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Transcriptomics of plant responses to apical damage reveals no negative correlation between tolerance and defense

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Abstract While one may expect the loss of plant tissue by animal herbivores to be universally detrimental to a plant's fitness, a wide range of tolerance responses exists, including undercompensation (lower fitness when damaged), equal compensation, and even overcompensation (increased fitness when damaged). Theory predicts that these responses could be constrained by the investment into defensive chemicals and structures produced for improving resistance in damaged tissues, and thus tolerance and induced defense could be considered alternative strategies to the selection pressure imposed by herbivory. To determine which genetic pathways underlie differences in compensatory (i.e., growth and fitness) responses to damage by tissue loss, and to test their relation with pathways involved in defense, we performed a controlled greenhouse study to measure total gene expression via RNA-sequencing of undamaged and mechanically damaged plants of three *Arabidopsis thaliana* genotypes that differ in their compensatory performances: Columbia-4, Landsberg *erecta*, and a recombinant inbred line (RIL) from a Columbia-4 × Landsberg *erecta* cross. Among the many genetic pathways that responded to clipping, Columbia-4 significantly up-regulated genes involved in secondary defense chemistry and equally compensated for fitness while Landsberg *erecta* and the RIL both undercompensated and significantly down-regulated secondary defense pathways. The genotypes' different compensatory performances are thus positively correlated with their differential investments into secondary metabolism following tissue loss. This study identifies differential post-damage gene regulation of growth, developmental signaling, and environmental response pathways in *A. thaliana*, and provides the first transcriptomic evidence counter to the presumed tradeoff between tolerance and defense in plant-herbivore interactions.

Keywords Herbivory · Resistance · Compensation · Chemistry · Glucosinolate · RNA-seq

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Introduction

A broad, consequential issue in the ecology and evolution of plant-animal interactions regards the ways in which plants respond to herbivore-induced

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damage. Plants exhibit a wide variety of responses to damage by animal herbivores, including defense strategies that aim to prevent damage, and tolerance strategies that aim to prevent the loss of reproductive output upon sustaining damage (Rosenthal and Kotanen 1994; Stowe et al. 2000; Agrawal 2011; note that while we use these terms in accordance with their historical usage, "resistance" and "defense" are often used conversely to their presentation here (Strauss and Agrawal 1999)). Defensive traits often entail preventing damage via structural impediments or by decreasing the palatability of the plant tissue, and may include the production of thorns, trichomes, lignin, latex, numerous classes of secondary metabolites, or the sequestration of silicon, as examples (Levin 1973; Ma 2004; Mithöfer and Boland 2012). These traits may be produced constitutively, often as defensive structures and baseline levels of secondary compounds, or they may be induced upon the detection of a potential herbivore or after sustaining damage (Kaplan et al. 2008). Defense traits may also be general or specific with regard to the herbivore against which they act—for example, there is substantial evidence for coevolution between plants and their specialized herbivores for defense compound toxicity and detoxification, respectively, in a number of systems (Fraenkel 1959; Ehrlich and Raven 1964; Berenbaum 1983).

Tolerance refers to a plant's ability to maintain fitness despite sustaining damage. Tolerance mechanisms are generally theorized to include the reallocation of stored resources to replace the tissues removed, increased stem production and/or branching from the hormonal release of apical dominance, and increases in photosynthetic output and growth rates following damage, among others (Strauss and Agrawal 1999). Precise mechanisms underlying tolerance are not fully understood and are likely variable among populations, species, types of damage, and environments (Maschinski and Whitham 1989; Strauss and Agrawal 1999). Tolerance outcomes range from undercompensation (reduced fitness when damaged), equal compensation (i.e., complete tolerance), and overcompensation (increased fitness when damaged), with compensatory abilities varying across genotypes and populations of a wide range of species (Rosenthal and Kotanen 1994; Strauss and Agrawal 1999; Stowe et al. 2000; Tiffin 2000).

Tolerance and defense strategies have traditionally been considered alternative forms of resistance to herbivory with plants primarily exhibiting either tolerance or defense due to the necessity of allocating limited resources (Coley et al. 1985; Mauricio et al. 1997; Wise and Abrahamson 2005, 2007). For example, fitness following apical damage of *Solidago altissima* decreased nearly tenfold when plants were nutrient-stressed versus fertilized, a result consistent with the limiting resources model of tolerance (Wise and Abrahamson 2008). The production of secondary metabolites may also be resource-limited, where the constitutive level and inducibility of such defenses are negatively impacted by low nutrient availability (e.g., Sampedro et al. 2011). There is also theoretical and empirical evidence of a negative genetic correlation between tolerance and defense strategies (van der Meijden et al. 1988; Fineblum and Rausher 1995; Stowe 1998). Specifically, van der Meijden et al. (1988) argued that because defended plants were likely to experience a lower frequency and intensity of herbivore attack, selection for tolerance ability would be reduced relative to less defended plants. Conversely, tolerant plants would not necessarily face selective pressure to invest in defense if they do not incur fitness losses from herbivory (van der Meijden et al. 1988). The dynamics of the tolerance/defense tradeoff have been theorized from varied perspectives (Stamp 2003), but ultimately under these circumstances tolerance and defense are considered redundant resistance strategies such that they would be mutually exclusive if either trait received a high level of investment (van der Meijden et al. 1988).

Despite the theoretical tradeoff between tolerance and defense strategies, recent research into the genetic underpinnings of tolerance provides evidence that tolerance and defense may not be alternative strategies after all. For example, the pentose phosphate pathway (PPP) is a metabolic pathway that provides five-carbon sugars and NADPH for generalized biosynthesis in the Calvin cycle (Kruger and von Schaewen 2003), but it also provides the raw materials for secondary metabolite production via the shikimate pathway (Maeda and Dudareva 2012). Siddappaji et al. (2013) demonstrated that the *A. thaliana* genotype Columbia-4 had higher expression of *GLUCOSE-6-PHOSPHATE DEHYDROGENASE 1* (*G6PD1*), which encodes the enzyme that catalyzes the first and rate-limiting step of the PPP, when damaged compared to when undamaged and that it overcompensated for fruit production. Landsberg *erecta*, in contrast, had reduced *G6PD1* expression and undercompensated (Siddappaji et al. 2013). The experimental

knockout and complementation of this gene confirmed *G6PD1*'s, and therefore the PPP's, influence in the compensatory performance of these genotypes. This influence may arise in part via endoreduplication, the replication of the genome without mitosis such that cellular genome copy number increases with each replication. This process is assumed to play important roles in plant development, growth, fitness, and general stress tolerance through its effects on cell size, metabolism, and gene expression (Sugimoto-Shirasu and Roberts 2003; Lee et al. 2009; Breuer et al. 2014; for review see Scholes and Paige 2015). Along with the PPP, Columbia-4 is known to increase its endoreduplication during regrowth following damage while Landsberg *erecta* has no such increase (Scholes and Paige 2011). Further, experimentally increasing the endopolyploidy of an *A. thaliana* genotype that typically undercompensates allows it to completely mitigate the otherwise detrimental effects of damage on its fitness (Scholes and Paige 2014). Since the PPP also produces DNA nucleotides, the ability of a genotype to increase its cellular ploidy during regrowth is likely constrained by its ability to increase the PPP (Kruger and von Schaewen 2003). Given these known roles in generalized primary metabolism, endoreduplication, and the production of secondary defense metabolites, the PPP may, therefore, not only underlie the abilities of these plants to tolerate apical damage but also provide a mechanism for the concurrent production of defensive compounds.

In this study, we provide a test of the hypothesis that tolerance and defense strategies to herbivory are not necessarily in opposition, but rather are positively correlated via the co-regulation of their genetic pathways. Specifically, using three genotypes of *A. thaliana* that vary in their degree of tolerance, we initially ask whether a correlation exists between the response in expression of genes in the PPP, the endocycle, and secondary metabolite biosynthesis following damage. We additionally ask whether the expression of genes in those pathways also correlates with measures of fitness compensation and endopolyploidy following damage. To address these questions, we performed quantitative transcriptome sequencing of *A. thaliana* Columbia-4, Landsberg *erecta*, and a Columbia-4 × Landsberg *erecta* recombinant inbred line (RIL) to determine whether the genetic expression of tolerance and defense correlate with their compensatory performances following damage. Our results demonstrate that compensatory performance positively corresponds with the expression of secondary defense metabolic pathways in these genotypes, providing transcriptomic evidence that tolerance and defense metabolism are not necessarily mutually exclusive strategies and that they may in fact be employed concurrently following damage.

Materials and methods

A. thaliana genotypes, growth, and experimental clipping

Columbia-4 (Col-4; TAIR stock number: CS933; The Arabidopsis Information Resource 2014), Landsberg *erecta* (L*er*-0; TAIR stock number: CS20), and one RIL from their crossing (TAIR stock number: CS1936) were selected for this study based on their previously demonstrated fitness compensatory responses (Scholes and Paige 2011; Siddappaji et al. 2013). All genotypes were inbred a minimum of eight generations to achieve full homozygosity (Lister and Dean 1993).

Sixty-five individuals of each genotype (Col-4, L*er*-0, CS1936) were grown, with a randomized distribution, in a greenhouse at approximately 21 °C and on a 12 h light/dark cycle. When bolting inflorescences reached 6 cm in height, the inflorescences of 35 plants of each genotype were clipped with scissors, leaving 1 cm of inflorescence tissue as has been performed in previous studies with these genotypes (Scholes and Paige 2011). This clipping regimen is comparable to natural apical damage of *A. thaliana* observed throughout its native range, stimulates a similar change in architectural traits following damage, and approximately coincides with *A. thaliana*'s maximum rate of inflorescence elongation (Scholes et al. unpublished). Thirty of the remaining plants of each genotype remained unclipped as controls. Five of the clipped plants of each genotype were utilized for transcriptomic analysis (see "Transcriptomic analysis" section for details). Rosette diameter at the time the inflorescence reached 6 cm in height was recorded as a measure of plant size at this stage.

Phenotypic analysis

Measurement of endopolyploidy

At the induction of senescence, all inflorescence tissue of 20 plants (10 unclipped, 10 clipped) of each

genotype was analyzed for nuclear DNA content via flow cytometry. Tissue for cytometric analysis was chopped with a razor blade, sheared in a nuclear isolation buffer (sodium citrate, 3-morpholinopropane-1-sulfonic acid, magnesium chloride, Triton X-100; Galbraith et al. 1983), filtered for debris removal, and stained with the DNA fluorophore propidium iodide. Isolated nuclei were then measured for DNA content via a BD Biosciences (San Jose, CA, USA) FACScanto flow cytometer. Nuclei population gating was performed using De Novo Software FCS Express (v.3; Los Angeles, CA, USA) to measure the number of nuclei at each ploidy level (2C, 4C, 8C, 16C) for each plant sample. The cycle value, interpreted as the average number of endocycles that a nucleus in the sample has undergone and thus an overall measure of endoreduplication (Barow and Meister 2003), was calculated by the following equation:

$$\text{Cycle value} = (0 \times n_{2C} + 1 \times n_{4C} + 2 \times n_{8C} + 3 \times n_{16C})/(n_{2C} + n_{4C} + n_{8C} + n_{16C})$$

where the cycle value is the sum of the number of nuclei at each ploidy level multiplied by the number of endocycles required to achieve each corresponding ploidy level, divided by the total number of nuclei measured.

Measurement of fitness

At the completion of senescence, 40 plants of each genotype (20 unclipped, 20 clipped) were analyzed for fitness. Inflorescence dry biomass and silique yield were measured for each plant. Seed yield for each plant was estimated by multiplying the number of siliques by the average number of seeds per silique for the respective genotype × treatment group (calculated by counting the number of seeds for five representative siliques from each plant).

Statistical analysis of phenotypic measures

Analyses for phenotypic measures were conducted as general linear models with SAS (v.9.3; Cary, NC, USA). Genotype and treatment (clipping) were tested as fixed effects via ANOVA for silique yield, seed yield, inflorescence dry biomass, and cycle values. For silique yield, seed yield, and biomass measures, each plant's rosette diameter at an inflorescence height of 6 cm (to control for differences in plant size) and the number of days from planting to an inflorescence height of 6 cm (to control for differences in developmental timing) were used as covariates to standardize between genotype × treatment groups. Counts of silique yield, seed yield, and biomass were also square-root transformed to approximate normality to satisfy the assumptions of ANOVA. Linear contrasts were used to test for differences between unclipped and clipped plants of each genotype for each measure.

Transcriptomic analysis

Sample preparation, library construction, and sequencing

Five of the clipped plants of each genotype were randomly selected for transcriptomic analysis, with the clipped inflorescence tissue collected for RNA-sequencing. Upon the generation of axillary stems, the first regrown axillary inflorescence to reach a height of 6 cm was likewise collected from each plant for transcriptomics. Initial and lateral stem tissues collected for transcriptomics (i.e., stems collected at the time of and after clipping, respectively) were comparable in mass, chronological age, and developmental stage, allowing for the comparison of gene expression before versus after clipping for individual plants while controlling for differences between individuals. Tissues for transcriptomic analysis were flash-frozen in liquid nitrogen immediately upon clipping from the plant. Total RNA was extracted from each sample via an RNeasy Plant Mini Kit (Qiagen; Venlo, Netherlands). Quality of extracted RNA was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA; software v.1.3) with the assay class designated as Plant RNA Nano. RNA integrity values averaged 9.0 (range 7.7–10.0), 25S/18S rRNA ratios averaged 1.9 (range 1.3–2.7), and adequate quality was confirmed upon visual inspection of electrophoretic images. cDNA libraries were constructed by the High-Throughput Sequencing and Genotyping Unit, Roy J. Carver Biotechnology Center of the University of Illinois at Urbana–Champaign (Urbana, IL, USA), using a TruSeq Stranded Total RNA Sample Prep Kit (Illumina; San Diego, CA, USA). All libraries were quantified by qPCR and sequenced on three lanes (one for each genotype,

comprising ten multiplexed samples per lane), distributed randomly within a single flow cell, for 101 cycles each on a HiSeq 2000 sequencer using a TruSeq SBS Sequencing Kit (v.2) and CASAVA software (v.1.8.2; Illumina; San Diego, CA, USA). On average, 20,717,090 reads were generated per sample.

Read mapping, count normalization, and identification of differentially expressed genes

Reads were trimmed to remove adapter sequences, sequences with low quality scores (limit = 0.05, Phred scores >20; Ewing and Green 1998), and sequences with multiple ambiguous bases (maximum two ambiguous nucleotides per read) using CLC Genomics Workbench (v.6.5; CLC bio; Aarhus, Denmark). After these quality control measures, an average of 19,303,545 reads per sample were mapped to the TAIR10 *Arabidopsis thaliana* annotated reference genome of 28,642 genes (The Arabidopsis Information Resource 2014).

After mapping, genes with fewer than five uniquely mapped reads per treatment on average were removed from the analysis given the increased measurement error typically associated with low read counts (Robinson and Smyth 2007). An average of approximately 21,707 genes remained per sample. Statistical analysis for differential gene expression was performed using the edgeR (Robinson et al. 2010) package in R (i386 of v.3.0.3; R Core Team 2014). The numbers of trimmed reads mapped uniquely to each gene were normalized via the edgeR function calcNormFactors to control for differences in sequencing depth among samples (Robinson and Oshlack 2010). Normalized read counts of each gene were compared between treatments (before clipping, after clipping) via a generalized linear model (GLM; McCarthy et al. 2012), with samples coming from the same individual paired to control for individual variation. Model dispersion was estimated for each gene separately via the function estimateTagwiseDisp in edgeR (Robinson and Smyth 2007, 2008). Genes whose normalized read counts differed significantly before vs. after clipping were identified using GLM likelihood ratio tests via the function glmLRT in edgeR (Lund et al. 2012). Genotypes were analyzed independently with no direct comparisons made among them. p values resulting from the GLM likelihood ratio tests were then corrected to control the false discovery rate (FDR) at 5% in edgeR based on the total number of genes analyzed for the respective genotype, generating FDR-corrected p values (i.e., q values; Benjamini and Hochberg 1995).

Genome-wide GO term enrichment, GO term redundancy reduction, and targeted GO term analysis

Gene ontology (GO) analysis was performed to determine pathway-level patterns of gene expression enrichment among the genes deemed significantly differentially expressed (i.e., $q < 0.05$) relative to the entire set of analyzed genes. GO terms are descriptors of genes based on the cellular component, molecular function, and biological process for which they are associated, and are often determined by experimental demonstration or by sequence similarity to genes of known function. GO terms may therefore be used to infer the functions of genes of interest (e.g., differentially expressed genes) or for targeted analysis of genes with specific functions (e.g., to determine which genes within a pathway of interest are differentially expressed, and/or if the pathway as a whole is differentially regulated). Gene identities and q values were imported into GOrilla (Eden et al. 2009), which analyzes a user-supplied "target" gene list for significant GO term enrichment relative to a user-supplied "background" gene list. The significantly up-regulated genes and the significantly down-regulated genes were analyzed separately for each genotype. GO analyses were performed with *Arabidopsis thaliana* selected as the experimental organism, with two unranked lists of genes (target: either up-regulated or down-regulated genes; background: all genes analyzed for the respective genotype), for the biological process ontology, and with a p value threshold for significant GO term enrichment of 10^{-3}. Briefly, for each GO term for which *A. thaliana* genes are associated (2340 GO terms at the time of analysis), GOrilla uses the total number of genes in the user-supplied background gene list (N), the total number of genes associated with the GO term (B), the total number of genes in the user-supplied target gene list (n), and the number of genes in the target gene list associated with the GO term (b) to calculate an enrichment score [defined as $(b/n)/(B/N)$]; Eden et al. 2009). GOrilla then uses the hypergeometric method to calculate a p value from the enrichment score and produces an FDR-corrected p value (i.e., q value) to

adjust for the number of GO terms in the analysis (Benjamini and Hochberg 1995; Eden et al. 2009). Genes in either user-supplied gene list not associated with a GO term were omitted from the analyses (among the three genotypes, 13,261 genes on average were analyzed for GO enrichment). Across all six GO enrichment analyses (up- and down-regulated genes analyzed separately for three genotypes), an average of 19.7 GO terms were deemed significantly enriched (Supplementary materials 1, 2, 3).

GO terms deemed significantly enriched (i.e., $q < 0.05$) were imported with their q values into REViGO to reduce the redundancy of the GO term list (Supek et al. 2011). REViGO uses q values, semantic similarity, and GO term parent-child relationships to calculate a "dispensability" score (ranging from 0 to 1 with greater values indicating greater dispensability) for each GO term and produces a shortened GO term list based on a user-indicated threshold (Supek et al. 2011). Analyses in REViGO were performed with a dispensability threshold (i.e., C value) of 0.5, imported q values for each user-supplied GO term, the *Arabidopsis thaliana* database selected to provide GO term relationships and sizes, and SimRel as the semantic similarity measure (Supek et al. 2011). After redundancy reduction, an average of 5.8 GO terms remained across the six analyses (Supplementary materials 1, 2, 3).

Given the nature of our clipping treatment and our interest in the integration of the PPP, secondary defense chemistry, and endoreduplication following plant damage, seven GO terms were selected *a priori* (Table 1). These include overall secondary metabolite biosynthesis as well as the biosynthesis of specific classes of secondary compounds of *A. thaliana* (Table 1; Kliebenstein 2004). We acknowledge that the classes of secondary compounds assessed may comprise a variety of compound sub-classes (e.g., sub-classes of phenylpropanoids include coumarins, flavonoids, lignins, etc., which may or may not be specifically involved in herbivore defense; Solecka 1997); however, limitations in the availability of sub-class GO terms, the annotation of genes to these GO terms, and ambiguity in the involvement of specific compounds in herbivore defense prevent the assessment of more finely defined compound sub-classes. The "pentose phosphate shunt" and "DNA endoreduplication" GO terms were also selected for analysis due to their previously reported involvement in tolerance (Scholes and Paige 2011, 2014; Siddappaji et al. 2013). To assess the effects of the treatment on specific GO terms of interest selected *a priori*, lists of *A. thaliana* genes with direct and indirect associations with each *a priori* GO term were obtained using the AmiGO 2 (http://amigo2.geneontology.org) Grebe search wizard. For each gene associated with a given GO term of interest, the p value calculated by edgeR was corrected for a false discovery rate of 5 % based on the total number of genes associated with the GO term that remained after the quality control measures described above (Benjamini and Hochberg 1995). An exact binomial test was then performed in R via the binom.test function to determine if the number of up-regulated genes differed significantly from the number of down-regulated genes for each GO term.

Results

Fitness and nuclear DNA content

Landsberg *erecta* and the Col-4 × L*er*-0 RIL CS1936 both significantly undercompensated for silique yield [$t(67) = 2.88$, $p < 0.01$, and $t(67) = 2.52$, $p < 0.05$, respectively; Fig. 1]. CS1936 also significantly undercompensated for seed yield [$t(67) = 3.08$, $p < 0.01$), while L*er*-0 nearly undercompensated for seed yield [$t(67) = 1.93$, $p = 0.058$; Fig. 1). Columbia-4 equally compensated for both silique and seed yield [$t(67) = 0.41$, $p = 0.682$, and $t(67) = 0.16$, $p = 0.874$, respectively; Fig. 1]. There were no differences between unclipped and clipped plants in inflorescence dry biomass for any genotype [Col-4: $t(67) = 0.30$, $p = 0.762$; L*er*-0: $t(67) = 0.95$, $p = 0.348$; CS1936: $t(67) = 1.20$, $p = 0.234$; Fig. 1], though the change in their means closely approximates the silique and seed compensatory performance for each genotype (Fig. 1).

Although cycle values of unclipped and clipped plants did not differ significantly for any genotype, the changes in direction of their means did correspond roughly to their fitness compensatory responses with a trend toward decreased endopolyploidy for L*er*-0 and CS1936 [$t(39) = 0.66$, $p = 0.514$, and $t(39) = 0.25$, $p = 0.807$, respectively] and a trend toward increased ploidy for Col-4 [$t(39) = 1.67$, $p = 0.103$; Fig. 1].

Table 1 Gene ontology terms of interest selected *a priori*

ID	Term	# Genes	Col-4	L*er*-0	CS1936
GO:0044550	Secondary metabolite biosynthetic process	310	56	11	51
GO:0016114	Terpenoid biosynthetic process	261	6	1	2
GO:0019761	Glucosinolate biosynthetic process	169	19	10	21
GO:0009699	Phenylpropanoid biosynthetic process	135	40	1	30
GO:0052315	Phytoalexin biosynthetic process	11	0	0	2
GO:0006098	Pentose phosphate shunt	182	0	2	2
GO:0042023	DNA endoreduplication	111	0	0	1

Gene ontology IDs, terms, and numbers of *Arabidopsis thaliana* genes annotated to each GO term were compiled from the AmiGO 2 gene ontology browser (http://www.geneontology.org). Due to data trimming for quality, the number of genes annotated to each GO term is not necessarily equal to the number of genes that constituted the family for which the false discovery rate was corrected. Values in the Col-4, L*er*-0, and CS1936 columns are the numbers of differentially expressed genes for each respective genotype after false discovery rate (FDR) correction ($q < 0.05$). See Supplementary materials 1, 2, and 3 for full results

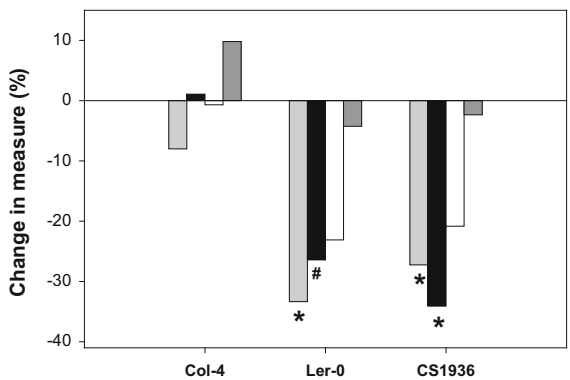

Fig. 1 Percent change in phenotypic measures of clipped Columbia-4, Landsberg *erecta*, and CS1936 plants relative to unclipped controls. *Asterisks* (*) significant ($\alpha = 0.05$) differences between unclipped and clipped plants for the given genotype and measure; $^{\#}\, p = 0.0584$. *Light gray shading* silique yield, *black shading* seed yield, *white shading* mass, *dark gray shading* cycle value

Gene expression

Differential gene expression and genome-wide GO term enrichment

For those genes differentially expressed between samples before and after clipping, we conducted a gene ontology (GO) analysis that identifies biological processes (i.e., GO terms) for which the genes are associated. Here we describe broad themes from our gene regulation and biological process analyses (see Supplementary materials 1, 2, and 3 for detailed analyses and full lists of significant genes and GO terms). Upon clipping, 302 genes were significantly up-regulated in Col-4 while 663 were significantly down-regulated. When considering Col-4 up-regulated genes, 21 GO terms were significantly enriched (Fig. 2; Supplementary material 1). Upon redundancy reduction, the three remaining GO terms were all involved in cell wall biosynthesis (Fig. 2; Supplementary material 4). The 30 GO terms significantly enriched with down-regulated Col-4 genes were generally involved in pollen tube growth and cell wall modification (Supplementary material 1). After redundancy reduction, eight GO terms remained (Fig. 2; Supplementary material 4).

Only four GO terms were significantly enriched with L*er*-0's 38 up-regulated genes, with three remaining after redundancy reduction (Fig. 2). These terms were all related to the response to various stimuli (e.g., chitin, nitrogen, fungi, oxygen-containing compound; Supplementary materials 2, 4). Although 27 genes were significantly down-regulated, no GO terms were significantly enriched with L*er*-0 down-regulated genes (Fig. 2; Supplementary material 4).

The Col-4 × L*er*-0 RIL CS1936 exhibited 124 significantly up-regulated genes and 148 significantly down-regulated genes. CS1936 demonstrated a varied transcriptomic response to the clipping treatment relative to its parents, with 25 and 38 GO terms significantly enriched with up- and down-regulated genes, respectively, relative to the genomic background (Fig. 2; Supplementary material 3). Upon redundancy reduction of up-regulated GO terms, only five remained and collectively were associated with a

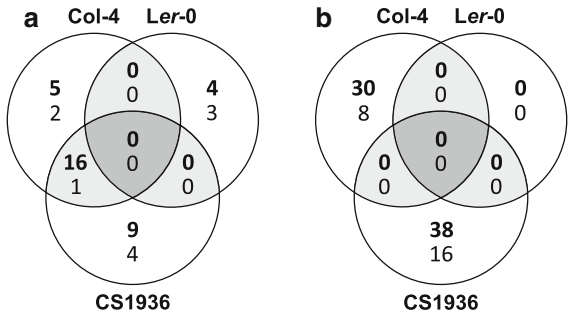

Fig. 2 The number of gene ontology terms significantly enriched with **a** up- and **b** down-regulated genes for Columbia-4, Landsberg *erecta*, and CS1936 following clipping in the genome-wide analysis. *Bold values* indicate the numbers of significantly enriched GO terms identified by GOrilla in total; normal-weight values indicate the numbers of significant GO terms following redundancy reduction by REViGO. See "Materials and methods" section for details. Values in regions of overlap indicate the numbers of GO terms in common among genotypes. Values are additive for each genotype (e.g., there were 21 GO terms significantly enriched with up-regulated genes for Col-4 in total, with three remaining after redundancy reduction). See Supplementary material 4 for the identities of these significant GO terms

wide variety of processes, including xylan biosynthesis, transition metal ion transport, and cellular response to iron ion starvation (Fig. 2; Supplementary material 4). Of the 38 down-regulated GO terms, 16 remained after redundancy reduction, with many involved in growth and developmental signaling (e.g., ethylene biosynthesis and phosphorelay signal transduction) and the response to the environment (including "response to wounding," "response to mechanical stimulus," "response to external stimulus," etc.; Fig. 2; Supplementary material 4).

Differential regulation of GO terms of a priori interest

GO terms selected *a priori* generally fall into two groups: the biosynthesis of secondary metabolites (i.e., chemical defenses; Fig. 3a) and processes predicted to influence compensatory performance (the pentose phosphate shunt and endoreduplication; Table 1; Fig. 3b). Overall, Columbia-4 significantly up-regulated secondary metabolite biosynthesis while Landsberg *erecta* significantly down-regulated this process (Fig. 3a). Specifically, Columbia-4 significantly up-regulated the biosynthesis of glucosinolates and phenylpropanoids with no significant changes in the regulation of phytoalexins or terpenoids. Landsberg *erecta* significantly down-regulated glucosinolates with no significant changes in the regulation of the other metabolite groups (Fig. 3a). The Col-4 × L*er*-0 RIL CS1936 displayed intermediate secondary metabolite regulation relative to its parents, with significant down-regulation of glucosinolates, significant up-regulation of phenylpropanoids, and no significant changes in the regulation of biosynthesis of phytoalexins, terpenoids, or secondary metabolites overall (Fig. 3a). No genotype had significant changes in the regulation of the pentose phosphate shunt or endoreduplication upon clipping (Fig. 3b).

Discussion

Our results yield insight into the transcriptional response of plants to apical damage, using the model species *Arabidopsis thaliana*. We demonstrate that Columbia-4, Landsberg *erecta*, and the RIL generated from a cross between these two accessions differ in their regulation of genes associated with the response to stimuli, cell wall biosynthesis, and a variety of other processes. The most striking results, however, are differences between the genotypes in their regulation of secondary defensive chemistry that are positively correlated with their compensatory performances. In the context of these three genotypes, our results indicate that tolerance and defense are not necessarily alternative responses to the removal of apical dominance since one genotype appeared to employ both strategies while the other two employed neither. While the generalities of our results remain unassessed across populations and species, our methods were sufficient to produce robust, reliable transcriptomic data that provide the basis for conclusions for these three commonly studied genotypes that could be more thoroughly investigated in future genetic and natural field studies. Together, this study contributes the first transcriptomic data counter to the presumed tradeoff between tolerance and defense, as well as transcriptomic characterization of the plant regrowth process broadly, adding a new perspective to this consequential issue in plant damage dynamics.

The *A. thaliana* genotype Columbia-4 experienced no significant change in attributes of fitness when damaged relative to when undamaged (i.e., it equally compensated). Col-4's complete tolerance of damage corresponded with the significant up-regulation of genes involved in secondary metabolism, and particularly the

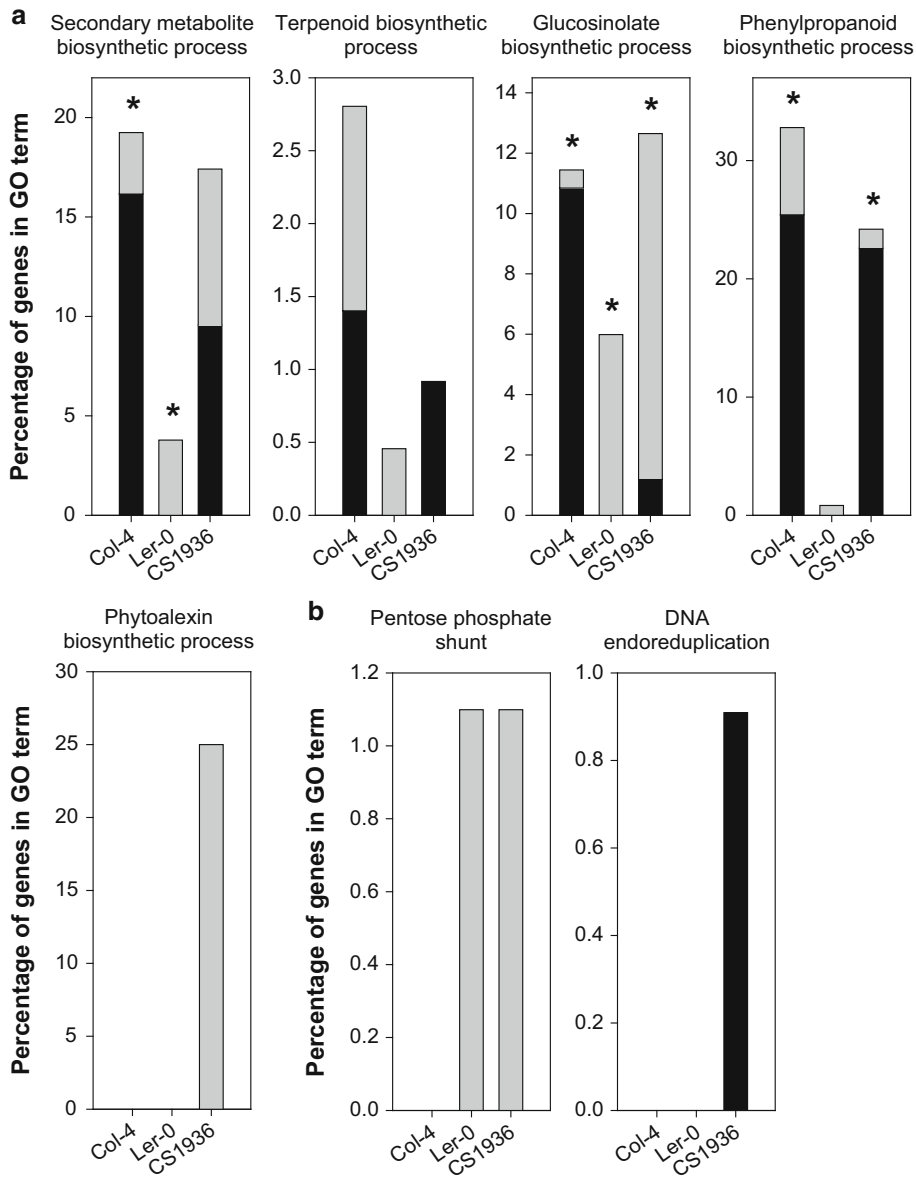

Fig. 3 Differential gene regulation of *a priori* gene ontology terms of interest. Gene ontology terms include **a** the biosynthesis of secondary metabolites and **b** the pentose phosphate shunt and endoreduplication. *Asterisks* percentage of up- and down-regulated genes in the GO term differs significantly (exact binomial test, $p < 0.05$; see Supplementary materials 1, 2, and 3 for full results). *Black shading* up-regulated, *gray shading* down-regulated

production of phenylpropanoids and glucosinolates. Landsberg *erecta*, which suffered reduced fitness when damaged relative to when undamaged (i.e., it undercompensated), displayed significant down-regulation of generalized secondary chemistry and glucosinolate biosynthesis. The Col-4 × L*er*-0 RIL CS1936 also undercompensated and displayed a mix of defensive chemistry responses relative to its parents, including significant down-regulation of glucosinolates, the primary anti-herbivore compounds produced by *A. thaliana* (Kliebenstein 2004; D'Auria and Gershenzon 2005; Tzin and Galili 2010).

Theory and historical perspective often consider tolerance and defense to be alternative strategies to

damage based on the necessity of allocating limited resources and a presumed negative genetic correlation between the strategies. Across the literature, there is no conclusive evidence for the generality of this tradeoff, however, and in fact there is growing evidence supporting the co-occurrence of these strategies. For example, classical resource availability theory predicts that limited resources would promote the investment of a plant into anti-herbivore defenses given the plant's likely impaired growth rate, and subsequently its impaired ability to rapidly regrow and maintain fitness following damage (Coley et al. 1985). However, mathematical models suggest that plant tolerance could actually be maximized when resources are limiting since growth rates are reduced for undamaged plants as well, allowing damaged plants with multiple stems to match undamaged plants in total biomass and fitness production (Hillbert et al. 1981; Alward and Joern 1993). Clearly theory does not provide adequate generalizations for the employment of tolerance versus defense strategies, and empirical evidence often supports mixed-strategy hypotheses. Specifically, numerous studies have discovered that genotypes of many species generally display a mix of resistance strategies and that these strategies are thus not a product of diversifying selection (i.e., a genetic tradeoff between strategies; Leimu and Koricheva 2006; Núñez-Farfán et al. 2007; Carmona and Fornini 2013; Turley et al. 2013). For example, studies of tolerance (measures of fitness) versus defense (trichome and glucosinolate production) in *A. thaliana* revealed that natural populations exhibited mixed resistance strategies due to selection for the maintenance of both traits, and thus no negative genetic correlation exists for these plants (Mauricio et al. 1997). In fact, mixed resistance strategies may be expected when considering the concurrent selection pressures applied by a diverse herbivore community and/or a genetically diverse plant population, as are commonly present in natural systems (Fornoni et al. 2004; Agrawal 2011; Carmona and Fornini 2013). The growing number of similar examples has even spawned a revision of the semantics surrounding the discussion of plant resistance strategies, with Stowe (2013) expressing that the distinction between defensive and tolerance traits is not useful in practice due to their often interdependent phenotypic expression and evolution. For example, many species allocate defensive chemicals to valuable tissues, like seeds, such that non-defended tissues are preferred by their animal herbivores (Coley 1983; Berenbaum et al. 1986; Steele et al. 1993; Newman et al. 1996; Shroff et al. 2008; Stowe 2013). This trait prevents damage to valuable structures, and is thus a form of "defense," yet it also allows plants of these species to tolerate damage of other structures since there is no evident cost to reproduction (Stowe 2013).

Because the correlation between compensation and the regulation of secondary chemistry is present at both extremes in this study (i.e., we observed a genotype that invested in both, as well as genotypes that invested in neither, at least in terms of the primary anti-herbivore compounds; Kliebenstein 2004; D'Auria and Gershenzon 2005; Tzin and Galili 2010), a genetic pathway that influences both could be underlying the resistance strategies of these plants. Our prior studies have indicated that the induction of the pentose phosphate pathway (PPP) and endoreduplication following apical damage is important for the compensatory performance of *A. thaliana* plants, with consistent differences in the responses of these pathways between genotypes that differ in their compensation (Scholes and Paige 2011, 2014; Scholes et al. 2013; Siddappaji et al. 2013). Although we did not observe a concurrent regulatory response of the PPP or endoreduplication, this does not necessarily indicate that these processes are not integrated with secondary metabolism, as we theorize. Because we did not observe overcompensation in Columbia-4, nor significant changes in ploidy (though trends were in the expected directions) as in our previous studies (Scholes and Paige 2011, 2014; Scholes et al. 2013; Siddappaji et al. 2013), a primary constraint may be variation in the year-to-year environment to which correlations in pathway regulation might have been weak or absent. Further, previous studies have observed a delay in the induction of the PPP and endoreduplication ranging from a few to several days after clipping (Scholes and Paige 2011; Siddappaji et al. 2013), yet these pathways clearly influence fitness compensation as measured at senescence. While we used all available data to design our experimental protocol to maximize our detection of differential gene regulation, it is also possible that effects of clipping on gene regulation could have been missed if the timing of sampling did not correspond with differential expression. There thus remains a theoretical framework by which the PPP supports

generalized metabolism for growth, secondary metabolism for defense, and endoreduplication for further increases in growth, metabolism, and gene expression, though a high-resolution time series of gene expression would be necessary to fully assess this connection.

Given the variation in fitness compensation observed among the genotypes here, what insights can be gained with regard to Col-4's ability to completely compensate for apical damage? The genome-wide analysis of Col-4 gene expression revealed many enriched GO terms involved in cell wall processes that were not enriched to the same degree in L*er*-0 or CS1936 (Supplementary material 4). Cell wall biosynthesis may be expected to be important during the regrowth of tissue following damage, and Col-4's compensation for biomass is in accordance with its up-regulation of biosynthetic pathways of hemicellulose, glucuronoxylan, and other cell wall polysaccharides (Supplementary material 1). The GO terms "cellular macromolecule biosynthetic process" and "carbohydrate metabolic process" also refer to cell wall biosynthetic processes (Supplementary material 1). Landsberg *erecta*, in contrast, significantly up-regulated genes involved in the response to various stimuli (e.g., chitin, fungus, nitrogen, oxygen-containing compound) while the RIL CS1936 differentially regulated a wide variety of terms not seen in either parent. The importance of any given differentially regulated GO term (e.g., investment into cell wall structures, which may be related to regrowth and/or fitness), however, is difficult to assess with respect to the entire transcriptome. Our most explicit explanation for differences in the compensatory performance of Col-4, L*er*-0 and CS1936 is thus limited to the genotypes' differential investments into secondary metabolism, with other intriguing and potentially important processes identified.

Why does CS1936 exhibit a transcriptomic response so varied and different from either parent, and how does its response relate to its compensatory performance? Upon the crossing of Col-4 and L*er*-0, new allelic combinations arose that evidently produced transcriptomic responses unlike those of either parent. Specifically, when combining the GO enrichment analyses for up- and down-regulated genes before redundancy reduction, 47 of CS1936's 63 significantly enriched GO terms were novel to Col-4's 51 and L*er*-0's four significantly enriched GO terms (Fig. 2; Supplementary material 1, 2, 3). In fact, CS1936's 63 significantly enriched GO terms are based on the differential expression of only 272 genes, whereas Col-4's 51 significantly enriched GO terms are based on 965 differentially expressed genes (Supplementary materials 1, 3), indicating that CS1936's differentially expressed genes are highly variable in function relative to its parents. CS1936 does share a substantial number of its significant GO terms with Col-4, however, with 16 of its total 25 up-regulated GO terms in common with Col-4 (though these terms were reduced to one following redundancy reduction; Fig. 2). Even though both Col-4 and CS1936 had a relatively large number of GO terms enriched with down-regulated genes upon clipping (30 and 38, respectively), none were shared between them (Fig. 2). These GO terms in particular may thus provide some suggestion to the genes and pathways that led to CS1936's undercompensation while Col-4 tolerated damage. Interestingly, the RIL displays a greater reduction in fitness upon clipping than even its parent L*er*-0, which may be a symptom of these new epistatic allelic interactions that are potentially maladaptive with regard to fitness compensation. Because individuals of *A. thaliana* are highly inbred and largely self-fertilizing (Abbott and Gomes 1988), genetic lineages are often effectively considered populations. Perhaps the reduced compensatory performance of CS1936 here is analogous to that of outbreeding depression of other species. Studies of the effect of such outbreeding depression on tolerance and defense of *Lychnis flos-cuculi* populations have determined that defense is reduced in plants from between-population crosses, while the effect on tolerance was population-dependent (Leimu and Fischer 2010). Here, this notion is in part supported by the identity of CS1936's significantly down-regulated GO terms, which are primarily involved in growth and developmental signaling (e.g., phosphorelay, ethylene, and brassinosteroid processes) or the response to stimuli (e.g., chitin, nitrogen, wounding; Supplementary material 4). Because the down-regulation of these genetic pathways would not be expected given the treatment we applied, these results in the context of maladaptive allelic combinations would suggest that CS1936 is not able to sufficiently maintain the expression of these important pathways following damage, causing it to undercompensate.

Overall, these results provide an overview of the transcriptional response of plants to apical damage with

intriguing patterns emerging when considered with regard to the genotypes' compensatory performances. Most importantly, we report that the response of secondary defense metabolism actually corresponds positively with the damage tolerance of these genotypes. Inclusion of a wider selection of genotypes and/or species will be a costly but valuable endeavor in assessing the generality of the patterns reported here. Further, with growing evidence to the importance of post-transcriptional gene regulation, future studies could focus on protein activity and abundance for the genes and pathways identified here, with particular emphasis on secondary metabolite accumulation over time and their anti-herbivore defense properties via herbivore bioassays. In sum, this study provides the first transcriptomic evidence that tolerance and the induction of defense pathways are not always employed as mutually exclusive alternative responses to damage. We also provide a transcriptomic characterization of the regrowth responses of these three commonly studied genotypes, including the identification of numerous differentially regulated gene pathways. Together, these results establish a basis for future investigations using more targeted molecular genetic methods and direction in assessing individual responses to damage within and between natural populations.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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