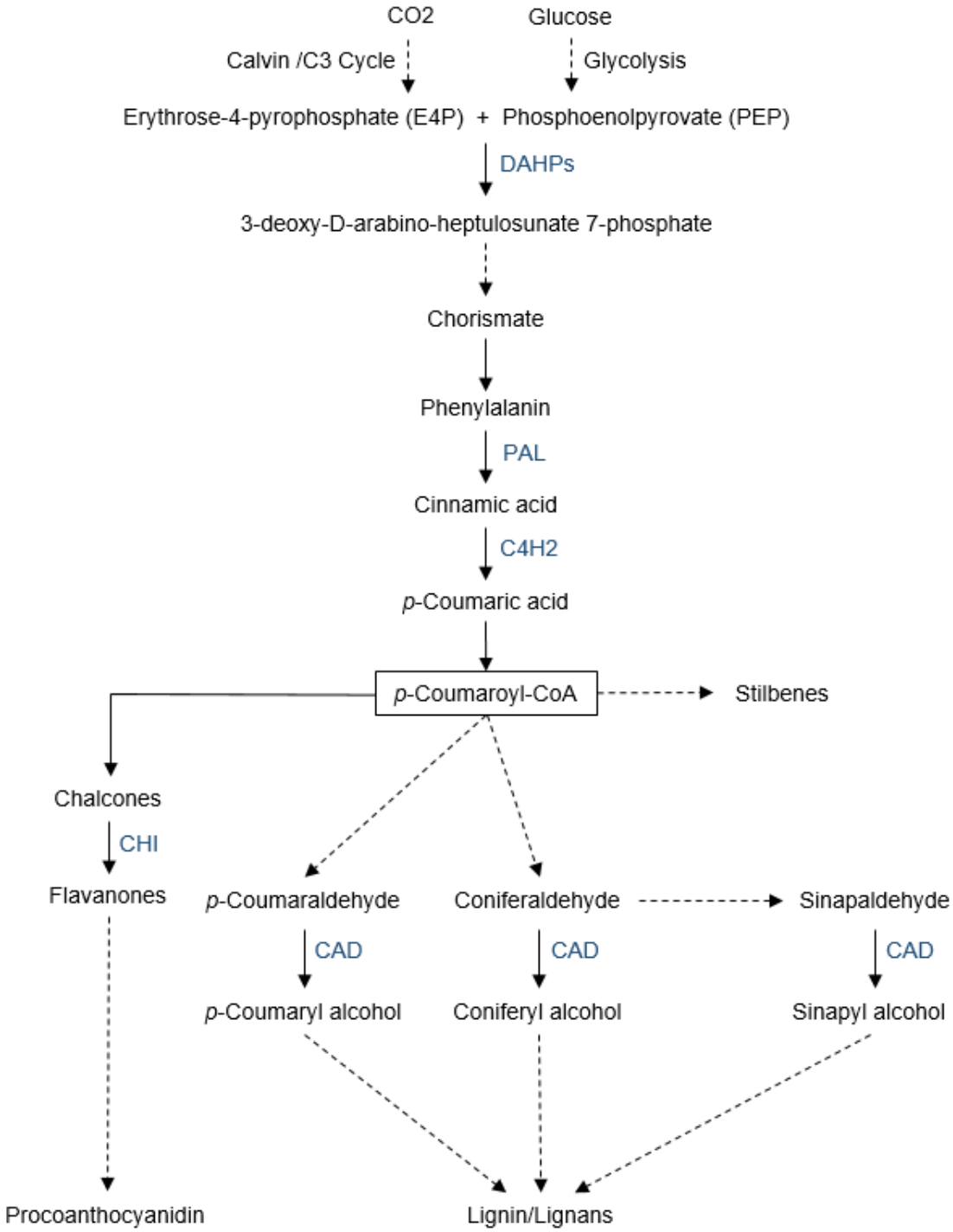

Figure 1| Schematic display of relevant aspects of the shikimate and phenylpropanoid pathway. Both pathways belong to the secondary metabolism in conifers. Blue colour indicates key enzymes that were studied in this experiment. Dashed errors represent multiple intermediate steps within the pathway. DAHPs – 3-deoxy-D-arabino-heptulosonate 7-phosphate synthases; PAL – phenylalanine ammonia-lyase; C4H2 – cinnamic acid 4-hydroxylase 2; CHI – chalcone isomerase, CAD – cinnamoyl alcohol dehydrogenase. DAHPs belong to the shikimate pathway, whereas PAL and the enzymes downstream belong to the general phenylpropanoid pathway.

Lignin is located in the plant cell wall and provides a physical barrier against pathogen and insect attacks (Koutaniemi *et al.*, 2007; Vogt, 2010), whereas lignans function as defence compounds against natural enemies (Federolf *et al.*, 2007). An essential enzyme in lignin and lignan biosynthesis is cinnamyl alcohol dehydrogenase (CAD) which is needed to produce different hydroxycinnamyl alcohol subunits or monolignols (Figure 1) that polymerize to form lignin or dimerize to form lignans (Federolf *et al.*, 2007; Koutaniemi *et al.*, 2007; Vogt, 2010). Chalcone isomerase (CHI) is a rate limiting enzyme of flavonoid biosynthesis and catalyses the conversion of chalcones to flavanones. Flavanones provide the structural skeleton for most other flavonoid compounds (Winkel-Shirley, 2001; Jez & Noel, 2002; Ferrer *et al.*, 2008).

Both, flavonoids and lignins are modified by methylation in most plants. These alterations specify the contribution of single monolignols to lignin formation or change the reactivity and interaction of flavonoid compounds with cellular targets, as well as their solubility. Enzymes contributing to these modifications are S-adenosyl-*L*-methionine (AdoMet)-dependent *O*-methyltransferases (OMTs) (Ferrer *et al.*, 2008; Vogt, 2010; Cheynier *et al.*, 2013). As their name implies, the substrate of AdoMet-dependent OMTs is AdoMet which is generated by the enzyme S-adenosylmethionine synthase (AdoMetS) in the methionine metabolism (Tehlivets *et al.*, 2013).

Both pathways mentioned above are essential for plant defence and are therefore, interesting targets to study maternal effects or epigenetic memory in plants.

1.2 Maternal Effects/Epigenetic Memory in Plants

In 1983, the impact of maternal environments on progeny phenotypes and performance was first investigated in tobacco plants (*Nicotiana tabacum*). In this study, progenies of tobacco plants infected with *tobacco mosaic virus* (TMV) exhibited an elevated resistance to TMV in comparison to control plants that were also infected with TMV (Roberts, 1983). These results indicated a maternal effect or epigenetic memory of the progeny. A discovery in Norway spruce opened up the investigation of maternal effects in forest trees (Kohmann & Johnsen, 1994). At the end of the 1980s, Norway spruce ecotypes, adapted to the cold climate of northern Norway, were transferred to a seed orchard located further south. The progenies of these trees showed phenotypes that were rather similar to trees present within this warmer region, even though their parents originated in cold climate areas. Eleven years later, a study investigating this phenomenon was conducted by Johnson *et al.* (2005a). Within this study, genetically identical mother trees were exposed to different temperatures (warm and cold) during their reproductive cycle. All progenies were full-sibs. Progenies of cold maternal environments showed an altered phenology, such as advanced timing of dehardening in spring, advanced bud burst, earlier leader shoot growth cessation and timing of bud set equivalent to 4°-6° latitudinal ecotypic difference, and an advanced cold acclimation in autumn compared to progenies of warm maternal environments. These differences in phenotypes where most pronounced when cold or warm temperatures were present during zygotic embryogenesis and seed maturation. It was further shown that photoperiod also played an essential role in the phenotypic adaptation of the progenies (Johnsen *et al.*, 2005b). However, photoperiod and temperature changes during meiosis and microsporogenesis of the male reproductive organs did not alter progeny performance (Johnsen *et al.*, 1996). Furthermore, progenies originated from warm maternal environments had a higher degree of methylation of their DNA than progenies originated from cold maternal environments (Johnsen *et al.*, 2005a). Interestingly, the progenies' memory effects lasted more than 20 years (Yakovlev *et al.*, 2011).

Similar results were observed in Scots pine, *Larix* spp., longleaf pine, and progenies from white spruce crosses (reviewed by Bräutigam *et al.*, 2013).

The concept of an epigenetic memory especially in perennial conifers seems reasonable because these plants are woody, long-lived, and stationary organisms. Conifer seeds are not very widely dispersed, making a previous adaptation of the progeny to the prevailing environmental conditions advantageous. However, epigenetic memory effects were also observed in ornamental and herbacious plants. A similar experiment to that of Johnsen *et al.* (2005a) was performed in *Arabidopsis thaliana* (Blödner *et al.*, 2007). Progenies originating from warm (25 ºC) and cold (15 ºC) parental environments were grown under stable environmental conditions (20 ºC). Progenies of warm parental environments showed faster germination rates, faster root elongation growth, higher leaf biomass, and increased seed production, whereas progenies of cold parental environments recovered faster from freezing and chilling stresses. Seeds from warm parental environments had also higher nitrogen contents which Blödner et al (2007) explained to be the reason for their progenies phenotypic advantages. However, this reasoning fails to explain the adaptive advantage of progenies of cold parental environments to chilling and freezing stresses and suggests another underlying mechanism, such as epigenetic mechanisms.

The exhibited plasticity of plant phenotypes both in angio- and gymnosperms across one or two generations cannot exclusively be explained by genetic changes, since mutation frequencies are very low (10^{-7} - 10^{-8} / nucleotide) (Kunkel, 2004). Furthermore, Besnard *et al*. (2008) showed that in full-sib Norway spruce progeny, maternal environments induced only minor segregation distortions of single gene loci concluding that the resulting effects are unlikely to account for the observed phenotypic changes in progenies of warm and cold maternal environments. Therefore, those results may be attributed to maternal effects or an epigenetic memory. Yakovlev *et al.* (2010, 2011) suggested the putative partition of micro (mi) RNAs in the regulation of temperature-dependent epigenetic memory in Norway spruce and verified the differential expression of genes belonging to the small RNA biogenesis pathway, supporting the concept of an epigenetic memory. The studies by Johnsen *et al.* (2005a) and Blödner *et al.* (2007) also show similarities in temperature-dependent epigenetic memory between angiosperms and gymnosperms and open up the question to the presence of other conformities. Nevertheless, it is likely that epigenetic inheritance mechanisms/patterns will differ between species.

1.2.1 Epigenetics and Epigenetic Regulation of Gene Expression

Differentially inherited gene expression patterns mediated by epigenetic changes seem to be the leading mechanism behind maternal effects or epigenetic memory. Russo *et al.* (1996) defined epigenetics as “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence.” There are two classic systems in epigenetics, the Polycomb/Trithorax system and DNA methylation. Whereas the Polycomb/Tritorax system focuses on proteins involved in the transcriptional regulation of developmental genes, DNA methylation mostly focuses on the methylation of CG sites in the DNA (Bird, 2007).

In plants, most DNA methylation occurs in areas of the genome rich in transposable elements (TEs) and other repetitive DNA to repress their expression and transposition. Furthermore, DNA methylation in flowering plants is realized via CG and non-CG methylation, the latter including CHG and CHH (asymmetric) methylation (H denotes to A, C, or T) (Law & Jakobsen, 2010), which are aided and regulated by decrease in DNA methylation 1 (DDM1) and RNA-directed DNA methylation (RdDM), a specialized branch of the RNA interference (RNAi) pathway (Zemach *et*

al., 2013). *DDM1* codes for a chromatin-remodelling ATPase involved in DNA methylation of transposable elements (TEs) and specifically opens up heterochromatin linked by histone H1, so that methyltransferases, especially chromomethylase 2 (CMT2), can maintain DNA methylation or *de novo* methylate DNA (Zemach *et al.*, 2013). Furthermore, DDM1 and RdDM separately facilitate most of the *de novo* or asymmetric methylation in plants (Law & Jakobsen, 2010; Zemack *et al.*, 2013). Maintenance of DNA methylation is accomplished by enzymes of different methyltransferase families. CG epigenetic marks are only present on one side of the complementary DNA strand after replication, therefore termed hemi-methylated, and must be applied to the other strand, as well, which is realized and maintained by methyltrantransferase 1 (MET1) (Bird, 2007; Law & Jakobsen, 2010). Methylation of CG sites can lead to stable gene silencing because transcription factors (TFs) are unable to bind to promoter regions or repressor proteins are recruited and bind to the methylated sites (Bird, 2007). It was further suggested that CG methylation is essential to coordinate epigenetic memory and that CG epigenetic marks will be restored if lost (Mathieu *et al.*, 2007). Chromomethylase 3 (CMT3), an enzyme unique to plants, maintains CHG methylation, whereas domains rearranged methyltransferase 2 (DRM2) (in RdDM) or CMT2 (facilitated by DDM1) maintain CHH methylation via continuous *de novo* methylation. It is important to mention that DNA methylation can be reversible through demethylation by a number of enzymes (Law & Jakobsen, 2010).

In plants, gene expression is not only altered and regulated by DNA methylation, but also via histone modification and the expression of small RNAs that target mRNAs. Post-translational modifications of histone proteins (methylation/acetylation/phosphorylation of the N-terminal segment) recruit downstream effector protein complexes that change the accessibility of DNA to transcription, concluding in a changed gene expression pattern (reviewed by Berger, 2007). Small RNAs (siRNA, miRNA), generated by the RNAi pathway, target and degrade mRNAs, causing a change in mRNA as well as protein abundance within the cell and are often generated upon received stress signals (Henderson & Jakobsen, 2007). They are further phloem mobile and might be able to reach gamets and influence their DNA methylation patterns through *de novo* methylation (Boyko & Kovalchuk, 2011).

1.2.2 Putative Mechanisms of Epigenetic Inheritance

Trans-generational and maternal effects in plants are most pronounced when parental plants are exposed to mild stresses and are suggested to be accounted for by trans-generational hardening or priming effects (Kovalchuk & Kovalchuk, 2010). Plant progenies exhibiting trans-generational priming effects have the same level of constitutive defence expression as unprimed progenies. However, upon insect or pathogen attack the defence response of primed progenies will be faster and stronger, whereas plants exhibiting trans-generational induced resistance have an increased innate level of resistance (reviewed by Holeski *et al.*, 2012).

A study by Agrawal (2002) suggested the presence of an inherited induced resistance in progenies of wild reddish plants (*Raphanus raphanistrum*) treated with an herbivorous caterpillar (*Pieris rapae*) and MeJA. It seems that the destabilization of plant genomes due to abiotic and biotic stresses, resulting in an increased somatic homologous recombination frequency (HRF), is essential for trans-generational induced stress responses in plants (Kovalchuk & Kovalchuk, 2010; reviewed by Boyko & Kovalchuk, 2011). Progenies of susceptible tobacco plants treated with TMV showed hypermethylated genomes in comparison to parental plants as well as an inherited locus specific increased HRF (Boyko *et al.*, 2007). The authors of this study suggested that genome hypermethylation in plants stabilizes the genome and acts as a protection mechanism against

stresses. This was also suggested for gymnosperms, namely *Pinus sylvestris*, grown in the Chernobyl area. Trees exposed to different levels of radiation showed varying levels of hypermethylation (Kovalchuk *et al.*, 2003). However, it seems that single loci or gene regions, such as of potential R-genes, can simultaneously be hypomethylated, resulting in genome destabilization and an increased HRF, potentially leading to trans-generational adaptive responses in the progeny (Boyko *et al.*, 2007). This view is further strengthened by findings in *A. thaliana*. Kovalchuk *et al.* (2004) collected seeds near the Chernobyl area at sites with low and high levels of radioactive contamination. Progeny grown from these seeds were able to tolerate higher concentrations of different mutagens and showed a 10-fold lower frequency of extrachromosomal homologous recombination (HR) which was very likely due to the progenies' hypermethylation status and the resulting stability of their genomes. Two other studies on *A. thaliana* and tobacco plants further support the importance of somatic HRF for trans-generational stress adaption. In these studies, parental plants were either treated with UV-B or UV-C radiation. Progenies of UV treated plants exhibited higher frequencies of HR. Furthermore, progenies of plants treated with UV-B radiation simultaneously showed an induced expression of photolyases and *Rad51*, a gene involved in DNA and recombination repair as well as photoreactivation (Ries *et al.*, 2000; Molinier *et al.*, 2006).

2 Objectives

The aim of this study was to test whether progenies of *P. pinaster*, derived from differentially stressed maternal environments, exhibit changes in gene expression of selected candidate defence genes, either constitutively or upon induction with MeJA across one generation. The experiment was designed to answer the following questions:

i) Do offspring of stressed mother trees express enhanced constitutive and induced defences?
ii) Are essential epigenetic genes directly affected by different maternal environments?
iii) Is there variation in maternal effects between different maternal environments?
iv) Are maternal effects dependent on the maternal genotype?

To answer these questions, we studied the gene expression of key enzymes involved in the shikimate and phenylpropanoid pathway, the methionine metabolism, as well as in epigenetics.

3 Material and Methods

3.1 Plant Material

The plant material utilized within this experiment originated from and was treated by the GENECOLPINE group (genecolpines.weebly.com), located at the CISC Pontevedra, Spain.

3.1.2 Maternal Plants

In 2006, three different *Pinus pinaster* genotypes (1002, 1043, and 2062) were clonally propagated via grafting terminal buds of two-year old seedlings. Ramets of each genotype were grown for two years and, thereafter planted in 50 L pots with an automated irrigation system arranged in a split-plot design replicated in three blocks. These blocks were divided into five whole plots. Each whole plot contained one of the three genotypes, respectively. In 2009, seedlings of all genotypes were subjected to controlled pollination by a fourth genotype (1052). Early in 2010, part of the clones was designated as maternal control environment and left untreated (CNT), whereas the rest of the trees were subjected to two different, randomized induction treatments to mimic two distinct biological stresses. The treatments were i) mechanical wounding, in which the bark and phloem of six circular, evenly distributed, mechanical wounds with a diameter of 5 mm was removed along the stem (MW), and ii) a chemical induction treatment with 25 mM MeJA solution which was homogenously applied to the bark of the tree's stem (MJ). The induction treatments were repeated in intervals of 15 to 30 days between early 2010 until autumn 2011. After a two year period of cone maturation the seeds were harvested, weighed, and stored at -30 ˚C.

3.1.3 Plant Progenies and Treatment

Seeds of the three different genotypes and three maternal environments were grown in a greenhouse for 18 weeks at 14 ºC at night (12 hours) and 25 ºC during day (12 hours) in 2 l pots. At the end of the growing season, the full-sib progenies were randomly assigned to one of three different treatments within each genotype and maternal environment. The first subset of plants was left untreated and designated as control. The second and third subsets were sprayed with 5 mM MeJA and randomly divided into two batches. One batch was harvested three hours (MJ3) – the other six hours post treatment (MJ6) and the fresh weight of each seedling was determined. Thereafter, the plants were weighed and stored at 0 ˚C in RNA*later*® Soln. (Ambion®, Austin, TX) and sent to the Swedish University of Agricultural Science, Uppsala where they were stored at -20 ˚C within a week after harvesting. Table 1 provides an overview of progeny treatments and their corresponding abbreviations.

Table 1| Overview of the different maternal environments, progeny treatments, and corresponding sample codes.

Maternal Environment	Progeny Treatment		
	Control (CNT)	*MeJA 3h (MJ3)*	*MeJA 6h (MJ6)*
Control (CNT)	CNT-CNT	CNT-MJ3	CNT-MJ6
Mechanical Wounding (MW)	MW-CNT	MW-MJ3	MW-MJ6
MeJA (MJ)	MJ-CNT	MJ-MJ3	MJ-MJ6

While the progenies were grown and infection decimated plants in genotypes 1002×1052 and 2062×1052 so that only CNT-CNT and MW-CNT progenies remained for genotype 1002×1052

and CNT-CNT, MW-CNT, and MJ-CNT plants for genotype 2062×1052. During the infection, growing conditions were changed to 10 ºC at night (12 hours) and 19.5 ºC during the day (12 hours).

3.2 RNA Isolation, Quantification, Quality Analysis and cDNA Synthesis

A total of 85 plants were extracted, quantified, and qualitatively analysed for this experiment.

3.2.1 RNA Isolation

The RNA extraction protocol of Chang *et al.* (1993) was optimized and modified for this experimental design. RNA extraction buffer (2 % CTAB, 2 % PVP, 100 mM Tris-HCl (pH 8.0), 2 M NaCl, 25 mM EDTA, H_2O, 2 % β- mercaptoethanol) was incubated at 65 ˚C for 30 min while whole plants (root material excluded) were transferred from RNA*later*® Soln. to liquid nitrogen and ground until sufficiently pulverized and kept frozen. The pulverized plant material was mixed with 3 ml of the extraction buffer and incubated at 65 ˚C for 10 min. Thereafter, the samples were extracted twice with equal volumes of chloroform : isoamyl alcohol (24 : 1) and centrifuged at 7000 rpm for 20 min. The RNA was precipitated from the aqueous phase by adding 0.25 volumes of 8 M LiCl (Sigma-Aldrich®, St. Louis, MO) and subsequent incubation at 4 ˚C overnight. The precipitation step was followed by a centrifugation step at 4 ˚C for 40 min. The pellet was briefly dried and dissolved in 100 µl H_2O. Hereafter, the RNA was further precipitated via sodium acetate – ethanol precipitation. Therefore, the solution was mixed with 0.1 volumes of 3 M NaAc (Sigma-Aldrich®, St. Louis, MO) and 2 volumes of cold, 95 % ethanol (Solveco, Rosersberg, Sweden) and precipitated at -20 ˚C for 1 to 2 hours. After centrifugation at 4 ˚C and 14000 rpm for 20 min, the RNA was washed with 100 µl 70 % cold ethanol and centrifuged at 7000 rpm for 5 min. The pellet was dried for several minutes and dissolved in 32 µl H_2O.

3.2.2 RNA Quantification and Quality Analysis

Genomic DNA contamination was removed via DNase 1 Amplification Grade Kit (Sigma-Aldrich®, St. Louis, MO) according to the manufacturer's instructions. Furthermore, all the samples were tested for DNA residues by PCR and agarose gel electrophoresis. RNA concentration was quantified via NanoDrop (Spectrophotometer ND 1000, Saven Werner). Due to very high RNA concentrations, part of the samples was diluted 1:10 before RNA quality, as well as quantity was further analysed using the Aligent RNA 6000 Nano Kit (Aligent Technologies, Santa Clara, CA) in an Aligent 2100 bioanalyzer (Aligent Technologies, Santa Clara, CA) following the manufacturer's instructions. RNA samples were stored at -70 ˚C.

3.2.3 Complementary DNA (cDNA) Synthesis

One µg of each RNA sample was reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions and diluted 1:1 with H_2O. Only plants with sufficient RNA quality and quantity were selected for cDNA synthesis. cDNA of a total of 68 plants was further used for qPCR.

3.3 Primer Design, Cloning, and Sequencing of Candidate Genes

One of the intentions of this experiment was to study genes that are known to be directly involved in epigenetic processes. However, these genes have not been the object of previous studies in

conifers and primers were not publicly available. Due to the lack of a completely sequenced *P. pinaster* genome an alternative genome and various data bases were utilized to obtain candidate gene sequences. Hence, orthologous candidate genes were identified by using the sequenced *Arabidopsis thaliana* and *Picea abies* genomes.

3.3.1 Primer Design

Protein sequences of the enzymes S-adenosyl-L-homocysteine hydrolase 1 (SAH1), methyltransferase 1 (MET1), and decreased DNA methylation 1 (DDM1) were obtained from The Arabidopsis Information Resource (TAIR) site (http://www.arabidopsis.org/). To identify orthologous sequences in gymnosperms, the selected protein sequences were used to conduct a database search in ConGenIE (http://congenie.org/). *P. abies* orthologous were selected based on the average identity (≥ 60 %) and the presence of important catalytic domains. Furthermore, a GBrowse search was performed to obtain trinity transcript assemblies of each gene. Thereafter, the orthologous protein sequences were retrieved from ConGenIE and run through a second database search in BLAST® (NCBI, National Center for Biotechnology Information; http://blast.ncbi.nlm.nih.gov/Blast.cgi) within the family of Pinaceae (taxid:3318) (E-value cutoff = $1e^{-4}$). For sequencing purposes, adequate sequences from ConGenIE and BLAST were aligned in the MEGA5 software (http://www.megasoftware.net/) and all primers were designed against specific gene regions using the web-based Primer3 software (Untergasser *et al.*, 2007). Fragment lengths varied between 1 to 2 kbp. A primer pair was also designed against a *phenylalanine ammonia-lyase (PAL)* gene (AY641535.1) and the *cinnamoyl alcohol dehydrogenase (CAD)* gene (FN824799.1). All primers were ordered at TAG Copenhagen.

3.3.2 Molecular Cloning and Sequencing of Candidate Genes

The amplicon of interest was amplified using cDNA from *P.pinaster* seedlings as template and PCR. The PCR products were separated via agarose gel electrophoresis and purified from the gel using the GeneJet™ Gel Extraction Kit (Fermentas Life Science, Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. The purified amplicons were quantified using NanoDrop (Spectrophotometer ND 1000, Saven Werner). Depending on the amplicon concentration, 1 µl to 4.5 µl of insert were added to 1 µl salt solution and 0.5 µl pCR2.1®-TOPO® vector, included in the TOPO® TA Cloning® Kit (Invitrogen™, Life Technologies, Carlsbad, CA), to obtain a total reaction volume of 6 µl (the remaining units of volume were filled with H_2O_{DEPC}). The ligation reaction was then incubated for 15 min at RT.

One Shot® Top 10 Chemically Competent *Escherichia coli* cells (Invitrogen™, Life Technologies, Carlsbad, CA) were transformed via the heat shock technique. Therefore, the *E. coli* cells were incubated with the ligation product (10 % of final volume) on ice for 30 min, heat shocked at 42 ˚C for exactly 30 sec, cooled down on ice for 3 min, carefully mixed with 100 µl S.O.C. Medium (Invitrogen™, Life Technologies, Carlsbad, CA), and incubated on a shaker at 250 rpm and 37 ˚C for 1 h. Thereafter, 75 µl of the cell suspension were streaked onto LB-medium (pH 7) (Sambrook & Russel, 2001), mixed with ampicillin (100 µg/ml) and X-gal (40 µg/ml) for blue – white screening, and incubated at 37 ˚C overnight. The white colonies were screened using M13 primers supplied by the manufacturer (Invitrogen™, Life Technologies, Carlsbad, CA) in a PCR reaction (instead of DNA a few bacterial cells were used as template) and the product was analysed via agarose gel electrophoresis. After colony screening, cells carrying the correct insert were selected and propagated in 3 ml of liquid LB-medium, mixed with ampicillin (100 µg/ml) and incubated on a shaker at 250 rpm and 37 ˚C overnight. Thereafter, *E. coli* cells were pelleted and the plasmids were isolated using the GeneJet™ Plasmid Miniprep Kit (Thermo Scientific, Waltham, MA)

according to the manufacturer's instructions. Plasmid concentration was quantified via NanoDrop (Spectrophotometer ND 1000, Saven Werner). The inserts were sequenced by Macrogen (Macrogen, Amsterdam, Netherlands).

3.4 Quantification of Transcript Levels via qRT-PCR

Oligonucleotides were designed against sequenced genes using the web-based Primer3 software (Untergasser *et al.*, 2007). If possible, primers were positioned in a conserved and 3'-UTR region of the gene. Genes and corresponding primer pairs analysed within this expression study are displayed in Table 2. The specificity of each primer pair was verified via agarose gel electrophoresis and gene specific standard curves were prepared using 10^3 to 10^7 copies of amplicon to determine the efficiency of the reaction. The copy number was calculated using the DNA Copy Number Calculator web-tool (Thermo Scientific, Waltham, MA).

Table 2| Oligonucleotide primers used for the quantification of the transcript levels.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon Size (bp)	T_m (˚C)
DAHP1 [a]	AGATCCCCAAAGGATGGTTC	TGAAATCCAAGTTCCACTGAA	122	58
DAHP2 [f]	AATGGGGACACCTTTGATGA	AAGCACGGAGCAAATTCAGT	100	58
DAHP3 [f]	TGCAACTGGAGGTTATGCA	TGCCATGAATCCAAGAGCTT	130	58
PAL	GATTTGCATCCCACTGGATT	GCAGTACACGATCACCCAACT	126	60
C4H2 [e]	CACCGTTGGAAGGCTGGTT	TTGTCACTTACATCCACCTTCGATT	-	60
CHI [b]	ATGGGTTGCAACTGGAGAAC	TCCAGTGCTTCCTCTTCCTC	82	60
CAD	AGGATTTGCAAGCAGTATGGT	TGCTTCATTGGGCTGAAAACT	123	60
CesA [c]	CCAGCCTGTTCTGTCTCTGTGTG	TGCATGAGAAGCACTGTCCCTTTG	133	60
AdoMetS [b]	GTAAACGGCAGGTTCCAAAA	TGACAATCTCCCAGGTGAAA	79	60
SAH1	GCCTAAGTTGGGTGCTCATC	GATTCGTCCTTCTGGCAACT	175	60
MET1	ATCCTGAACAAGACCGCATT	AGTGAACAGGTTATCTTTCTCAAGC	210	60
DDM1	TGTCGCATTGATGGAAGTGT	AGGTCGTGTTTGTCCAATCC	225	60
TUB1 [d]	GGCATACCGGCAGCTCTTC	AAGTTGTTGGCGGCGTCTT	67	60
MPP [c]	ACACCATTAACCATGGCCTC	CCTGCTCACGATCAATTCTTG	168	58
ACT [c]	ATCTCTCAGCACATTCCAACAG	TATTGCCACCATCATCTCAAGC	167	58

[a] Arnerup *et al.*, 2011
[b] Heller *et al.*, 2008
[c] Flores-Monterroso *et al.*, 2013
[d] Karlsson *et al.*, 2007
[e] Koutaniemi *et al.*, 2007
[f] provided by Jenny Arnerup

The qPCR was performed on an iQ5™ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA) for the quantification of transcript levels. The primer concentration was set to 500 nM and 1 µl cDNA was used per reaction. Three technical and five biological replicates (only four biological replicates for CNT-MJ6 (1043×1052) and MJ-CNT (2062×1052)) were run with the following thermal-cycling condition parameters: 95 ˚C for 30 sec; 40 cycles of 95 ˚C for 5 sec, 58/60 ˚C for 10/15 sec. A melting curve analysis was performed to verify single product amplification. The constitutively expressed genes *actin* (*ACT*), *mitochondrial processing peptidase* (*MPP*), and *α-tubulin* (*TUB1*) were used as reference genes and utilized to generate the BestKeeper (Pfaffl *et al.*, 2004). All genes were normalized to the BestKeeper and relative expression values were calculated using the Pfaffl equation (http://pathmicro.med.sc.edu/pcr/pcr-pfaffl.htm).

3.5 Statistical Analysis

The experiment considered three fixed factors – treatment (CNT, MJ3, MJ6), genotype (1002×1052, 1043×1052, 2062×1052), and maternal environment (CNT, MW, MJ). However, due to the fact that an infection reduced the number of plants in genotypes 1002×1052 and 2062×1052 significantly, progeny treatment with MeJA was only performed for genotype 1043×1052 (see Materials and Methods 3.1.3).

Therefore, four different comparisons were conducted – i) comparison of the relative constitutive expression level of genes between different maternal environments in genotype 1043×1052, ii) comparison of the gene expression induction upon treatment with MeJA between maternal environments at different time points in genotype 1043×1052, iii) comparison of the constitutive relative expression level of genes in the maternal environments CNT and MW between all three genotypes, iv) comparison of the constitutive relative expression level of genes in the maternal environments CNT, MW, and MJ between two genotypes (1043×1052, 2062×1052).

Data were square root transformed to meet the normality assumption of the residuals and a two-way ANOVA was conducted in Minitab software (Minitab Inc., State College, PA) for ii) – iv). Backtransformed means were utilized to generate graphs and compare maternal environments via a one-tailed t-test for i) – ii) in the Prism® 5 software (GraphPad Software, San Diego, CA). P-value cutoffs were set to 0.05 (*), 0.01 (**), 0.001 (***).

4 Results

4.1 Constitutive Gene Expression

The constitutive relative gene expression was determined by comparing untreated seedlings (genotype 1043x1052), differing in their maternal environment [control (CNT), mechanical wounding (MW), and methyl jasmonate (MJ)]. Progenies originating from the MJ maternal environment showed significant higher levels of steady-state mRNA for *DAHP2* (shikimate pathway), *CHI* (flavonoid pathway), *CAD* (lignin/lignan biosynthesis), *cellulose synthase* (*CesA*) (cellulose biosynthesis), *MET1* (epigenetics), and *DDM1* (epigenetics) relative to progenies of the CNT maternal environment (Figure 2).

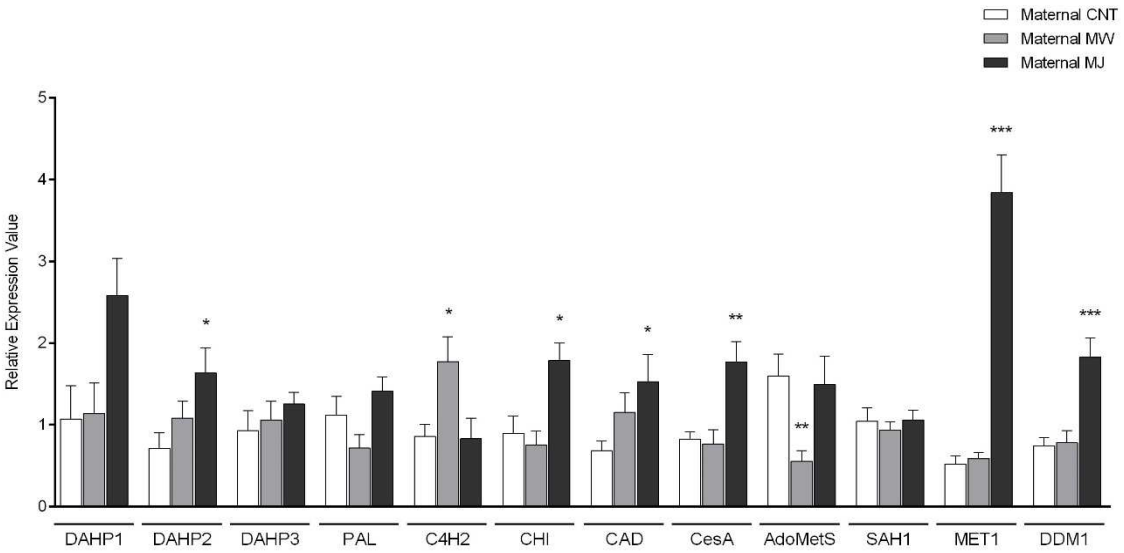

Figure 2| Relative constitutive expression levels of genes involved in the shikimate pathway (*DAHP1*, *DAHP2*, and *DAHP3*), the general phenylpropanoid pathway (*PAL*, *C4H2*), the flavonoid pathway (*CHI*), lignin/lignan biosynthesis (*CAD*), methionine metabolism (*AdoMetS*, *SAH1*), cellulose biosynthesis (*CesA*), and epigenetics (*AdoMetS*, *SAH1*, *MET1*, *DDM1*). qRT-PCR was conducted in progenies (genotype 1043×1052) of different maternal environments using three technical and five biological replicates for each gene. Genes were normalized against the average Ct-value of CNT-CNT, MW-CNT, and MJ-CNT progenies and data were statistically analysed using a t-test and p-value cutoffs were set to 0.05 (*), 0.01 (**), and 0.001 (***). Maternal environments: CNT – control (white); MW – mechanical wounding (grey); MJ – MeJA (black). Genes: *DAHP – 3-deoxy-D-arabino-heptulosonate 7-phosphate synthases*; *PAL – phenylalanine ammonia-lyase*; *C4H2 – cinnamic acid 4-hydroxylase 2*; *CHI – chalcone isomerase*; *CAD – cinnamoyl alcohol dehydrogenase*; *AdoMetS – S-adenosylmethionine synthase*; *SAH1 – S-adenosyl-L-homocysteine hydrolase 1*; *CesA – cellulose synthase*; *MET1 – methyltransferase 1*; *DDM1 – decrease in DNA methylation 1*.

The level of steady-state mRNA was significantly higher for *C4H2* (general polypropanoid pathway) and was significantly lower for *AdoMetS* (methionine metabolism, epigenetics) in progenies of the MW maternal environment relative to progenies of the CNT maternal environment.

The constitutive expression of all three *DAHP* genes was analysed and compared. The data (not shown) revealed that *DAHP3* was constitutively the highest expressed gene. *DAHP1* showed the second highest expression, whereas *DAHP2* expression was the lowest in comparison.

4.2 Induction of Gene Expression

The induction of different key genes of several defence pathways was studied (Table 3). Thereby, progenies of the genotype 1043×1052 and three different maternal environments, control (CNT), mechanical wounding (MW), and methyl jasmonate (MJ), were either left untreated (CNT), or were treated with MeJA for three hours (MJ3) or six hours (MJ6). To analyse the significance of maternal environment and treatment, as well as their interaction, a two-way ANOVA was conducted. Only transformed data were used to generate the ANOVA (Table 3).

The maternal environment was significant for all genes, except for *DAHP3*. This indicated an influence of the maternal environment on the observed gene expression patterns when subjected to MeJA. The treatment was significant for all genes, except for *CAD*, indicating that MeJA was responsible for the induction of gene expression and that *CAD* expression was only dependent on the maternal environment. *DAHP3* gene expression was solely dependent on the treatment. However, when evaluating the interaction of maternal environment and treatment, it was only significant for *DAHP1*, *PAL*, *CHI*, *CesA*, *AdoMetS*, *MET1*, and *DDM1*. This indicated a qualitative difference in gene expression patterns between different maternal environments upon treatment with MeJA.

Table 3| Two-way ANOVA of progenies of the genotype 1043×1052 with the fixed factors Maternal (Levels: CNT, MW, and MJ) and Treatment (Levels: CNT, MJ3, and MJ6). Bold values indicate qualitative differences in genes expression. P-value cutoffs were set to 0.05, 0.01, and 0.001.

Gene	Maternal		Treatment		Maternal×Treatment		R^2
	F-Value	*P-Value*	*F-Value*	*P-Value*	*F-Value*	*P-Value*	(%)
DAHP1	8.16	0.001	4.33	0.021	3.14	**0.026**	52.23
DAHP2	4.59	0.017	19.67	0.000	1.01	0.416	60.66
DAHP3	0.39	0.683	15.18	0.000	1.07	0.386	50.15
PAL	16.27	0.000	66.40	0.000	4.48	**0.005**	84.41
C4H2	3.60	0.038	15.45	0.000	0.86	0.496	54.54
CHI	34.77	0.000	72.87	0.000	5.89	**0.001**	87.75
CAD	3.63	0.037	0.02	0.978	0.52	0.721	21.96
CesA	6.01	0.006	25.81	0.000	3.67	**0.013**	69.93
AdoMetS	27.77	0.000	188.44	0.000	18.80	**0.000**	93.82
SAH1	3.89	0.030	9.92	0.000	2.03	0.112	51.71
MET1	114.82	0.000	7.19	0.002	15.34	**0.000**	89.77
DDM1	28.41	0.000	16.62	0.000	4.03	**0.009**	74.84

In order to understand the possible influence of phenotypic variables on the observed patterns, the effect of covariates was tested, including the number of days until plants germinated, seed weight, as well as the fresh weight of the progenies when harvested. Even though the raw data showed a decline in fresh weight in progenies of the CNT to MJ to MW maternal environments (even in different genotypes) neither of the covariates, either when introduced individually or when included combined, showed a change in the observed expression patterns following MeJA treatment.

4.2.1 The Shikimate Pathway

The enzyme DAHP marks an entry point to the secondary metabolism. It is the first enzyme of the shikimate pathway which precedes the phenylpropanoid pathway. Therefore, analysis of *DAHP* expression might give some indication about the allocation of carbon to the secondary metabolism.

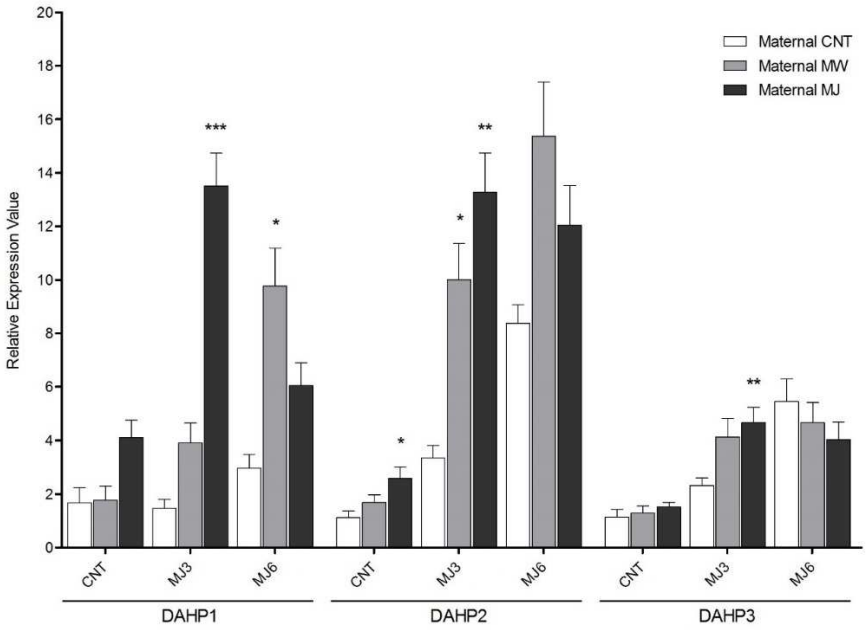

Figure 3| Relative induced expression levels of genes involved in the shikimate pathway (*DAHP1*, *DAHP2*, and *DAHP3*). qRT-PCR was conducted in progenies (genotype 1043×1052) of different maternal environments using three technical and five biological replicates (4 biological repeats for MJ6) for each gene. Genes were normalized against the average Ct-value of CNT-CNT progenies and data were statistically analysed using a t-test and p-value cutoffs were set to 0.05 (*), 0.01 (**), and 0.001 (***). Maternal environments: CNT – control (white); MW – mechanical wounding (grey); MJ – MeJA (black). Treatments: CNT – control; MJ3 – MeJA 3 h; MJ6 – MeJA 6 h. Genes: *DAHP – 3-deoxy-D-arabino-heptulosonate 7-phosphate synthases*.

Upon application of MeJA to the plant, gene expression of all three *DAHP* paralogues was induced in treatments MJ3 and MJ6 for progenies of all maternal environments (Figure 3, Table 3). All three *DAHP* paralogues showed stronger induction in progenies of the MJ maternal environment in treatment MJ3, whereas *DAHP1* and *DAHP2* displayed stronger induction in progenies of the MW maternal environment in treatment MJ6 compared to control. Generally, progenies of MW/MJ maternal environments showed significantly higher and faster induction of *DAHP* gene expression relative to progenies of the CNT maternal environment. Furthermore, there seemed to be a qualitative difference in gene expression patterns between progenies of CNT/MW and MJ maternal environments. Whereas there was a continuous increase in gene expression for all three *DAHPs* across the 6 hours of treatment in progenies of CNT/MW maternal environments, there was a stronger and faster response in progenies of the MJ maternal environment that showed higher expression at MJ-MJ3 and decreased towards MJ-MJ6.

4.2.2 The Phenylpropanoid Pathway and Cellulose Biosynthesis

The phenylpropanoid pathway succeeds the shikimate pathway and generates a wide array of different defence related molecules, alternatively carbon is derived to cellulose biosynthesis which stabilizes and strengthens the cell walls. To analyse the carbon flow within the phenylpropanoid pathway and cellulose biosynthesis and its dependence on different maternal environments and treatments, the gene expression for *PAL* (general phenylpropanoid pathway), *C4H2* (general phenylpropanoid pathway), *CHI* (flavonoid pathway), *CAD* (lignin/lignan biosynthesis), and *cellulose synthase* (*CesA*) (cellulose biosynthesis) was analysed (Table 3).

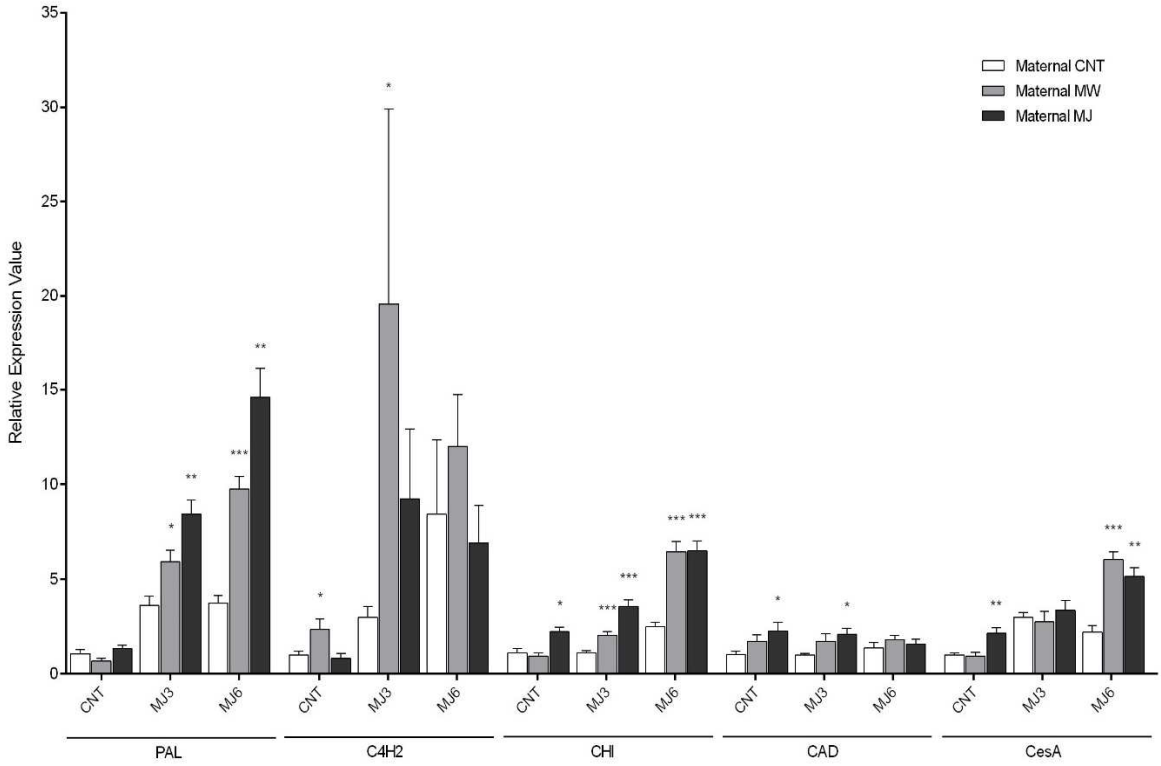

Figure 4| Induced relative expression of genes involved in the general phenylpropanoid pathway (*PAL*, *C4H2*), the flavonoid pathway (*CHI*), lignin/lignan biosynthesis (*CAD*), and cellulose biosynthesis (*CesA*). qRT-PCR was conducted in progenies (genotype 1043×1052) of different maternal environments using three technical and five biological replicates (4 biological repeats for MJ6) for each gene. Genes were normalized against the average Ct-value of CNT-CNT progenies and data were statistically analysed using a t-test and p-value cutoffs were set to 0.05 (*), 0.01 (**), and 0.001 (***). Maternal environments: CNT – control (white); MW – mechanical wounding (grey); MJ – MeJA (black). Treatments: CNT – control; MJ3 – MeJA 3 h; MJ6 – MeJA 6 h. Genes: *PAL – phenylalanine ammonia-lyase*; *C4H2 – cinnamic acid 4-hydroxylase 2*; *CHI – chalcone isomerase*; *CAD – cinnamoyl alcohol dehydrogenase*; *CesA – cellulose synthase*.

MeJA induced the expression of all studied genes, except for *CAD*, in progenies of all maternal environments (Figure 4). The relative gene expression of *PAL* and *CHI* showed a higher and progressing up-regulation in MJ3/MJ6 treatments in MW/MJ maternal environments when compared to control. The relative expression values for *CHI* only increased after six hours of MeJA treatment in progenies of the CNT maternal environment, whereas *CHI* was already induced three hours after MeJA treatment in progenies of MW/MJ maternal environments. *C4H2* also showed a stronger and faster induction response in progenies of MW/MJ maternal environments relative to control. However, this response decreased in intensity towards treatment MJ6 in progenies of MW/MJ maternal environments, whereas progenies of the CNT maternal environment showed a continuous increase in *C4H2* from three to six hours after application of MeJA. The results for *C4H2* in progenies of the MW maternal environment were very variable which was mirrored by a large standard error. *CesA* displayed a continuous increase in relative expression across the six hours of treatment (Figure 4). However, differences in relative expression were only significant for treatment MJ6 in progenies of MW/MJ maternal environments in comparison to progenies of the CNT maternal environment.

C4H2, *CHI*, and *CesA* displayed the strongest induction in fold in progenies of the MW maternal environment after six hours of MeJA treatment, whereas *PAL* showed the strongest induction in progenies of the MJ maternal environment (Figure 4).

CAD displayed significantly higher levels of steady-state mRNA in progenies of the MJ maternal environment (Figure 2). Upon treatment with MeJA the expression patterns of *CAD* did not change in progenies of any of the maternal environments (CNT, MW, and MJ) (Figure 4). It seems that MeJA treatment did not have any influence on *CAD* expression, but that rather the maternal environment was responsible for the observed differences in relative gene expression (see Table 3).

4.2.3 Genes Involved in Methionine Metabolism and Epigenetics

AdoMetS and *SAH1* are involved in the methionine metabolism, whereas *MET1* and *DDM1* are responsible for methylation maintenance. Therefore, studying these genes might give some indication of how epigenetics and maternal effects are connected.

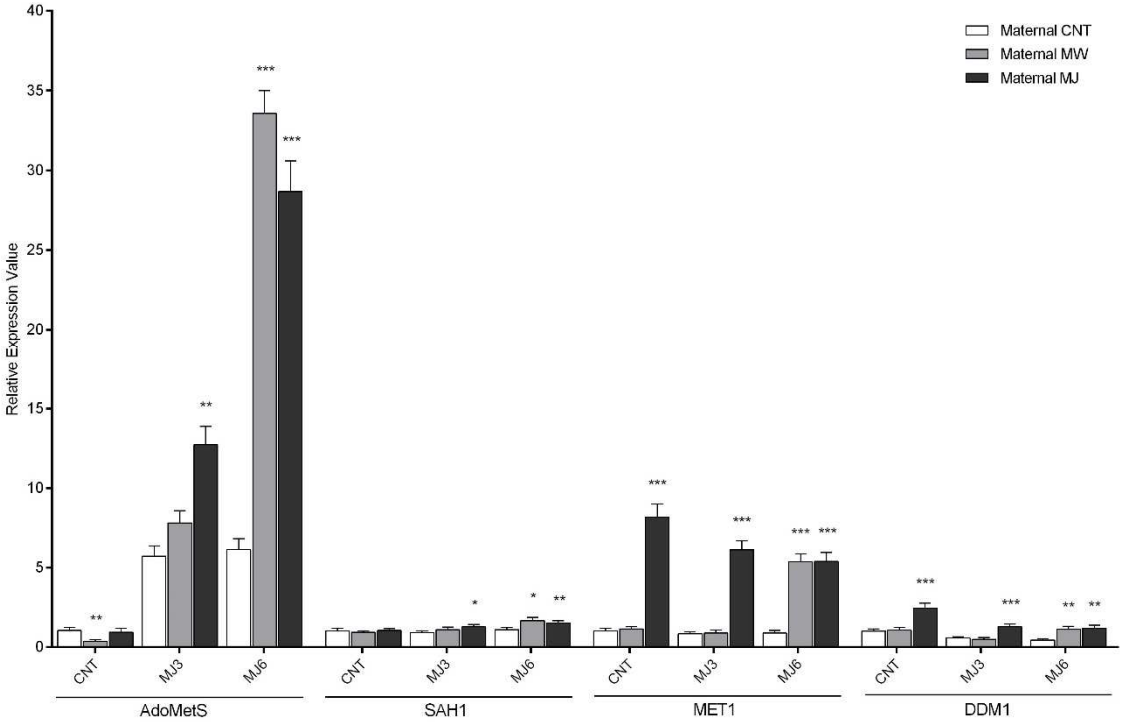

Figure 5| Induced relative expression of genes involved in methionine metabolism (*AdoMetS*, *SAH1*) and epigenetics (*AdoMetS*, *SAH1*, *MET1*, *DDM1*). qRT-PCR was conducted in progenies (genotype 1043×1052) of different maternal environments using three technical and five biological replicates (4 biological repeats for MJ6) for each gene. Genes were normalized against the average Ct-value of CNT-CNT progenies and data were statistically analysed using a t-test and p-value cutoffs were set to 0.05 (*), 0.01 (**), and 0.001 (***). Maternal environments: CNT – control (white); MW – mechanical wounding (grey); MJ – MeJA (black). Treatments: CNT – control; MJ3 – MeJA 3 h; MJ6 – MeJA 6 h. Genes: *AdoMetS – S-adenosylmethionine synthase*; *SAH1 – S-adenosyl-L-homocysteine hydrolase 1*; *MET1 – methyltransferase 1*; *DDM1 – decrease in DNA methylation 1*.

MeJA treatment significantly induced *AdoMetS* in progenies of MW/MJ maternal environments when compared to progenies of the CNT maternal environment (Figure 5). At treatment MJ3 progenies of the MJ maternal environment showed stronger induction, whereas after six hours of MeJA treatment the induction in fold of *AdoMetS* was higher in progenies of the MW maternal environment. *SAH1* expression was significantly induced in progenies of MW/MJ maternal environments after treatment with MeJA. However, the greatest difference in relative expression was observed in treatment MJ6. There was no induction of *SAH1* in progenies of the CNT maternal environment at all. When comparing transcript abundance of *AdoMetS* and *SAH1* directly (data not

shown), *SAH1* is continuously higher expressed than *AdoMetS*, in progenies of all maternal environments and in all treatments. However, induction with MeJA increased *AdoMetS* much faster in relation to *SAH1* in treatments MJ3 and MJ6.

MET1 and *DDM1* displayed a similar expression pattern. Both genes showed a significantly increased relative constitutive expression in progenies of the MJ maternal environment relative to control. However, upon application of MeJA, the relative expression of both genes was progressively down-regulated after three and six hours of treatment in progenies of all maternal environments (CNT, MW, and MJ). The only exceptions were progenies of the MW maternal environment that were treated with MeJA for six hours. These progenies showed a significant up-regulation of both genes. Nevertheless, progenies of the MJ maternal environment showed significant differences in relative *MET1* and *DDM1* expression values for all treatments (CNT, MJ3, and MJ6) relative to control.

4.3 Influence of the Genotype on Constitutive Expression

To analyse the significance of maternal environment and genotype, as well as their interaction, a two-way ANOVA was conducted. Only transformed data were used to generate the ANOVA.

When studying differences in constitutive gene expression between CNT, MW, and MJ maternal environments across genotypes 1043×1052 and 2062×1052, fresh weight had a significant contribution to *DAHP2*, *PAL*, *CAD*, *AdoMetS*¸ *MET1*, and *DDM1* expression (Table 4). This indicated that in contrast to progenies within one genotype, differences in relative constitutive gene expression between genotypes were related to the size of the seedlings for some genes. Furthermore, the value for the coefficient for fresh weight was checked (either positive or negative). The coefficient was negative for *DAHP2*, *CAD*, *MET1*, and *DDM1*, referring to a stronger expression in smaller plants, and positive for *PAL* and *AdoMetS*, indicating a stronger expression in larger plants. Data on seed weight and the number of days until germination were introduced to the ANOVA (data not shown) and resulted in no significant changes.

The statistical analysis showed that there were quantitative and qualitative differences in constitutive gene expression between the two studied genotypes (Table 4). *PAL* and *CAD* showed quantitative differences in steady-state mRNA levels between the genotypes. *CesA*, *MET1*, and *DDM1* displayed quantitative differences in steady-state mRNA levels between maternal environments, as well as both genotypes. Furthermore, *CHI*, *AdoMetS*, *MET1* and *DDM1* were significant for the interaction of Maternal and Genotype, indicating qualitatively different constitutive gene expression patterns between maternal environments in different genotypes. In case of *CHI* these differences were due to significantly lower levels of steady-state mRNA for *CHI* in progenies of CNT/MW maternal environments in genotype 1043×1052 when compared to genotype 2062×1052 (data not shown). *AdoMetS* displayed significantly lower levels of steady-state mRNA in progenies of the MW maternal environment in genotype 1043×1052 compared to genotype 2062×1052 (data not shown). *MET1* steady-state mRNA levels were significantly lower in progenies of CNT/MW maternal environments and significantly higher in progenies of the MJ maternal environment in genotype 1043×1052 in comparison to genotype 2062×1052 (data not shown). *DDM1* steady-state mRNA levels were significantly higher in progenies of the MJ maternal environment in genotype 1043×1052 compared to genotype 2062×1052 (data not shown). These differences in steady-state mRNA levels caused the significant interaction of Maternal and

Genotype. *DAHP1*, *DAHP2*, *DAHP3*, *C4H2*, and *SAH1* showed no significant difference between maternal environments and genotypes and were not significant for their interaction, either.

Table 4| Two-way ANOVA of progenies of different genotypes with fixed factors Maternal (Levels: CNT, MW, and MJ) and Genotype (Levels: 1043×1052 and 2062×1052). Fresh Weight is viewed as covariate. P-value cutoffs were set to 0.05, 0.01, and 0.001.

Gene	Fresh Weight		Maternal		Genotype		Maternal×Genotype		R^2
	F-Value	*P-Value*	*F-Value*	*P-Value*	*F-Value*	*P-Value*	*F-Value*	*P-Value*	(%)
DAHP1	0.53	0.476	0.20	0.823	0.01	0.939	1.75	0.197	15.95
DAHP2	8.12	0.009	0.51	0.605	4.03	0.057	2.90	0.076	52.58
DAHP3	2.89	0.103	0.05	0.947	0.01	0.918	0.97	0.395	22.40
PAL	13.61	0.001	0.04	0.961	4.90	0.038	2.22	0.132	55.08
C4H2	0.28	0.604	0.51	0.606	0.12	0.734	2.02	0.156	21.01
CHI	3.98	0.059	2.67	0.092	3.33	0.082	4.63	0.021	52.09
CAD	6.23	0.021	1.23	0.310	43.65	0.000	2.07	0.150	72.83
CesA	4.28	0.051	8.75	0.002	7.73	0.011	3.20	0.060	65.26
AdoMetS	6.38	0.019	0.32	0.730	1.83	0.189	3.68	0.042	46.15
SAH1	2.75	0.111	0.17	0.845	0.59	0.452	0.13	0.878	20.54
MET1	11.31	0.003	50.13	0.000	9.87	0.005	53.24	0.000	91.65
DDM1	12.80	0.002	8.70	0.002	27.77	0.000	16.44	0.000	80.47

Fresh weight was also significant for *DAHP3*, *PAL*, *CesA*, *AdoMetS*, *MET1*, and *DDM1*, when studying differences in constitutive gene expression between CNT and MW maternal environments across genotypes 1043×1052, 1002×1052, and 2062×1052. The coefficient was negative for *DAHP3*, *MET1*, and *DDM1*, and positive for *PAL*, *CesA*, and *AdoMetS* (Table 5). Data on seed weight and the number of days until germination were introduced to the ANOVA (data not shown) and resulted in no significant changes between the genotypes.

The statistical analysis showed that there were quantitative and qualitative differences in constitutive gene expression between the three studied genotypes (Table 5). *DDM1* showed quantitative differences in steady-state mRNA levels between the maternal environments. *PAL*, *CHI*, *CAD*, and *CesA* displayed quantitative differences in steady-state mRNA levels between the genotypes. *AdoMetS*, and *MET1* showed quantitative differences in steady-state mRNA levels between maternal environments, as well as the three genotypes. Furthermore, *DAHP1*, *DAHP2*, *PAL*, *C4H2*, *CAD*, *AdoMetS*, *MET1* and *DDM1* were significant for the interaction of Maternal and Genotype, indicating qualitatively different constitutive gene expression patterns between maternal environments in different genotypes. In case of *CAD* the qualitative differences in quantitative expression were due to higher levels of steady-state mRNA in progenies of the CNT maternal environment compared to the MW maternal in genotype 1043×1052, as well as in comparison to the other two genotypes (Figure 6). Qualitative differences in constitutive expression between genotypes for *DAHP1*, *DAHP2*, *PAL*, *C4H2*, and *AdoMetS* were due higher levels of steady-state mRNA of these genes in progenies of the CNT maternal environment relative to progenies of the MW maternal environment in genotype 1002×1052, as well as compared to the other genotypes. *MET1* and *DDM1* showed different levels of steady-state mRNA in all genotypes. *DAHP3* and *SAH1* show no significant difference between maternal environments and genotypes and are not significant for their interaction, either.

Table 5| Two-way ANOVA of progenies of different genotypes with fixed factors Maternal (Levels: CNT and MW) and Genotype (Levels: 1043×1052, 2062×1052, and 1002×1052). Fresh Weight is viewed as covariate. P-value cutoffs were set to 0.05, 0.01, and 0.001.

Gene	Fresh Weight		Maternal		Genotype		Maternal×Genotype		R^2
	F-Value	*P-Value*	*F-Value*	*P-Value*	*F-Value*	*P-Value*	*F-Value*	*P-Value*	(%)
DAHP1	0.14	0.710	2.08	0.162	1.71	0.203	3.47	0.048	42.54
DAHP2	2.00	0.170	0.53	0.473	2.30	0.123	8.59	0.002	50.56
DAHP3	5.57	0.027	0.60	0.447	0.51	0.606	0.57	0.576	36.41
PAL	15.44	0.001	1.69	0.207	7.73	0.003	8.58	0.002	81.28
C4H2	1.61	0.217	0.00	0.969	0.64	0.537	5.32	0.013	43.15
CHI	1.39	0.251	1.96	0.175	7.33	0.003	2.24	0.129	59.59
CAD	1.61	0.217	0.74	0.398	21.09	0.000	11.75	0.000	74.83
CesA	4.27	0.050	0.78	0.386	12.50	0.000	0.40	0.675	71.44
AdoMetS	12.06	0.002	7.06	0.014	11.83	0.000	11.00	0.000	84.44
SAH1	4.06	0.056	0.11	0.742	0.26	0.771	0.09	0.914	24.10
MET1	7.35	0.012	5.70	0.026	88.42	0.000	3.41	0.050	89.56
DDM1	11.50	0.003	9.47	0.005	0.95	0.402	3.99	0.033	51.86

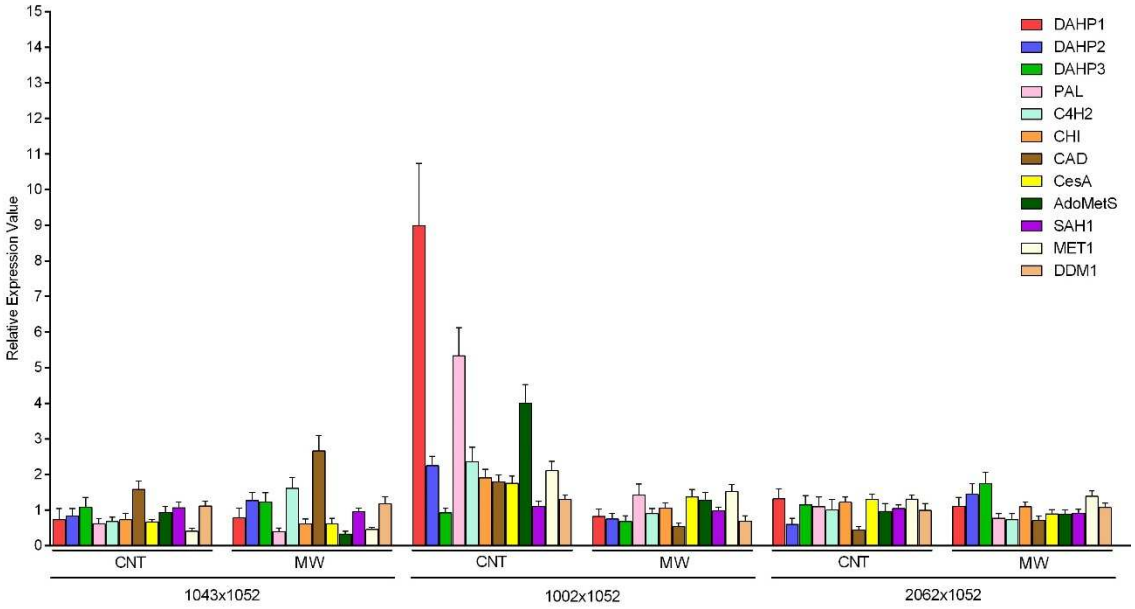

Figure 5| Relative constitutive expression levels of genes involved in the shikimate pathway (*DAHP1*, *DAHP2*, and *DAHP3*), the general phenylpropanoid pathway (*PAL*, *C4H2*), the flavonoid pathway (*CHI*), lignin/lignan biosynthesis (*CAD*), methionine metabolism (*AdoMetS*, *SAH1*), cellulose biosynthesis (*CesA*), and epigenetics (*AdoMetS*, *SAH1*, *MET1*, *DDM1*). qRT-PCR was conducted in progenies of different genotypes (1043×1052, 1002×1052, and 2062×1052) and different maternal environments using three technical and five biological replicates for each gene. Genes were normalized against the average Ct-value of CNT-CNT, MW-CNT, and MJ-CNT progenies of all genotypes. Maternal environments: CNT – control; MW – mechanical wounding. Genes: *DAHP – 3-deoxy-D-arabino-heptulosonate 7-phosphate synthases*; *PAL – phenylalanine ammonia-lyase*; *C4H2 – cinnamic acid 4-hydroxylase 2*; *CHI – chalcone isomerase*; *CAD – cinnamoyl alcohol dehydrogenase*; *AdoMetS – S-adenosylmethionine synthase*; *SAH1 – S-adenosyl-L-homocysteine hydrolase 1*; *CesA – cellulose synthase*; *MET1 – methyltransferase 1*; *DDM1 – decrease in DNA methylation 1*.

5 Discussion

In this study, the constitutive and induced gene expression of a number of candidate genes of the shikimate and general phenylpropanoid pathway, the flavonoid pathway, lignin/lignan biosynthesis, methionine metabolism, as well as epigenetics was analysed in *P. pinaster* progenies originating from different maternal environments and genotypes.

Causing stress to mother trees with MeJA or mechanical wounding produced consistent inherited constitutive effects in defence-related and epigenetic genes in *P. pinaster* progenies. Constitutive gene expression was quantified in full-sib progenies originating from different maternal environments (Figure 2). Since those progenies remained untreated, differences in constitutive gene expression were expected to be exclusively dependent on the maternal environments. The results showed that higher levels of steady-state mRNA were more common for progenies of the MJ maternal environment than the MW maternal environment, relative to progenies of the CNT maternal environment. The volatile plant hormone MeJA is a more effective stressor than mechanical wounding, thereby inducing stronger and more lasting defence responses (Moreira *et al.*, 2012; Yaqoob *et al.*, 2012). Thus, it is possible that the inherited changes of the basal level of defence gene expression, termed inherited induced resistance (Holeski *et al.*, 2012), are more distinct after treatment with MeJA and, as a result, were observed more frequently in progenies of the MJ maternal environment. Genes directly involved in epigenetics, *MET1* and *DDM1*, showed the greatest difference in steady-state mRNA levels in progenies of the MJ maternal environment compared to control. Since both genes code for enzymes involved in methylation status maintenance, these results suggest an increase in genome wide methylation, maybe causing an increase in genome stability that is usually triggered by trans-generational stress responses (Kovalchuk *et al.*, 2003; Kovalchuk *et al.*, 2004; Boyko *et al*, 2007). However, the results concerning *MET1* and *DDM1* differ from findings by Ou *et al.* (2012) who exposed *Oryza sativa* plants to heavy metal stress and examined *MET1* and *DDM1* expression in S0 and S1 generations. They were able to show that plants of the S0 generation exposed to heavy metals induced *MET1* but significantly down-regulated *DDM1*. The up-regulation of *MET1* was retained in S1, whereas *DDM1* was further down-regulated. These contrasting patterns support the idea of a high degree of specificity of the maternal environment in regulating maternal effects or even trans-generational responses.

The genotype seemed to predetermine maternal effects especially in genes involved in epigenetics. The constitutive gene expression and thereby maternal effects were studied in different genotypes (Table 4 and 5, Figure 5). The results showed that quantitative differences in constitutive gene expression for some genes that were solely dependent on the genotype (*PAL, CAD*), whereas others were dependent on the genotype and the maternal environment (*CesA, AdoMetS, MET1, DDM1*). Several defence related genes and especially *MET1* and *DDM1* showed qualitative differences in expression between genotypes. This is in line with findings of other studies (Pecinka *et al.*, 2009; Holeski *et al.*, 2013). Pecinka *et al.* (2009) used two different *A. thaliana* recombination lines, and exposed them to 10 different physical and chemical stresses and monitored somatic homologous recombination (SHR) events in the treated and two subsequent untreated generations. They found that distinct stresses stimulated SHR differently in both lines, especially in the two subsequent generations, and that one line was more respondent than the other. However, one needs to consider that different genotypes or lines are not only genetically diverse, but also epigentically. Latzel *et al.* (2012) showed a significant heritable epigenetic variation in epigenetic recombinant inbred lines

(epiRILs) that caused phenotypic changes such as in growth rate and intensity of response to salicylic acid (SA) and jasmonic acid (JA). Therefore, the observed differences in constitutive gene expression between genotypes might not solely be accounted for by genetic variation, but also by epigenetic variation.

Treatment with MeJA induced most of the studied genes and resulted in quantitative and qualitative differences in gene expression between maternal environments (Table 3). Interestingly, treatment with MeJA caused a much faster and stronger induction response for all genes (except *MET1*, *DDM1*, and *CAD*) in progenies derived from MW/MJ maternal environments in comparison to progenies of the CNT maternal environment, suggesting an inherited effect corresponding to defence induction. This is in accordance with findings by Luna *et al.* (2012) who showed that progenies of *A. thaliana* exposed to *Pseudomonas syringae* were primed to activate salicylic acid (SA)-inducable genes in response to attack. Further, progenies showed increased resistance to *Hyaloperonospora arabidopsis* and displayed reduced responsiveness to jasmonic acid (JA). Luna *et al.* (2012) were able to trace these changes back to acetylation and methylation events in key regulatory units of SA/JA responsive genes. However, another study by Holeski *et al.* (2013) described the opposite effect in *Populus* in response to herbivory. They found that phytochemical resistant traits were reduced by 10-18 % in offspring of trees exposed to herbivory in comparison to offspring of untreated trees and suggested an allocational trade-off. They assumed that maternal damage favoured maintenance of seed size and growth at the expense of phytochemical defences. In *P. pinaster*, seed size was insignificant for any of the maternal environments and fresh weight was only significant when comparing between genotypes. This suggests the absence of an allocational trade-off as described by Holeski *et al.* (2013). However, it remains to be seen if there are other allocational trade-offs as *P. pinaster* progenies mature.

Whereas progenies of the MJ maternal environment displayed an inherited induced resistance, progenies of the MW maternal environment showed a priming effect. Contrary to progenies of the MJ maternal environment that showed higher steady-state mRNA levels for several genes at the basal level, progenies of the MW maternal environment generally did not. Higher initial transcript abundance might have supported induction, resulting in a significantly stronger response compared to progenies of the CNT maternal environment. Interestingly, progenies of the MW maternal environment showed a similarly strong defence induction relative to progenies of the MJ maternal environment and both were significantly up-regulated in comparison to control. Since mechanical wounding is a weaker stressor than MeJA (see above), progenies derived from trees exposed to mechanical wounding likely display a priming response which is often triggered by weaker stressors and is represented by a stronger induction of gene expression when compared to progenies of the CNT maternal environment (Boyko & Kovalchuk, 2011; Holeski *et al.*, 2012). These results are in accordance with findings by Boyko *et al.* (2010) who studied transgenerational effects and genome stability in progenies of *A. thaliana* exposed to different NaCl concentrations (25 mM, 75 mM, 100 mM). Interestingly, 100 mM concentrations of NaCl had no effect on transgenerational genome rearrangements in the progenies, whereas 25 mM, a weaker stress, showed the strongest increase. Therefore, mild stresses might be necessary to induce transgenerational priming. Furthermore, the qualitatively different responses in progenies of the MJ and MW maternal environments suggest

that different maternal environments are able to specifically influence defence induction. We describe these findings figuratively in a hypothetical model (Figure 6).

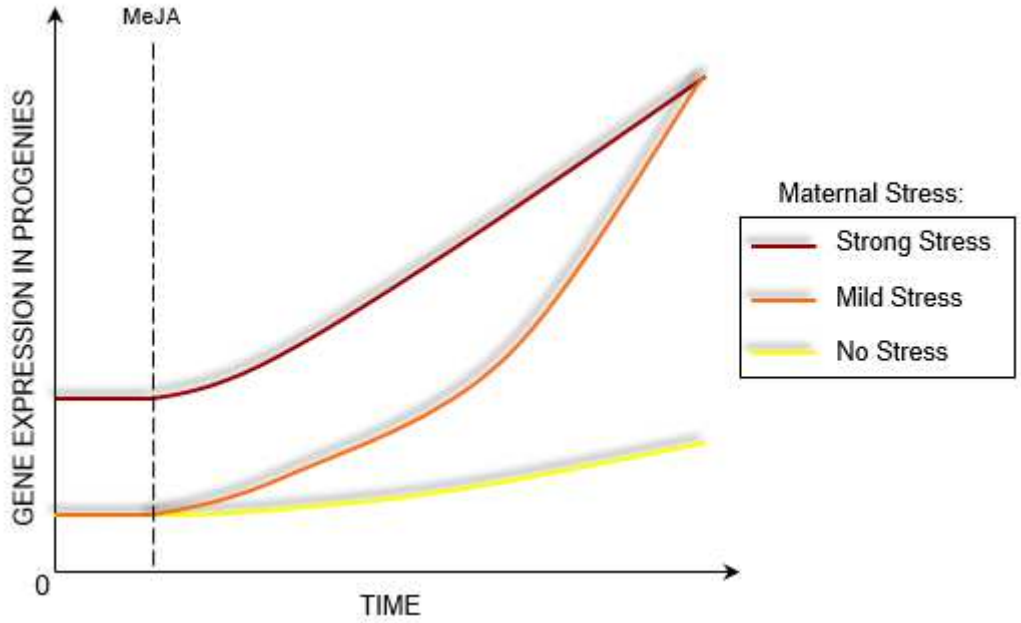

Figure 6| The hypothetical model shows qualitative differences in gene expression between maternal environments observed in several of the studied genes. The dotted line represents MeJA application. Carbon was allocated to various pathways previous to the defence induction in progenies originating from a maternal environment exposed to a strong stressor (i.e. the MJ maternal environment), causing an inherited induced resistance. In contrast, progenies derived from a maternal environment exposed to a mild stressor (i.e. the MW maternal environment) showed a similar level of steady-state mRNA as the control. As a result, progenies of a mildly stressed maternal environment had to increase their pool of metabolites much faster in response to a novel stress (MeJA) than progenies of a severely stressed maternal environment, to reach the same level of transcript abundance. This indicates an inherited priming effect caused by a mild stressors. In the end, both, progenies of a strongly stressed and mildly stressed maternal environment, displayed higher induction relative to progenies of the control maternal environment.

Three *DAHP* paralogues, previously described in Norway spruce (Arnerup *et al.*, 2011), were also present in *P. pinaster* and were responsive to MeJA, shifting the carbon flow from primary to secondary metabolism. All three *DAHP* paralogues showed significant up-regulation upon treatment with MeJA. Furthermore, *DAHP3* was constitutively the highest expressed *DAHP* paralogue in all maternal environments. A study by Adomas *et al.* (2007) also showed up-regulation of *DAHP2* after *Pinus sylvestris* had been inoculated with *Heterobasidion annosum*. The observations in *P. pinaster* differ from findings in Norway spruce by Arnerup *et al.* (2011), who observed the down-regulation of *DAHP1* and up-regulation of *DAHP2*, while *DAHP3* expression remained unchanged after inoculation of bark with *H. annosum*. They also observed that *DAHP1* was constitutively the highest expressed *DAHP* paralogue. These contrary results suggest differences in *DAHP* regulation and activity across species, as well as different organs and/or tissues.

As expected, the shikimate and the phenylpropanoid pathway were co-induced (Logemann *et al.*, 2000) and genes of the phenylpropanoid pathway responded in accordance to observations confirmed by several other studies analysing defence responses in conifers (Adomas *et al.*, 2007; Koutaniemi *et al.*, 2007; Vogt, 2010; Arnerup *et al.*, 2011; Danielsson *et al.*, 2011; Yaqoob *et al.*, 2012). Furthermore, *PAL* and *CHI* were also co-induced, suggesting co-regulation of the phenylpropanoid and flavonoid pathway which is in accord with findings by Danielsson *et al.*

(2011). Lignin/lignan biosynthesis seemed to be independently regulated. The unresponsiveness of *CAD* to MeJA is in accordance with findings in Norway spruce that showed no significant up-regulation for *CAD* upon inoculation with *H. annosum* (Danielsson *et al.*, 2011). However, *CAD* displayed quantitative differences in steady-state mRNA levels across distinct maternal environments (Figure 2). Furthermore, the results in *P. pinaster* suggested that differences in *CAD* expression were also dependent on the genotype (Table 4 and 5). A study by Fossdal *et al.* (2012) in Norway spruce also observed differential *CAD* expression in different genotypes. Since there are several members in the *CAD* family and only one member was studied in this experiment, it is likely that distinct genotypes rely differently on *CAD* isozymes that are important for lignin composition. Carbon was also allocated to cellulose biosynthesis and composition in response to stress, since *CesA* was induced by MeJA and up-regulated in all maternal environments. Furthermore, *CesA* showed expression patterns as described by the hypothetical model (Figure 6).

One could speculate that the progressive down-regulation of *MET1* across six hours of MeJA treatment in progenies of the MJ maternal environment might have caused demethylation of histone complexes, resulting in the activation of defence-related genes as a direct response to stress. *MET1* is an S-adenosin-*L*-methionine (AdoMet)-dependent *O*-methyltransferase (OMT) that is responsible for methylation maintenance of CG methylation after replication (Bird, 2007). Furthermore, it has been shown that *met1* mutants displayed a significant loss in H3K9 histone methylation which is needed to reinforce heterochromatin formation. H3K9 histone methylation was further assumed to act down-stream of CG methylation (Sahu *et al.*, 2013). Therefore, weakening of heterochromatin, caused by a decrease in *MET1* mediated methylation as a direct response to stress, might fail to repress otherwise active defence genes, suggesting a connection between changes in methylation patterns and defence induction. This is supported by finding of Dowen *et al.* (2012) who provided evidence of dynamic changes in DNA methylation in *A. thaliana* upon pathogen attack, leading to the transcriptional activation of defence genes that resulted in elevated resistance in plants. Therefore, alterations in the methylation patterns are likely to play a role in defence induction in plants.

Interestingly, the expression patterns of *MET1* and *DDM1* were similar in progenies of all treatments and maternal environments, pointing towards similar regulation mechanisms or a dependency of both genes.

A shift in the AdoMet/AdoHcy ratio might be related to the progressive down-regulation of *MET1* in treated progenies of the MJ maternal environment. AdoMetS synthesizes AdoMet, the substrate for AdoMet-dependent OMTs (Tehlivets *et al.*, 2013). In this experiment, *AdoMetS* strongly increased its pool of metabolites in all treatments and maternal environments. The induction of *AdoMetS* upon stress is in accordance with findings in *P. sylvestris* by Adomas *et al.* (2007). Whereas AdoMetS synthesizes the educt for AdoMet-dependent OMT mediated methylation, SAH1 reversibly hydrolizes their product S-adenosyl–*L*–homocysteine (AdoHcy) to homocysteine (Hcy) and adenosine. AdoHcy simultaneously acts as an inhibitor to AdoMet-dependent OMTs. Furthermore, the ratio of AdoMet to AdoHcy seems to give an indication about the methylation status of the cell (Caudill *et al.*, 2001; Tehlivets *et al.*, 2013). Interestingly, *SAH1* displayed a higher transcript abundance in all maternal environments and treatments in relation to *AdoMetS* (data not shown). Even though, direct comparison between *SAH1* and *AdoMetS* expression after induction (data not shown) displayed stronger induction for *AdoMetS* in comparison to *SAH1*. This might have shifted the AdoMet/AdoHcy ratio towards AdoHcy, assuming higher levels of AdoMet increased the methylation by AdoMet-dependent OMTs as well as the overturn of AdoMet to AdoHcy. Since OMTs not only methylate DNA, but also RNA, lipids, proteins, and defence

chemicals (Tehlivets *et al.*, 2013), the shift in AdoMet/AdoHcy ratio might be reflected by the progressive decrease of *MET1* in reference to time and treatment in progenies of MJ maternal environments. However, the AdoMet/AdoHcy ratio needs to be experimentally determined in progenies of the MJ maternal environment to reliably correlate the observed expression pattern for *MET1* to an inhibitory effect of AdoHcy.

6 Conclusion

Overall, the observed differences in gene expression at a basal level and upon induction between different maternal environments for several of the studied genes strongly indicate the presence of a maternal effect in the analysed *P. pinaster* progenies. These effects differed between genotypes. Furthermore, the observed maternal effects operated on different levels in various pathways and biological processes. Additionally, the maternal environment seemed to qualitatively influence and/or regulate inherited responses, since progenies originating from trees treated with MeJA rather exhibited an inherited induced resistance, whereas progenies derived from trees exposed to mechanical wounding displayed a priming response in comparison to progenies of the CNT maternal environments. Furthermore, a stronger defence induction might correlate to an enhanced adaptational fitness in progenies of the MW and MJ maternal environments, resulting in an increased survival chance of these progenies on hazardous sites. Furthermore, maternal effects might allow trees to adapt to biotic/abiotic environmental stresses much faster than originally thought. However, more research has to be conducted on the mechanisms that cause maternal effects and the nature of how they are inherited.

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