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Intergenerational environmental effects: Functional signals in offspring transcriptomes and metabolomes after parental jasmonic acid treatment in apomictic dandelion

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Summary

- Parental environments can influence offspring traits. However, it is poorly understood how large the impact is of parental environments on offspring molecular phenotypes. Here we test the direct effects and the intergenerational effects of jasmonic acid (JA) treatment, which is involved in herbivory-induced defense signaling, on transcriptomes and metabolomes in apomictic common dandelion (*Taraxacum officinale*).
- In a full factorial crossed design with parental and offspring JA and control treatments, we performed leaf RNA-seq gene expression analysis, LC-MS metabolomics, and total phenolics assays in offspring plants.
- Expression analysis, leveraged by a de novo assembled transcriptome, revealed an induced response to JA exposure that is consistent with known JA effects. The intergenerational effect of treatment was considerable: 307 of 858 detected JA-responsive transcripts were affected by parental JA treatment. In terms of numbers of metabolites affected, the magnitude of the chemical response to parental JA exposure was ~10% of the direct JA treatment response. Transcriptome and metabolome analyses both identified the phosphatidylinositol signaling pathway as a target of intergenerational JA effects.
- Our results highlight that parental environments can have substantial effects in offspring generations. Transcriptome and metabolome assays provide a basis for zooming in on the potential mechanisms of inherited JA effects.

Key words: RNA-Seq, LC-MS, Jasmonic Acid, induced defenses, transgenerational effects, *Taraxacum officinale* (common dandelion), metabolomics, transcriptomics.

Introduction

The phenotype of a plant can be affected by its direct ancestors' environmental experiences through effects on the parents that are transmitted to the offspring. While parental (or intergenerational) effects can be non-adaptive, they sometimes 'prepare' offspring for enhanced performance when the offspring experiences similar environmental stresses as the parents (Galloway & Etterson, 2007; Holeski et al., 2012). In such cases, intergenerational effects may be evolved adaptive responses to environmental stresses, extending adaptive phenotypic plasticity across generations (Herman et al., 2014).

One area in which parental effects are thought to be particularly relevant is in plant-insect and plant-pathogen interactions. Within a single generation, priming of systemic tissue for enhanced defense has been well-documented and can be induced by pathogen attack or other cues (Fu & Dong, 2013; Pieterse et al., 2014). Priming of the defense response does not constitutively activate defense responses, but results in a more rapid activation of the defense response upon subsequent pathogen attack. Such effects that are induced by pathogens or herbivores can persist to offspring (Holeski, 2007) and in some cases effects are sustained for multiple generations (Luna et al., 2012; Rasmann et al., 2012). The underlying mechanisms are not completely understood, but mounting evidence for durable epigenetic changes in response to environmental cues (Feil & Fraga, 2012) indicates at least one possible mechanism.

Although parental environmental effects on offspring phenotypes have been shown repeatedly, there is little knowledge of the extent to which gene expression is affected by parental environment, and the limited available data to date show mixed results. Examples of transgenerational effects in plants are associated with histone modifications at defense gene promoters (Luna et al., 2012) and with siRNA (Rasmann et al., 2012), implicating epigenetic gene regulation in offspring after parental exposure. Exposure of *Arabidopsis thaliana* to the bacterial elicitor flagellin was reported to increase homologous recombination frequencies in this plant and several subsequent generations; however, whole-transcriptome microarray analysis revealed no effects on offspring gene expression (Molinier et al., 2006). In sharp contrast, artificial leaf herbivory in *Mimulus guttatus* triggers gene expression changes at nearly a thousand genes in untreated offspring (Colicchio et al., 2015).

In addition to up- or downregulation of specific genes in offspring, also *variability* in offspring gene expression may be affected. Increased variability can arise due to variable penetrance

among offspring individuals or due to epigenetic mutations that are triggered stochastically in germline tissue in response to stress. In either scenario the result is hypothesized to be a bet-hedging strategy to increase levels of phenotypic variation among offspring, which may be adaptive when environments are variable (Levy et al., 2012; Herman et al., 2014).

Better insight in the consequences of parental environmental effects on offspring gene expression is important for understanding the ecological role and evolution of phenotypic plasticity. From a practical perspective it is also relevant to determine whether parental environments should be taken into account in the set-up of transcriptomic studies in general, which do not always control for pre-experiment variation. Here, we use RNAseq expression profiling and LC-MS metabolomics in the apomictic common dandelion (*Taraxacum officinale*) to evaluate how leaf gene expression and metabolites are affected in offspring due to jasmonic acid treatment in the parental generation. Jasmonic acid (JA) is a plant signaling hormone involved in various processes including the regulation of growth and responses to biotic and abiotic stresses (Wasternack & Hause, 2013) and plays a major role in the induction of plant chemical defenses in response to herbivory. Application of JA solutions to plants generally elicits an induction of chemical defenses that is systemic (e.g. Schenk et al., 2000; van Dam et al., 2004; De Vos et al., 2005; Tytgat et al., 2013).

T. officinale is a convenient natural model system for such studies because of its apomictic reproduction through clonal seeds (van Dijk, 2003) that permits an evaluation of transgenerational effects in absence of genetic differences between experimental plants. In *T. officinale*, effects of parental JA treatment on offspring epigenetic profiles (Verhoeven et al., 2010) and on offspring resistance to caterpillar feeding (Verhoeven & van Gurp, 2012) were previously reported, showing a potential role for epigenetically mediated parental effects on herbivore resistance in this species. In this study, we specifically aimed to (1) evaluate the intergenerational gene expression response, in terms of effects on gene expression means and variances, after parental JA treatment; and (2) evaluate if a parental effect of JA is associated with modified offspring leaf (secondary) chemistry including defense compounds.

Materials and Methods

Plant material and experimental design

The common dandelion, *T. officinale*, is a widespread perennial plant species that has diploid sexual and polyploid (mostly triploid) obligate apomictic variants (van Dijk, 2003). Apomixis

in dandelion is through meiotic diplospory followed by parthenogenetic embryo development from unreduced egg cells and autonomous endosperm development (Koltunow, 1993) which is thought to result in seeds that are clonal copies of the heterozygous mother plant. For this study we used a single triploid apomictic genotype (A68), an accession collected near Heteren (The Netherlands) that had been propagated for multiple generations under common greenhouse conditions prior to the experiment. This genotype has been studied previously in the context of parental effects (Verhoeven & van Gurp, 2012). Some genomic resources are available for *Taraxacum officinale*, including an EST database (Compositae Genome Project, compgenomics.ucdavis.edu) and a de novo assembled transcriptome based on RNAseq data of a different apomictic genotype than used for the current study (Ferreira de Carvalho et al., 2016), but currently no annotated reference genome has been published. For this study we generated a new de novo assembled reference transcriptome specific for the A68 apomictic genotype (see below).

Parental generation: Eight 'control' and eight 'JA' parental treatment lineages were derived from a single A68 founder individual by subjecting plants for two subsequent generations either to JA or control treatments under common climate chamber conditions (14h light / 10h dark at 20C / 15C, fully randomized pots) using single-seed descent between the generations. Exposing two subsequent generations to the same environmental stress can enhance parental effects compared to single-generation parental exposure (Wibowo et al., 2016). Based on previous experience in dandelion (Verhoeven & van Gurp, 2012) JA treatment was applied as 10 mM JA solution (Sigma J-2500, dissolved in ethanol and diluted to the desired concentration with a 0.1% Triton X-100 surfactant solution) to the upper surface of 3-4 fully expanded leaves. In generation 1, 0.75 ml JA solution per plant was applied to 8-week old plants; in generation 2, a total amount of 0.75 ml was applied to each plant distributed over two application moments when plants were 5 and 7 weeks old. In both generations JA was applied during vegetative growth, approximately 1 month prior to first flowering.

Experimental generation: For each of the G2 'control' and 'JA' parental treatment lineages, seeds from a single seed head were weighed individually, surface-sterilized (0.5% sodium hypochlorite wash) and germinated on 0.8% agar plates. After 10 days, seedlings were transplanted to individual pots and grown under climate chamber conditions as described above in fully randomized blocks. Each block contained two G3 plants from each of the eight JA (J) and control (C) parental lineages; one of these two plants received a JA treatment (JJ or CJ,

depending on parental lineage) and the other a mock treatment (JC and CC) (2 parental treatments x 2 experimental treatments x 8 independent replicates = 32 plants per block; see Fig. 1 for an overview of the experimental design). (JA was applied to 8-week old plants by distributing 0.25 ml of a 10 mM JA solution (see above) over the surface of two standardized leaves. Mock-treated plants received 0.25 ml of a similar ethanol/Triton-X solution without JA. In one block of plants, 3h after treatment two standardized leaves (younger than the JA-treated leaves) were collected, discarding the latex-rich mid-vein, flash-frozen in liquid nitrogen and stored at -80°C for subsequent RNA analysis. In a second block of 32 plants, leaf tissue was sampled in a similar way (but including the mid-vein) 24h after treatment for subsequent leaf chemical analysis; these samples were flash-frozen in liquid nitrogen, freeze dried and stored at -80°C. Three additional blocks of 32 plants were grown for time-series RT-qPCR gene expression analysis of a known JA early response gene (LOX2) to validate the induced JA response (see Supporting Notes S1). Note that for chemical and RT-qPCR expression analysis all available replicate plants were used but for RNA-seq expression analysis only six replicates per group were used (see below).

RNA-seq expression analysis

RNA isolation: Total RNA was isolated from liquid nitrogen-ground tissue using Trizol (Ambion, Life Technologies) according to the manufacturer's protocol, with an additional chloroform phase separation. Quality and concentration were checked on agarose gels and on a NanoDrop 2000 spectrophotometer. For each sample 10 µg of total RNA was DNase treated using the TURBO DNA-free kit (Ambion, Life Technologies). Quality and concentration were checked again on agarose gels and the NanoDrop spectrophotometer and samples were stored at -80°C until further use.

RNA-seq library preparation: Based on RNA quality, six samples from each of the four experimental groups (CC, CJ, JC, JJ; see Fig. 1) were prepared and barcoded individually using the TruSeq RNA Sample Prep kit v2 with 24 available barcodes from index sets A and B (Illumina, catalog numbers RS-122-2001 and -2002). Prior to sample preparation, we added ninety-two synthetic ERCC RNA spike-in control sequences (Jiang et al., 2011) (Ambion, Life Technologies, catalog number 4456739) at 50% of the manufacturer's recommended concentration. Samples were quantitated (Kapa Biosystems, catalog number KK4824) and tested using PCR primers of Ambion ERCC controls (Life Technologies, catalog number 4456739). Two ERCC controls of low concentration (ERCC 85 and ERCC 28) and two ERCC

controls of high concentration (ERCC 130 and ERCC 4) were amplified and cycle numbers compared. All samples showed a qualitative difference between low and high spikes. Samples were pooled and run as a single multiplexed library on the Illumina Hiseq with one lane at Florida State University (HiSeq 2000, Single end 101 bp) and two lanes at Wageningen University (HiSeq 2000, Paired end 101 bp). After demultiplexing it appeared that one sample (of the CJ group) was represented by very few reads and this sample was excluded from further analysis. The remaining samples were examined for quality using the ERCC controls. Plots of the expected concentration *vs* the read count for each sample (Fig. S1) and Bland-Altman (BA) plots (Bland & Altman, 1986) between samples (Fig. S2) showed high quality libraries, supporting quantitative interpretation of sequence read output.

De novo transcriptome assembly: Raw fastq files were de-multiplexed and adapters were trimmed using fastq-mcf (version 488 with default settings) from ea-utils (Aronesty, 2011), which trims adapters and filters reads based on a minimum phred score of 20. Additionally the first 10 nucleotides of all reads (both forward and reverse) were trimmed using seqtk (https://github.com/lh3/seqtk) because this was shown to improve assembly of full-length transcripts (van Gurp et al., 2013). Overlapping paired-end reads were merged using fastq-join from ea-utils (Aronesty, 2011). *De novo* transcriptome assembly was done using Trinity version trinityrnaseq-r2013-02-16 (Haas et al., 2013) using default settings. The final assembly (…insert DRYAD Digital Repository DOI…) contained 192,951 contigs (unique Trinity comp_c_seq combinations) with minimum, median, mean and maximum lengths of 200, 809, 1,107 and 17,258 bp, respectively. The contigs clustered into 77,530 putative genes (unique Trinity comp_c combinations). All 192,951 contigs were mapped to the reference proteome of eudicots (NCBI RefSeq) consisting of 1,312,075 reference proteins using uBLASTx in usearch version 6.0.307 (minimum E-value of 1e-5); this algorithm has similar sensitivity to NCBI BLASTx but is much faster (Edgar, 2010). Command line output parameters were set to default, except for the output format which was set as "-userfields query+target+thi+bits+raw+evalue+qlo+qhi+tlo+thi+qframe+tframe+ids+gaps+alnlen+qrow+trow+pv+ql" in order to get a tabular file that was subsequently converted to a xml input file as required by BLAST2GO (Conesa et al., 2005). BLAST2GO was used for performing annotations. A maximum of 20 top blast hits (with E-value < 1e-5) were retained per contig with associated GO terms as determined by BLAST2GO. Within BLAST2GO, Interproscan was run, for which results were obtained for a subset of 110,016 contigs. GO terms were derived in BLAST2GO based on both the blast hits as well as the interpro results. We observed that

different contigs (comp_c_seq combinations) that belonged to the same putative gene (comp_c combination) did not always produce matching annotations, which indicates that pooling contigs for an analysis at the putative gene level would introduce an unknown amount of error because of imperfect assignment of contigs to genes. Rather than working with this unknown level of uncertainty, we decided to analyze at the contig level. Although it likely carries a multiple testing penalty, this allows for more certain interpretation of the significant results.

Differential gene expression analysis: As mapping algorithms are greedy, all contigs were used as the reference for alignments. Samples were aligned using Bowtie (Langmead et al., 2009) with the following settings: --best, --tryhard, --strata, -a, -v 3 and LAST (Kielbasa et al., 2011) with the -l 25 setting. Several normalization strategies were evaluated (Dillies et al., 2013) using BA plots of the ERCC controls. The log(RPKM) was selected as its related BA plots were the most consistent among all replicates (Fig. S2). Contigs were retained for quantitative analysis if they were expressed at an average of at least 10 reads per nucleotide in all four experimental groups (CC, CJ, JC, JJ) and at least 500 nucleotides long (n=65827). Across all samples, this set of analyzed contigs had an average read coverage per nucleotide of 84.1 per individual sample (median 25.3). Applying the 10x coverage criterion to each of the experimental groups enables robust statistical analysis using linear models and discarded 49658 contigs that had low expression in *all* of the experimental groups. This approach also excluded 11766 contigs that showed no or low expression (<10x) in some treatments but not in all treatments. While we do not provide statistical evidence for treatment effects in these 11766 contigs, this set may include contigs that are downregulated in response to treatment in one or more of the experimental groups (see Table S1 for the list of 11766 contigs).

Normalized expression estimates were modeled using the following model: $Y_{ij}=\mu + t_i+\varepsilon_{ij}$ where i=(CC, CJ, JC, JJ) and j=(1,…6). The ε_I were assumed ~$N(0,\sigma^2_i)$ (Law et al., 2014). Initial model fits with a common variance assumption did not satisfy model assumptions of residuals: The F test of the null hypothesis of homoscedastic error was rejected for 43% of the contigs at FDR=0.05 and 62% of the contigs at FDR=0.20. In addition, BIC for the homoscedastic model was worse than BIC for the heteroscedastic model 100% of the time. Thus, we fitted heteroscedastic models, for each contig separately, that allowed for different error variances for each experimental group. Individual contrasts were conducted to test the effect of parental JA treatment while controlling for the current (experimental) JA treatment ($H_o:\mu_{CC}-\mu_{JC}=0$; $H_o:\mu_{CJ}-\mu_{JJ}=0$) and to test the effect of the current JA treatment controlling for the parent treatment

(H_o:μ_{CC}-μ_{CJ}=0; H_o:μ_{JJ}-μ_{JC}=0). Additional contrasts for the interaction between the parental and current JA treatment (H_o:μ_{CC}-μ_{CJ}=μ_{JJ}-μ_{JC}) and the effect of parent treatment on current JA-response (H_o:μ_{CC}-μ_{JJ}=0; H_o:μ_{JC}-μ_{CJ}=0) were conducted. All 65,827 contrasts were simultaneously corrected for false discovery (Storey & Tibshirani, 2003). Unless otherwise specified we consider an FDR of 0.10 to be significant. The results were qualitatively similar at FDR 0.05 and FDR 0.20. We selected FDR 0.10 as a balance between type I and type II errors (Verhoeven et al., 2005). Results were merged with annotation and enrichment tests were performed using annotation from BLAST2GO (Conesa et al., 2005). Because the number of differentially expressed genes was relatively low at the FDR = 0.10 significance threshold, we performed GO enrichment tests based on a significance threshold for individual transcripts at FDR = 0.20. This relaxed significance threshold results in a larger set of significant genes while maintaining the expected proportion of false positive results <20%, potentially allowing for more robust enrichment analysis. Fisher's Exact enrichment tests were done at the putative gene level, pooling for each putative gene all unique GO annotations associated with its underlying contigs and comparing the list of significant putative genes to the list of all genes analyzed.

Untargeted metabolomic profiling

LC-MS analysis: 20 mg of the freeze dried and finely ground leaf material was extracted with 200 µl methanol, followed by a second extraction with 200 µl of 20 % methanol containing 0.1 % formic acid. Both supernatants were combined and dried in a vacuum concentrator. Pellets were re-dissolved in 60 µl of 20 % methanol with 0.1 % formic acid, of which 5 µl was injected onto the analytical column. For Liquid Chromatography – Mass Spectrometry analysis a Synapt G2 mass spectrometer equipped with an Acquity UPLC (Waters) was used. Chromatography was done with a flow rate of 200 µl per min on a Waters Acquity C18 HSS T3 column, 2.1 x 100mm, 1.8 µm. A 10 min gradient from 99 % water to 100 % methanol (both solvents with 0.1 % formic acid) was used to separate the different compounds. For ionization, positive and negative electrospray ionization modes were used. The mass spectrometer was operated in MS and MS^E mode in parallel with a scan range from m/z 50 to 2000. Extraction and alignment of the raw data was carried out using Waters MarkerLynx software.

Data analysis: Peak intensities were normalized to a total intensity of 10000 per sample and filtered to include only mass signals present in five or more samples. To analyze differences between treatment groups, Principal Component Analysis (PCA) and Partial Least Squares

Discriminant Analysis (PLS-DA) on normalized data were performed in SIMCA 13.0.3. PLS-DA models were cross-validated with permutation tests (999 permutations). To select m/z values for further identification we followed a two-step approach. First, we performed Orthogonal Partial Least Square-Discriminant Analyses (OPLS-DA) to obtain S-Plots and visually selected mass signals that showed the clearest association with JA treatment (either parental or direct JA treatment). All the (O)PLS-DA models showed evidence of overfitting: R^2 and Q^2 of permuted data were not different from the R^2 and Q^2 of the real data (full model negative mode $Q^2 = 0.16$, CV-ANOVA $P = 0.12$; positive mode $Q^2 = 0.19$, CV-ANOVA $P = 0.15$). We therefore considered evidence from S-Plots as suggestive but not as conclusive for detecting associations between mass signals and treatment.

Second, each mass signal was modeled using an ANOVA, testing the effects of direct JA treatment, parental JA treatment and the direct JA x parental JA interaction on mass signal scores. Normalized mass signals were ln-transformed prior to this analysis. P values were subjected to FDR correction for multiple testing (across all p values from all model factors simultaneously) and were considered significant at FDR=0.1. Only mass signals were considered for which at least three samples were present in the filtered data set (normalized signal >0) in each of the four experimental groups and for which analysis of model residuals showed that residuals did not deviate significantly from a normal distribution (Shapiro-Wilk test $p>0.05$).

We combined evidence from the visual OPLS-DA S-Plots and ANOVA statistical testing approaches. We report as the subset of signals with high-confidence treatment effects those mass signals that were identified in both approaches. The putative identification of these relevant metabolites was based on mass spectra and molecular formula.

Total phenolics assay

Total phenolics concentration was quantified using a Folin-Denis based protocol as described elsewhere (Engelkes et al., 2008). Briefly, freeze-dried samples were ground to a fine powder and phenolics were extracted in 50% aqueous methanol at 90°C for 2h. Total phenolic concentration was determined by exposing the samples to Folin-Denis reagent and subsequently quantified spectrophotometrically at 750nm by comparing absorbance to a tannic acid calibration curve. Concentrations were expressed as tannic acid equivalent per gram of dried sample. All leaf samples were quantified in two independent replicates, whose phenolic content estimates were averaged for subsequent ANOVA to test for effects of JA treatment in the

parental and experimental generations. Initial seed weight of experimental plants was included in the model as a covariate to correct for effects of initial size on phenolics concentration.

Results

Effects of direct JA exposure on gene expression

RT-qPCR expression analysis of the JA early response gene LOX2 confirmed that the experimental JA treatment elicited a systemic response that is detectable in tissue of leaves that were not themselves exposed to JA (see Notes S1). In offspring of control parents, RNA-seq analysis detected 149 contigs that were differentially expressed due to direct JA exposure; and in offspring of JA-treated parents, 440 contigs were differentially expressed due to direct JA exposure (Fig. 2A; see Table S2 for all RNA-seq test results). 38 contigs responded to direct JA exposure irrespective of parental treatment, and accounting for this overlap a total of 551 unique contigs showed a direct JA effect when controlling for parental treatment. Most expression differences observed were upregulations due to JA treatment (~70% of affected contigs, Table S2). The 551 contigs clustered into 244 putative genes. At a more relaxed significance threshold of FDR = 0.20 we detected a total of 1519 significant contigs, clustering into 664 putative genes, and enrichment analysis revealed that this set of 664 differentially expressed genes was significantly enriched for GO terms associated with JA responses (Table 1). Considering only the most specific GO terms, 24 terms were significantly enriched including biological processes related to jasmonic acid biosynthesis: “Response to wounding”, “Response to other organisms”, “Response to host immune responses”, Pathogen-associated induction of host innate immune response”, and “Response to jasmonic acid” (Table 1). This confirms that our RNA-seq approach was successful in capturing the elicited JA effect.

Effects of parental JA exposure on gene expression

When controlling for experimental treatment in offspring plants, 18 and 74 contigs were differentially expressed due to parental JA exposure, in offspring groups that received JA or control treatment respectively (Fig. 2B). Two additional contrasts, CC vs. JJ and CJ vs. JC, captured joint effects of direct JA treatment and parental JA treatment and were significant for 190 and 237 contigs (Table S2). In total, across the entire experimental design 858 different contigs were differentially expressed between groups, of which 551 were detected as direct JA effects and the remaining 307 were detected only when taking also the parental treatment into account.

Hierarchical clustering of the 92 contigs that were differentially expressed in the same offspring environment due to different parental treatments indicates that expression of these genes in JC plants is more similar to JJ plants than to CC plants (Fig. 3). This is consistent with direct JA effects that are sustained into the offspring generation. However, only five contigs (which clustered into three putative genes) overlapped between the genes that were significantly affected due to direct JA treatment and due to parental JA treatment (Table S2).

At a more relaxed significance threshold of FDR = 0.20 451 differentially expressed contigs were detected after parental JA treatment, clustering into 173 putative genes. No significant enrichment of GO terms was observed among this set of 173 genes.Lack of significant GO term enrichment may be due to the low number of significant genes. However, the list of top BLAST hits for the contigs that showed a significant parental JA effect (Table S2) indicated several genes that are consistent with reported JA responses or plant defense function, such as genes associated with phosphoinositide signaling (two different inositol phosphate kinases; Sheard et al., 2010, Laxalt & Munnik, 2002); defense-associated fatty acid epoxidation (CYP77A; Sauveplane et al., 2009); receptor-like serine threonine kinases (often involved in pathogen recognition and defense signaling; Afzal et al., 2008); an ethylene-responsive transcription factor (involved in pathogen defenses and the integration of hormonal signaling under stress; Müller & Munné-Bosch, 2015); and respiratory burst oxidases (Torres & Dangl, 2005).

Effects of JA treatment on between-replicate variation in expression

44% of all analyzed contigs showed significant differences in variances between the four experimental groups (CC, CJ, JC, JJ) as indicated by a significant difference in the estimated variances (Folded F, FDR 0.05) and all models had an improved fit based on the BIC when the group-specific error variances were included compared to models that assumed a common error. In these contigs with significant heteroscedasticity, almost always the CJ group had the highest variance (96.3% of contigs, see Table S2). Although there is a difference in sample size (n=5 in the CJ group and n=6 in the other groups), it suggests that JA treatment leads to large between-replicate variation in gene expression 3h after treatment. However, no trend was observed that JA treatment of parents leads to increased variance in offspring gene expression. Indeed, the estimated variance of expression for the CC group was larger than the estimated variance for the JC group in the large majority of contigs with significant heteroscedasticity (Fig. 4).

Effects of direct and parental JA exposure on leaf chemical composition

Untargeted LC-MS metabolomics profiling detected on average 968 mass signals per sample (in total 1,210 across all samples) in the negative ionization mode and an average of 5,151 mass signals per sample (in total 7,728 across all samples) in the positive ionization mode (data accessible at … insert DRYAD Digital Repository DOI…). The two ion modes show a slightly different selectivity based on the propensity of a molecule to gain or lose a proton. For example, phenolic compounds are detected well in the negative mode, while N-based metabolites like alkaloids are generally better detected in the positive mode. In both modes, PCAs clearly separated samples based on the direct JA treatment 24h prior to tissue sampling (CJ and JJ *vs* CC and JC, Fig. 5). An effect was visible due to parental JA treatment: CC and JC samples clustered with only limited overlap (red versus green dots, Fig. 5). Such separation based on parental treatment was not observed in plants that received JA-treatment 24h prior to sampling (i.e. CJ and JJ, Fig. 5). ANOVA analysis also indicated a strong induction of the leaf metabolome by the JA treatment where approximately 16% of the tested mass signals showed a significant effect of direct JA treatment (Table 2). Also parental JA treatment had a significant effect (either as main effect or in interaction with experimental treatment, Table 2) in 1.6% of mass signals.

Based on visual inspection of S-plots from OPLS-DA we selected 33 mass signals as potentially associated with direct JA treatment and/or with parental JA treatment (Fig. S3). Of these, 16 were also significant in the ANOVA tests and six of these overlapping results could be putatively assigned to known compounds (Table S3). Based on these putative assignments, the experimental JA treatment response involved changes in linolenic acid, caftaric acid, phosphatidylglycerol and phosphatidylinositol. A response to parental JA treatment was detected in phosphatidylinositol and glycosylated malonic acid (Table S3). Mass signals that were selected in the OPLS-DA S-plots that showed an effect of JA but not significant in the ANOVA test included putative assignments to caftaric acid, chicoric acid, phosphatidylcholine and a glycosylated flavone.

When looking at a specific class of compounds with known anti-herbivore and anti-microbial responses, total phenolics, an effect of parental JA treatment was observed as well (Fig. 6). In offspring of control parents, JA-treatment increased leaf phenolics concentration within 24h. However, phenolic concentration did not reach the same level upon JA-treatment in offspring of JA-treated parents (Fig. 6). Although the interaction between parental and offspring JA

treatment was not significant ($0.05 < p < 0.1$, see Fig. 6) this suggests an inhibition of JA-inducibility of phenolics in offspring after parental JA treatment.

Discussion

Although parental environmental effects are well documented in plants, the extent to which molecular phenotypes are affected by environmental exposures in previous generations is largely unknown. Very few transcriptome-wide evaluations have been performed and results have been ambiguous, ranging from complete absence (Molinier et al., 2006) to very widespread effects (Colicchio et al., 2015) after parental exposure to cues associated with pathogen or herbivore attack. Our study provides evidence that the inherited effect of parental exposure on molecular phenotypes can be substantial, and supports a previously noted trend that transgenerational effects of herbivore and pathogen attack may be a widespread phenomenon in plants (Holeski et al., 2012). The effect that we observed on offspring after parental JA treatment is consistent with functions related to the treatment, with both transcriptomic and metabolomics analysis converging on phosphatidylinositol signaling as a transgenerationally affected pathway. More generally, our results suggest that interpretation of gene expression and other molecular studies need to be mindful of effects on the seed source.

Gene expression analysis revealed a clear functional response to direct JA treatment that is consistent with known JA-induced processes (Table 1 and Notes S1). Because we putatively identified only a modest number of JA-affected chemical compounds, a detailed pathway analysis based on the chemical JA response was not possible. However, qualitative evaluation of the compounds that were putatively identified indicated that the functional signal identified in the gene expression data was mirrored in the metabolomics data. Compounds that were JA-induced included the precursor of JA biosynthesis (linolenic acid; Wasternack & Hause, 2013; consistent with the detected JA treatment effect on the JA biosynthesis pathway). Also identified were the hydrocinnamate phenolics caftaric acid and chicoric acid. These are major phenolic compounds in species from the Compositae family (Cheminat et al., 1988; Oh et al., 2009) including dandelion (Schutz et al., 2005) that are thought to function in plant defenses against pathogens and herbivores (Lee & Scagel, 2013). In dandelion, phenolic inositol esters, triterpene acetates and a sesquiterpene lactone taraxinic acid ester are important secondary metabolites in latex (Huber et al., 2015). The JA response of the hydrocinnamate phenolics in our experiment, which matches the pattern observed in total phenolics (Fig. 6), suggests that

these phenolics are inducible secondary metabolites involved in herbivore defenses in dandelion.

An important result of our study is that there is also a functional signal in the *inherited* JA-response. Several of the 40 putative genes that showed a significant parental JA effect have known functions in JA- or defense-related processes. Strikingly, the gene expression and metabolomics analyses converged on one pathway that was affected by parental JA treatment: the phosphatidylinositol signaling pathway. Two of the identified genes with parental JA effect were phosphatidylinositol phosphate kinases, and one of only two parental JA effect-metabolites that could be putatively assigned was phosphatidylinositol. Thus, JA treatment had a durable effect on phosphatidylinositol signaling. Phosphatidylinositol phosphate kinases are enzymes that phosphorylate the precursor inositol in a pathway that produces various phosphatidylinositol phosphates and inositol phosphates, which subsequently act as intracellular second messengers upon perceiving an extracellular signal (Munnik & Testerink, 2009). Enzymes in this pathway are inducible by stress and plant hormone treatments (Lin et al., 2004) and play an important role in defense response signaling upon herbivore and pathogen attack (Laxalt & Munnik, 2002; Mosblech et al., 2008; Hung et al., 2014). Phospholipid signaling is involved in various aspects of biotic defense signaling, including JA biosynthesis by affecting linoleic acid production from plasma membranes; potentiating the COI1-JAZ complex for jasmonate recognition via a specific inositol phosphate cofactor; and intracellular signaling to activate and later downregulate defense gene expression after pathogen elicitor recognition (Laxalt & Munnik, 2002; Sheard et al., 2010; Zhang & Xiao, 2015). While the exact role of this pathway after JA treatment in dandelion remains to be determined, our congruent RNAseq and LC-MS results provide a clear starting point for future work to pinpoint the (epigenetic) mechanisms that enable a JA response to persist across generations.

Genes that showed a significant effect of parental JA-treatment were largely different from genes that showed an expression response upon JA treatment in the experimental generation. This is counter to the idea that gene expression in offspring is limited to a sustained activity pattern in a subset of JA-responsive genes (Bruce et al., 2007). However, this is perhaps an overly simplistic view when the timing of the JA-induced expression response is considered. Upon JA application a rapid succession of transcriptional regulatory programs unfolds with different genes being involved in the immediate, intermediate and long-term responses (Acosta & Farmer, 2010; Wasternack & Hause, 2013). In our experiment, we tested the early expression

response 3h after JA-treatment, which involves mostly different genes, and is poorly correlated to, the expression response that is observed at later stages (Tytgat et al., 2013). A subset of the later-stage genes may correspond to the genes that are still affected in offspring. An alternative explanation for the lack of overlap of differentially expressed genes after JA treatment versus after parental JA treatment could be related to low statistical power to detect differentially expressed genes. If only a modest subset of genes that are affected by the treatments are recognized as statistically significant, then limited overlap between the two sets of detected genes is expected even when many of the genes are in fact affected by both treatments.

It has been proposed that exposure to stressful environments can trigger enhanced variability among offspring individuals, rather than a mean shift in trait values or gene expression levels (Rapp & Wendel, 2005; Verhoeven et al., 2010; Herman et al., 2014). Enhanced variability, which is potentially mediated by an increased rate of transgenerationally stable epigenetic mutations, might reflect a bet-hedging strategy that increases the probability of at least some progeny to survive or maintain high fitness. In our experiment, JA treatment did increase variability in gene expression 3h after induction, which may reflect subtle timing differences in the early JA response between replicated plants. However, increased variance was not sustained into the offspring generation. In contrast, gene expression among offspring of JA-treated plants showed reduced variance more often than increased variance compared to offspring of control plants. This is perhaps due to a conditioning of the response and a reduction in stochastic expression. Such apparent canalization of gene expression after parental JA treatment is interesting and it suggests that there is some constraint on expression changes that does not exist in the control plants. Reduced variability in gene expression among offspring individuals might also be related to crosstalk between plant defense pathways. For instance, transgenerational priming of salicylic acid (SA)-related plant defenses after parental treatment with SA or with other hormones or inducing agents (Slaughter et al., 2012; Luna et al., 2012) can enhance offspring expression of SA-related genes but at the same time, due to crosstalk between JA and SA pathways, can suppress the activity of JA-related defense responses (Luna et al., 2012). Thus, transgenerational activation of one pathway may result in transgenerational suppression of another pathway, and such suppression may be reflected as reduced offspring expression variability in a subset of genes.

This study revealed effects of parental treatment on the offspring transcriptome and metabolome but the underlying mechanism of the transmission of the environmental effect

between the generations remains to be elucidated. Parental environmental effects can be mediated by various mechanisms. In our experiment parental JA treatment occurred well before flowering; thus no direct induction of the germline occurred. Possible mechanisms therefore include maternal modification of the embryonic hormone balance or inherited epigenetic effects (Boyko et al., 2007; Rasmann et al., 2012; Luna et al., 2012; Bond & Baulcombe, 2014) which is consistent with previous observation of JA-induced heritable modification of DNA methylation patterns in dandelion (Verhoeven et al., 2010).

In conclusion, our findings demonstrate that parental environmental conditions can have long-lasting, functional effects that are visible in the transcriptome and metabolome of offspring individuals. Our results imply that in any gene expression study environmental conditions should be controlled not only in the experimental generation but also in the previous parental generation. The proportion of affected genes may be considerable, as evidenced in our study where a single JA application in parental plants during vegetative growth, well before the induction of flowering, affected the expression of approximately one third of the JA-responsive genes in offspring plants. This observation provides insight into the scope of parental environmental effects. Our results also provides a starting point for further unraveling of the underlying mechanisms that mediate transgenerational effects in plant interactions with herbivores, pathogens or parasites, where such inherited effects may be particularly common (Poulin & Thomas, 2008; Holeski et al., 2012).

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Author Contribution

KV conceived and designed the study, EV performed the experiment and the phenolics analysis, MS performed the metabolomics analysis and MM and KV performed the statistical analysis of the metabolomics data, CO performed RNA isolations and RT-qPCRs, CO and AM did the RNA-seq library preparation, TvG and JFC performed the *de novo* transcriptome assembly and annotation, and AM, KV and LM performed the analysis of differential gene expression. KV

and LM wrote the manuscript with input from all co-authors. All authors read and approved the final manuscript.

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The following Supporting Information is available for this article:

Fig. S1 Observed read counts for synthetic ERCC RNA spike-in control sequences

Fig. S2 Bland-Altman plots for within-group pairwise comparisons based on ERCC controls

Fig. S3 OPLS-DA selection of JA-responsive mass signals

Table S1 Contigs excluded from statistical analysis, but meeting the coverage threshold in at least one of the experimental groups

Table S2 RNA-seq test results for differential expression analysis

Table S3 Putative identification of LC-MS mass signals

Notes S1 RT-qPCR expression validation of the early JA-response candidate gene LOX2

Data for this study are deposited:

Dandelion leaf chemical data (LC-MS peak intensity signals and total phenolics) and the *de novo* assembled dandelion transcriptome are deposited in the DRYAD digital repository (…insert DRYAD Digital Repository DOI…).

Dandelion RNA-seq reads are deposited in the NCBI Sequence Read Archive (BioProject accession number PRJNA316842; samples SRS2047454 - SRS2047475).

Tables

Table 1. Gene Ontology terms enriched among the JA-responsive genes (most specific GO terms only). For this GO enrichment analysis, genes were considered differentially expressed at an FDR = 0.20 significance threshold.

GO ID	GO term	Type[1]	Number of genes		P value[4]
			Sign.[2]	Ref.[3]	
1. Direct JA treatment: significant JJ-JC and/or CJ-CC contrasts					
GO:0009695	jasmonic acid biosynthetic process	P	14	23	2.0^{-10}
GO:0009611	response to wounding	P	44	379	9.2^{-8}
GO:0042401	cellular biogenic amine biosynthetic process	P	17	78	7.0^{-7}
GO:0006571	tyrosine biosynthetic process	P	14	52	8.0^{-7}
GO:0009094	L-phenylalanine biosynthetic process	P	14	52	8.0^{-7}
GO:0000162	tryptophan biosynthetic process	P	15	63	1.2^{-6}
GO:0072329	monocarboxylic acid catabolic process	P	12	44	4.4^{-6}
GO:0009423	chorismate biosynthetic process	P	4	1	1.8^{-5}
GO:0006635	fatty acid beta-oxidation	P	9	27	1.9^{-5}
GO:0051707	response to other organism	P	92	1291	3.6^{-5}
GO:0050660	flavin adenine dinucleotide binding	F	9	31	4.6^{-5}
GO:0009821	alkaloid biosynthetic process	P	6	11	5.7^{-5}
GO:0005783	endoplasmic reticulum	C	51	609	7.9^{-5}
GO:0080167	response to karrikin	P	29	294	2.6^{-4}
GO:0004190	aspartic-type endopeptidase activity	F	6	16	2.9^{-4}
GO:0008970	phosphatidylcholine 1-acylhydrolase activity	F	3	1	3.3^{-4}
GO:0052572	response to host immune response	P	6	17	3.7^{-4}
GO:0034976	response to endoplasmic reticulum stress	P	6	17	3.7^{-4}
GO:0052033	pathogen-associated molecular pattern dependent induction by symbiont of host innate immune response	P	6	17	3.7^{-4}
GO:0030433	ER-associated ubiquitin-dependent protein catabolic process	P	4	5	3.9^{-4}
GO:0005777	peroxisome	C	29	304	5.5^{-4}
GO:0009415	response to water	P	40	482	6.2^{-4}
GO:0001676	long-chain fatty acid metabolic process	P	4	6	6.3^{-4}
GO:0009753	response to jasmonic acid	P	31	336	6.3^{-4}
2. Parental JA treatment: significant JJ-CJ and/or JC-CC contrasts					
No significant enrichment of GO terms detected					

[1] C: cellular component; P: biological process; F: molecular function

[2] Significant gene set for direct JA effect: 1519 significant contigs at FDR 0.20 = 664 putative genes, of which 526 with GO annotation included in enrichment analysis. Significant gene set for parental JA effect: 451 significant contigs at FDR 0.20 = 173 putative genes, of which 133 with GO annotation included in enrichment analysis.

[3] Reference gene set consists of 11961 analyzed and GO-annotated putative genes.

[4] 2-sided Fisher's Exact Test; all p-values are significant after FDR control at 0.05.

Table 2. Number of LC-MS mass signals affected by direct and parental JA treatment (ANOVA analysis, significance threshold FDR = 0.1).

	No. mass signals
Total	8938
ANOVA tested*	2821
Significant Direct JA effect	463 (16.4%)
Significant Parental JA effect	31 (1.10%)
Significant Direct x Parental JA interaction	15 (0.53%)

* At least three non-zero observations in each of the four experimental groups, and normally distributed residuals.

Figure legends

Fig. 1. Experimental design. Plants were exposed to JA treatment (grey boxes) or control (white boxes). G1 and G2 indicate the 'parental' treatment. Transcriptome and metabolome analysis was performed in G3 plants, assessing the effects of both experimental (direct) JA treatment and parental JA treatment. The four experimental G3 groups are coded as CC, CJ, JC and JJ, where the first letter denotes the parental treatment (J=JA, C=control) and the second letter denotes the treatment of the G3 experimental plants. For chemical analyses all 8 replicates per group were used but for RNAseq analysis only six replicates per group were used.

Fig. 2. Number of differentially expressed contigs in offspring plants due to JA-treatment in either the offspring generation (panel A: direct JA effect) or in the parental generation (panel B: parental JA effect). Differential expression was tested in a priori contrasts that control for parental treatment when testing the direct JA effect, or for offspring treatment when testing the parental JA effect (significance threshold FDR=0.1).

Fig. 3. Hierarchical clustering of experimental groups and contigs based on expression scores of contigs that are differentially expressed in offspring due to parental JA treatment. Analysis includes 92 contigs with significant JJ – CJ contrast test and/or JC-CC contrast test (FDR=0.1). Expression scores are log-RPKM values averaged across replicate plants within experimental groups.

Fig. 4. Between-plant variance in gene expression is reduced, not increased, in plants whose parents received JA treatment. Density distribution of the difference in variances between CC and JC groups, based on the subset of contigs (43.8% of total) with significant heteroscedasticity among the four experimental groups.

Fig. 5. LC-MS metabolomics analysis of leaf tissue from plants sampled 24h after JA treatment or control treatment and whose parents had received JA treatment (yellow and red dots respectively) or control treatment (blue and green dots respectively). Panel A: PCA clustering based on mass signals of the LC-MS negative ion mode; axis 1 and 2 explain 14% and 11% of the variation. Panel B: PCA clustering based on LC-MS positive mode; axis 1 and 2 explain 13% and 9% of the variation.

Fig. 6. Leaf total phenolics concentration in offspring of control plants and offspring of jasmonic acid-treated plants, 24h after exposure to jasmonic acid (dark grey box plots) or mock treatment (open box plots). Group codings (CC, CJ, JC, JJ) are as in previous figures. Boxes and whiskers denote the 25^{th}-75^{th} percentile and minimum-maximum observations, respectively; group mean values are indicated by the horizontal line. Inset table are ANOVA test results from a model that also accounted for possible plant size effects due to differences in initial seed weight.

Figure 1.

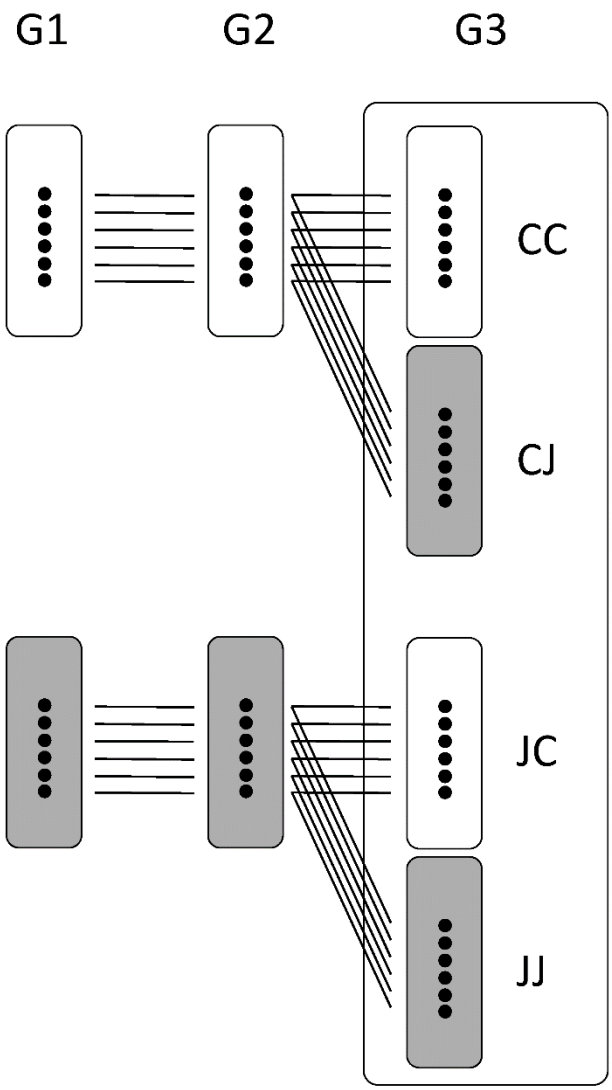


Figure 2.

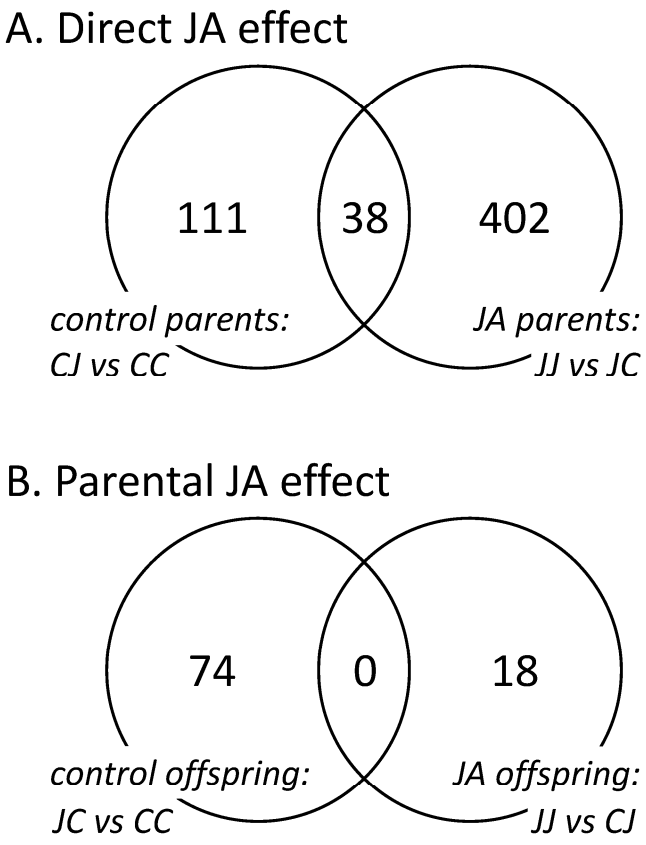

A. Direct JA effect
111
38
402
control parents:
CJ vs CC
JA parents:
JJ vs JC
B. Parental JA effect
74
0
18
control offspring:
JC vs CC
JA offspring:
JJ vs CJ

Figure 3.

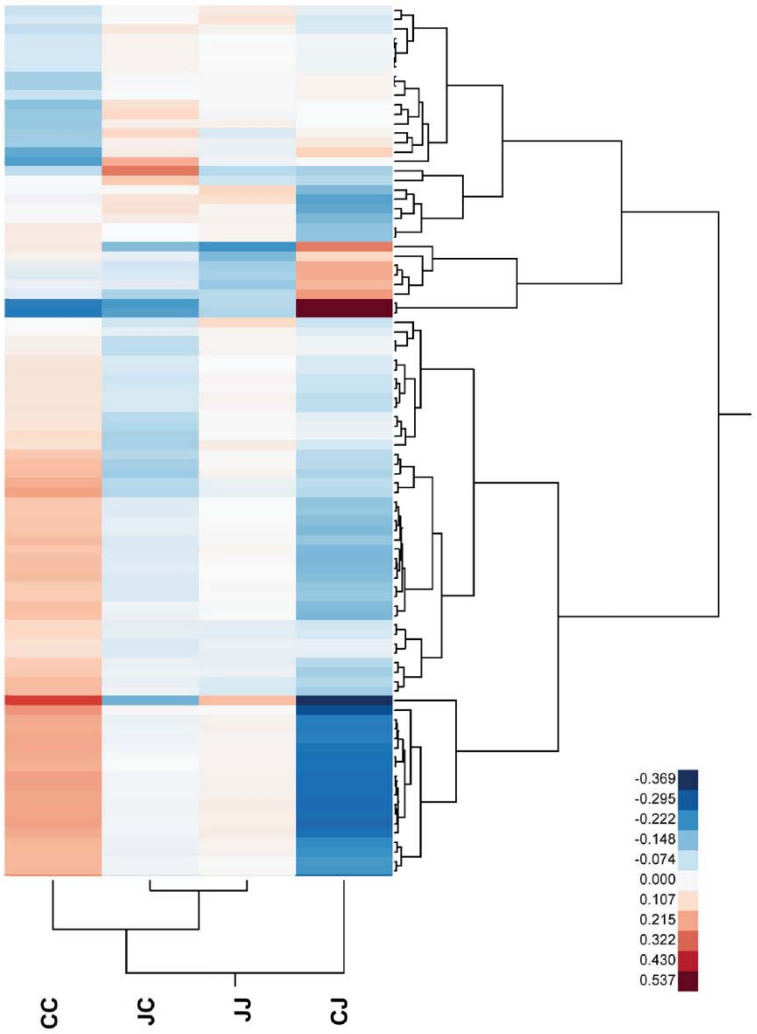

-0.369
-0.295
-0.222
-0.148
-0.074
0.000
0.107
0.215
0.322
0.430
0.537
CC
JC
JJ
CJ

Figure 4.

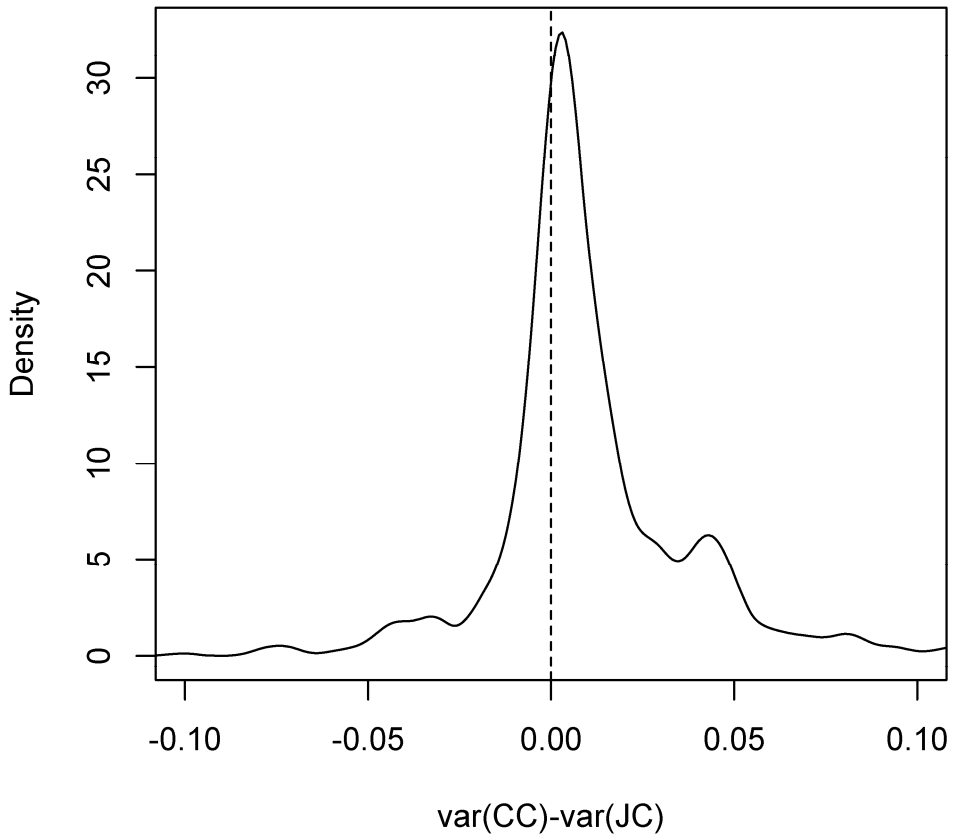


Figure 5.

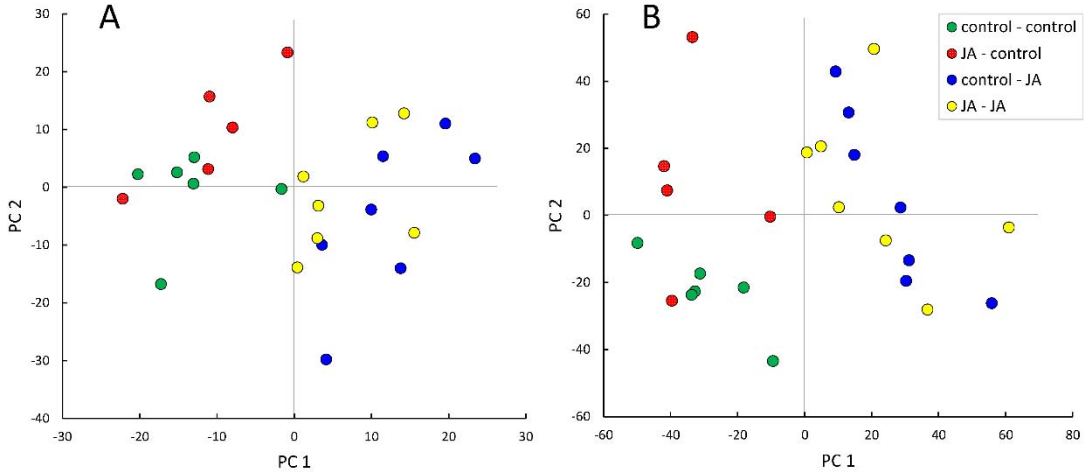
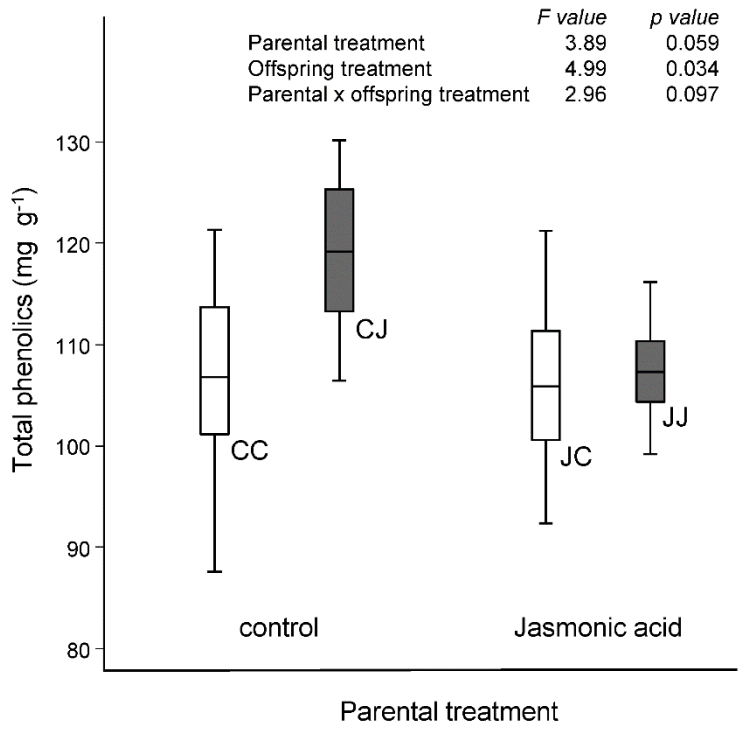


Figure 6.


F value
p value
Parental treatment
3.89
0.059
Offspring treatment
4.99
0.034
Parental x offspring treatment
2.96
0.097
Total phenolics (mg g-1)
130
120
110
100
90
80
CC
CJ
JC
JJ
control
Jasmonic acid
Parental treatment