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ORIGINAL PAPER

Local and systemic transcriptome responses to herbivory and jasmonic acid in *Populus*

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Abstract We used DNA microarrays to examine local and systemic transcriptional responses to herbivory by gypsy moth larvae (GM) and exogenous jasmonic acid (JA_{trt}) in leaves of *Populus nigra* L. to identify candidate signaling and defense genes and also to examine primary metabolism, as might relate to tolerance of damage. GM and JA_{trt} altered expression of over 800 genes, most of which have putative roles in defense signaling, secondary metabolism, and primary metabolism. Additionally, numerous uncharacterized genes responded to herbivory, providing a rich resource for future studies. There was limited overlap (14%) between the responses to GM and JA_{trt}. GM did, however, result in strong upregulation of genes involved not only in JA biosynthesis but also abscisic acid biosynthesis and other signaling pathways. GM induced known resistance transcripts, including polyphenolic biosynthetic genes, proteinase inhibitors, and amino acid deaminases. According to GOStats pathway level analysis, GM altered primary metabolism, including aromatic amino acid biosynthesis, fatty acid β-oxidation, and carbohydrate and organic acid metabolism. These alterations may be related to increased demands for substrate for secondary metabolites or may serve a tolerance-related role. Responses were more intense locally in treated leaves than in untreated (systemic) leaves and systemic responses were mostly a subset of the genes induced locally. A stronger local response might be needed to cope with localized stresses and wound healing. Since *Populus* in general and this clone in particular are known for their systemic induced resistance, genes induced both locally and systemically may be the highest quality candidates for resistance.

Keywords *Populus* · Herbivory · Jasmonic acid · Systemic induction · Tolerance · Induced resistance

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Introduction

Plants have evolved a multiplicity of induced defense traits to resist or tolerate damage by herbivores (Karban and Baldwin 1997; Kessler and Baldwin 2002; Tallamy and Raupp 1991). These include induction of antioxidant pathways (Park et al. 2006), changes in the allocation of limited resources to diverse antiherbivore traits, such as polyphenolics and proteinase inhibitors (Arnold and Schultz 2002; Karban and Baldwin 1997; Kessler and

Baldwin 2002), the production of volatiles that deter herbivores or attract natural enemies (Dicke et al. 2003; Gatehouse 2002), and changes in primary metabolism, such as mobilization of stored reserves, compensatory photosynthesis, and changes in source-sink dynamics (Bassman and Dickmann 1982; Hamilton and Frank 2001; Oleksyn et al. 1998; Pearson and Brooks 1996). These changes may occur locally within the damaged leaf or systemically in undamaged tissues. Both local and systemic changes typically involve activation of one or more signaling pathways that mediate plant responses to particular stresses and environmental conditions and coordinate defensive responses in different tissues within the plant (sensu Arnold and Schultz 2002; Babst et al. 2005; Schwachtje et al. 2006).

In *Populus*, induced defenses involve increases in many resistance traits locally and systemically, including tannins, polyphenol oxidases, and volatiles (Arimura et al. 2004; Arnold and Schultz 2002; Constabel et al. 2000; Ferrieri et al. 2005; Stevens and Lindroth 2005). There are also changes in photosynthesis and whole plant resource partitioning. For example, gypsy moths (GMs) induce increased export of carbon from undamaged systemic leaves (Babst et al. 2008) and a slight increase in photosynthesis (Babst 2006).

The diverse suite of induced traits both locally and systemically highlights the need for analyses that extend beyond specific tissues and specific traits (such as proteinase inhibitors, specific phenolics, photosynthesis, etc.). The complexity of the induced response locally and systemically can be evaluated with DNA microarrays (Baldwin 2001; Gibson 2002; Held et al. 2004; Schmidt et al. 2004; Taylor et al. 2004; Voelckel and Baldwin 2004) that simultaneously examine genes relating to signal transduction pathways (e.g., jasmonate, salicylic acid, ethylene), resistance (e.g., proteinase inhibitors, phenolics), and primary metabolism (e.g., carbohydrates, lipids, proteins). To date, there have been only a few small scale transcript profiling (Christopher et al. 2004; Major and Constabel 2006) or large scale microarray studies of tree responses to herbivory (Ralph et al. 2006a, b).

Here, we used 25K *Populus* DNA microarrays, covering about 16,500 *Populus* gene models (Sterky et al. 2004; Sjödin et al. 2006), to perform a transcriptome analysis on *Populus* leaves induced by GM larvae or by treatment with jasmonic acid (JA_{trt}). Ralph et al. (2006a) performed a DNA microarray analysis to examine the effects of forest tent caterpillar herbivory on *Populus trichocarpa x deltoides* gene transcription locally at the site of damage and reported a broad array of putative defense genes (Ralph et al. 2006a). Our study builds on this foundation, by comparing GM herbivory to an exogenously applied defense signal, JA, examining both treated and untreated leaves to try to distinguish metabolic modifications that are particular to the local response from those important to the systemic response and paying special attention to primary metabolism. We also used a clone of a different *Populus* species, *P. nigra* NC5271, which is reported to exhibit strong induced resistance to gypsy moth larvae in comparison with other *Populus* genotypes (Havill and Raffa 1999). We discuss the implications of these differences in terms of a metabolic reprogramming in *Populus* after herbivore attack.

Materials and methods

Plant material P. nigra clone NC5271 was chosen because it exhibited strong inducible resistance to gypsy moth (*Lymantria dispar*) caterpillars in previous experiments (Havill and Raffa 1999). The plants were grown from dormant wood cuttings in a glasshouse under mist with natural sunlight, partially attenuated with whitewash (~600 μmol m^{-2} s^{-1}; ~14:10 h ratio of day to night) at Brookhaven National Laboratory, NY, USA. Cuttings were dipped in 0.1% indole-3-butyric acid (TakeRoot, Schultz) and were rooted in 50:50 (*v*/*v*) sand/zeopro medium in 1.7 L pots. Once roots were established, a modified Hoaglands solution was applied every 2 days. All treatments were administered July 20, 2004 to ensure similarity of environmental conditions across treatments. Since plant heights ranged from ~35 to 55 cm at the time of the experiment, plants were grouped by height prior to treatment in blocks of four, to accommodate two treatments, herbivory and jasmonate, and their respective controls.

Herbivores Gypsy moth eggs obtained from Animal and Plant Health Inspection Service (APHIS; Otis Air National Guard Base, MA, USA) were hatched, and larvae were raised to third instars on artificial diet. A single third instar larva was held on each treated leaf using a spring-loaded clip cage.

Treatments For the herbivory treatment, gypsy moth caterpillars were caged overnight on three consecutive leaves (leaf plastochron index (LPI) 8, 9, and 10) on each plant to avoid within plant variability that may arise due to sectorial signal transport (Orians et al. 2005). These plants were compared with a set of control plants fitted with empty clip cages. For jasmonate elicitation, JA was solubilized in ethanol and then diluted in DiH_2O to a 1-mM JA solution, 0.1% triton-x 100 as a surfactant to increase penetration through the cuticle (Arnold and Schultz 2002). The JA solution was sprayed on three leaves per plant (LPI 8, 9, and 10), also in the evening. JA-treated plants were compared to plants sprayed with similar 1% ethanol 0.1% triton-x 100 solution without the JA. The leaves were sprayed only once and just until the leaf surface was wetted.

Harvest Plants were harvested the following evening (22 h after treatment); tissues were separated and flash-frozen in liquid nitrogen. The directly treated mature leaves (LPI 8–10) were pooled for analysis of local treatment effects, and young leaves (LPI 3–5) with the most direct vascular connections to the treated leaves were pooled for analysis of systemic effects, while recognizing that differences between the local and systemic leaves will also reflect developmental stage of the leaves. Leaf samples were kept frozen on dry ice before RNA extraction.

Microarrays For this experiment, we used *Populus* POP2 microarrays (Sterky et al. 2004). Details regarding the arrays, sequences, Joint Genome Institute poplar gene model numbers, GenBank accession numbers, and *Arabidopsis* Gene Ontology (GO) numbers for all expressed sequence tags (ESTs) can be found at PopulusDB (http://www.populus.db.umu.se; Sterky et al. 2004). The raw data are stored in the public poplar microarray database UPSC-BASE (http://www.upscbase.umu.se; Sjödin et al. 2006) as experiment UMA-0069. Transcript levels in leaves are determined by many different factors. First, leaf age has a most profound influence on the leaf transcriptome and environmental factors further modify gene expression patterns. To be able to separate true treatments effects from experimental noise, we employed the experimental design depicted in Fig. 1. Three treated and untreated leaves were sampled from each plant and pooled, and three biological replicates were analyzed as separate samples. Groups of plants for microarray analysis were the same as the blocks, based on plant height, described above. Since the massive developmental differences apparent between the transcriptomes of older (local) and younger (systemic) leaves are not the focus of this contribution, the experimental design aimed at optimizing the analytical power in finding differences within local leaves or within systemic leaves, but not between the two classes of leaves. Therefore, young untreated leaves from treated plants (i.e., systemic leaves) were subjected to microarray analysis separately from mature treated leaves (i.e., local leaves).

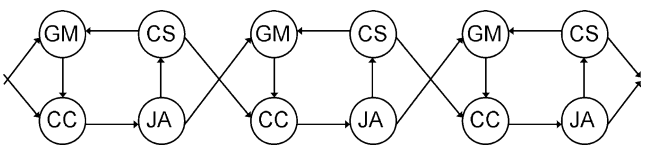

Fig. 1 Design of microarray experiment. Each *circle* represents a biological replicate. Each *arrow* represents a single microarray slide, on which relative transcription level was compared between two samples. We compared three biological replicates of four treatment groups, gypsy moth herbivory (*GM*), cage controls for herbivory treatment (*CC*), exogenous jasmonic acid application (*JA*), and plants receiving the control solution (*CS*), similar to the delivery solution for jasmonic acid application

Leaves were ground under liquid nitrogen, and mRNA was extracted using standard protocol (Chang et al. 1993), with the modifications described in (Bhalerao et al. 2003). Total plant RNA was reverse transcribed, and the resulting cDNA was labeled with fluorescent amino-allyl Cy3 or Cy5 dyes (Amersham Biosciences, Little Chalfont, UK), and hybridizations were performed on an automated slide processor (Amersham Bioscience, Little Chalfont, UK), as previously reported (Smith et al. 2004). Samples were heated to 95°C for 3 min, chilled on ice for 30 s, and injected into the slide processor, containing prehybridized POP2 slides. Each slide was scanned at 543 and 633 nm (for Cy3 and Cy5, respectively) using a ScanArray 4000 (PerkinElmer, Sverige AB, Sweden) at three different light intensities, to maximize the range over which spot intensity could be determined.

Microarray analysis Slide images were examined semiautomatically using GenePix Pro 5.0 (Axon Instruments, CA, USA), including a visual examination to exclude spots from further analysis where there were obvious artifacts or if there was clearly no fluorescence for either wavelength. Restricted linear scaling was applied to generate one data set from the three scans for each wavelength (http://www.umu.se/climi/bact/Microarray/R-libraries.htm). Spot intensities were step-wise normalized (Wilson et al. 2003) prior to calculation of P values and B statistics (Smyth 2004). We considered any spot with $B>0$ to exhibit differential expression (Taylor et al. 2005). When large numbers of transcriptional changes occur, multigenic pathway level responses take on more significance than single gene responses. Therefore, GO categories were tested for overrepresentation in the plant response as compared to their total representation in the transcriptome. A modified version of the GOstats Bioconductor package (Gentleman et al. 2004) implemented in UPSC-BASE (Sjödin et al. 2006) were used for all calculations. The *Populus* ESTs have been previously assigned Gene Ontology numbers according to their closest *Arabidopsis* ortholog. Upregulated and downregulated genes were tested separately. The Gene Ontology categories were important to give an unbiased broad overview of transcriptome response patterns, since a large set of genes were differentially expressed, but they were also useful as a roadmap to guide closer scrutiny of individual genes.

Forest tent caterpillar comparison We compared gene expression changes induced by GM feeding in *P. nigra* with the changes reported by Ralph et al. (2006a) for *P. trichocarpa x deltoides* in response to forest tent caterpillar (FTC). Since the two studies used different microarrays, we

used the closest *Arabidopsis* homolog for each gene to match the two gene lists. Ideally, gene lists should have been matched by corresponding *Populus* gene model but since these were not given for the genes mentioned in Ralph et al., this was not possible. In cases where two *Populus* genes from the different microarrays share the same *Arabidopsis* homolog, they were compared with each other. The majority of these close homologs probably have a similar regulation (Segerman et al. 2007), but in cases where they do not, gene lists will appear more dissimilar than they actually are.

Results

Expression of more than 800 genes was altered following either GM or JA_{trt} compared to controls (for full gene lists, see S1). GOstats analysis of multigenic pathways revealed many changes to signaling cascades, secondary metabolic pathways, and primary metabolic pathways (Table 1; for full list, see S2). The patterns revealed by the GO category analysis provided a guide for closer scrutiny of the changes in gene expression induced by the GM and JA_{trt} treatments. Below, we present some highlights of the pathways and

Table 1 Gene ontology categories up- or downregulated by herbivory or JA treatment in local and systemic leaves

Cat number	Category term	Herbivory		JA	
		Local	Systemic	Local	Systemic
GO:0009695	Jasmonic acid biosynthesis	Up	Up	–	–
GO:0009861	JA and ethylene-dependent systemic resist.	Up	Up	–	–
GO:0009688	Abscisic acid biosynthesis	Up	–	–	–
GO:0009850	Auxin metabolism	–	–	Down	–
GO:0006020	Myo-inositol metabolism	Up	–	–	–
GO:0009968	Negative regulation of signal transduction	–	–	–	Down
GO:0009934	Regulation of meristem organization	–	–	Down	–
GO:0009737	Response to abscisic acid stimulus	–	Up	–	Up
GO:0009611	Response to wounding	Up	Up	–	–
GO:0042828	Response to pathogen	Up	–	Down	–
GO:0009407	Toxin catabolism	Up	–	–	–
GO:0008299	Isoprenoid biosynthesis	Up	–	–	–
GO:0006721	Terpenoid metabolism	Up	–	–	–
GO:0009698	Phenylpropanoid metabolism	Up	Up	Up	–
GO:0009812	Flavonoid metabolism	Up	–	–	–
GO:0009809	Lignin biosynthesis	–	–	–	Down
GO:0009073	Aromatic amino acid family biosynthesis	Up	–	–	–
GO:0009074	Aromatic amino acid family catabolism	–	–	Down	–
GO:0019438	Aromatic compound biosynthesis	Up	Up	Up	–
GO:0006575	Amino acid derivative metabolism	Up	–	Up	–
GO:0006563	L-Serine metabolism	–	–	Down	–
GO:0044271	Nitrogen compound biosynthesis	Up	–	–	–
GO:0009769	Photosynthesis light harvesting PSII	–	–	–	Down
GO:0006082	Organic acid metabolism	Up	–	–	–
GO:0006084	Acetyl-CoA metabolism	Up	–	–	–
GO:0005975	Carbohydrate metabolism	Up	–	–	–
GO:0019318	Hexose metabolism	Down	–	–	–
GO:0006096	Glycolysis	Down	–	–	–
GO:0008643	Carbohydrate transport	Down	–	–	–
GO:0006631	Fatty acid metabolism	Up	Up	–	–
GO:0006635	Fatty acid beta-oxidation	Up	–	–	–
GO:0008618	7-Methylguanosine metabolism	–	Up	–	–
GO:0009119	Ribonucleoside metabolism	–	Up	–	–
GO:0009301	snRNA transcription	–	–	–	Down
GO:0007126	Meiosis	–	Down	–	–

Significant treatment effect was determined using GOstat, based on the number of significant transcriptional changes within a category and the proportional representation of the category in the entire genome. Since many GO categories are partially, or completely, overlapping, we extracted from the complete list a set of categories that were nonredundant and informative

examples of individual genes induced by GM and JA_{trt}, locally and systemically in *P. nigra*.

Herbivory and JA_{trt} strongly induce different gene sets in locally treated leaves There was some overlap of the list of genes up- or downregulated by the GM and JA_{trt} treatments (Fig. 2a), as would be expected, but considerably more unique changes in gene expression, particularly for downregulated genes. By measuring at one time point, it is possible that we underestimated the amount of overlap between GM and JA, since there may be many early-responsive signaling-related genes upregulated transiently and returned to uninduced levels before 22 h (e.g., Pauwels et al. 2008). However, the transcriptional differences between JA_{trt} and GM are consistent with previous reports that JA is not as effective as mechanical wounding or real herbivory in eliciting induced resistance or related traits in *Populus* (Constabel et al. 2000; Havill and Raffa 1999).

At the pathway level, upregulation of JA and abscisic acid (ABA) biosynthesis and myoinositol metabolism dominated the signaling-related response to GM, but not JA_{trt}, where downregulation of auxin metabolism was the only signal-related response. We also found that ethylene and gibberellic acid (GA) signaling were differentially altered by GM at the individual gene level, but GOStats did not indicate pathway level effects for ethylene or GA. Within the JA biosynthetic pathway, almost all of the genes were upregulated by GM (Fig. 3a), but only a few were upregulated by JA_{trt} (Fig. 3b). Also, distinct lipoxygenase 2 (LOX2) isoforms were upregulated by GM and JA_{trt}. In the ABA biosynthesis category, GM upregulated two important genes, LOS5/ABA3 (At1g16540) and ABA2/SDR1 (At1g52340). ABA2 and ABA3 are necessary to complete the last two steps of ABA biosynthesis (Schwartz et al. 1997). Although ethylene biosynthetic genes were unaffected by GM, GM upregulated several negative regulators of ethylene-responsive genes (EBF1, ERF3, and ERF4; Fujimoto et al. 2000; Potuschak et al. 2003). The affect of JA_{trt} on auxin metabolism and the regulation of meristem organization categories are consistent with JA's regulatory role in ordinary growth and development (Irving et al. 1999; Ulloa et al. 2002).

Since protein kinases and transcription factors play important roles in signaling cascades, but are not represented as individual GO categories, we used previously compiled gene lists and found many transcriptional changes (S3). There was only moderate overlap in the transcription factors, protein kinases, and phosphatase genes that were up- or downregulated by GM and JA_{trt}. For genes involved in protein folding (e.g., chaperonins and heat shock proteins), there was a strong tendency for upregulation by GM, but an equally strong tendency for downregulation by JA_{trt}. There was also a tendency toward upregulation of proteases by both GM and JA_{trt}, although these were mostly nonoverlapping sets of protease genes. Genes involved in protein ubiquitination also exhibited altered expression, but the trend toward upregulation was minor.

Overall, GM had much broader effects on signaling than did JA_{trt} treatment. Given the differences in signaling, it is not surprising that these treatments led to differential defense induction downstream, such as the upregulation of response to wounding and response to pathogen GO categories by GM but not JA (Table 1).

In particular GM-induced genes involved in secondary metabolism Putative defense-related pathways, such as phenylpropanoid and terpenoid metabolism, were strongly upregulated by GM and to a lesser extent by JA_{trt}, with few exceptions (Table 1). The individual phenylpropanoid genes, which also tended to be more strongly upregulated by GM than JA_{trt} (Fig. 4), included many early pathway genes, as well as many genes specific to condensed tannin biosynthesis. Phenylalanine ammonia lyase isoform 1, which is associated with nonlignin phenolics (e.g., condensed tannins, see Kao et al. 2002), was upregulated by both GM and JA_{trt}. GM also upregulated three genes that modify hydroxycinnamates prior to entering monolignol or

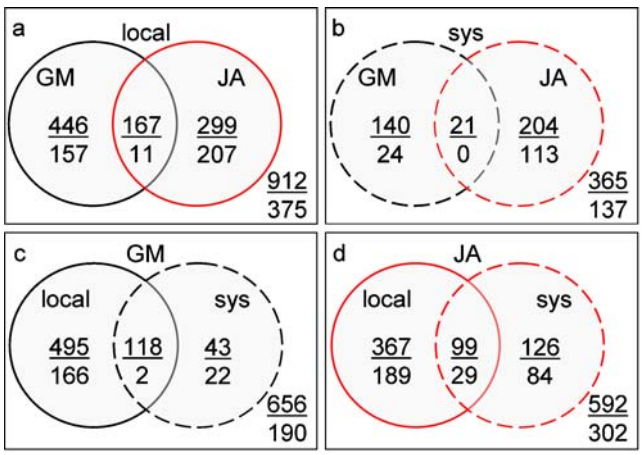

Fig. 2 Venn diagrams showing numbers of unique and overlapping changes in gene expression. In each compartment of the diagrams, the *number above the line* indicates the number of upregulated genes, and the *number below the line* indicates the number of downregulated genes. The expression of a gene was considered significantly different from its respective control (i.e., herbivory and cage control; JA and control solution), if $B>0$ (see "Materials and methods" section). Changes in gene expression were compared between herbivory and JA treatments for **a** local and **b** systemic leaves. Comparisons were also made between local and systemic gene expression changes, separately for herbivory **c** and jasmonic acid treatments **d**. Mature leaves on each plant were subjected to either gypsy moth herbivory, clip cage control, 1 mM jasmonic acid, or a spray control, and mature leaves as well as untreated younger leaves were analyzed for changes in gene expression 22 h after treatment

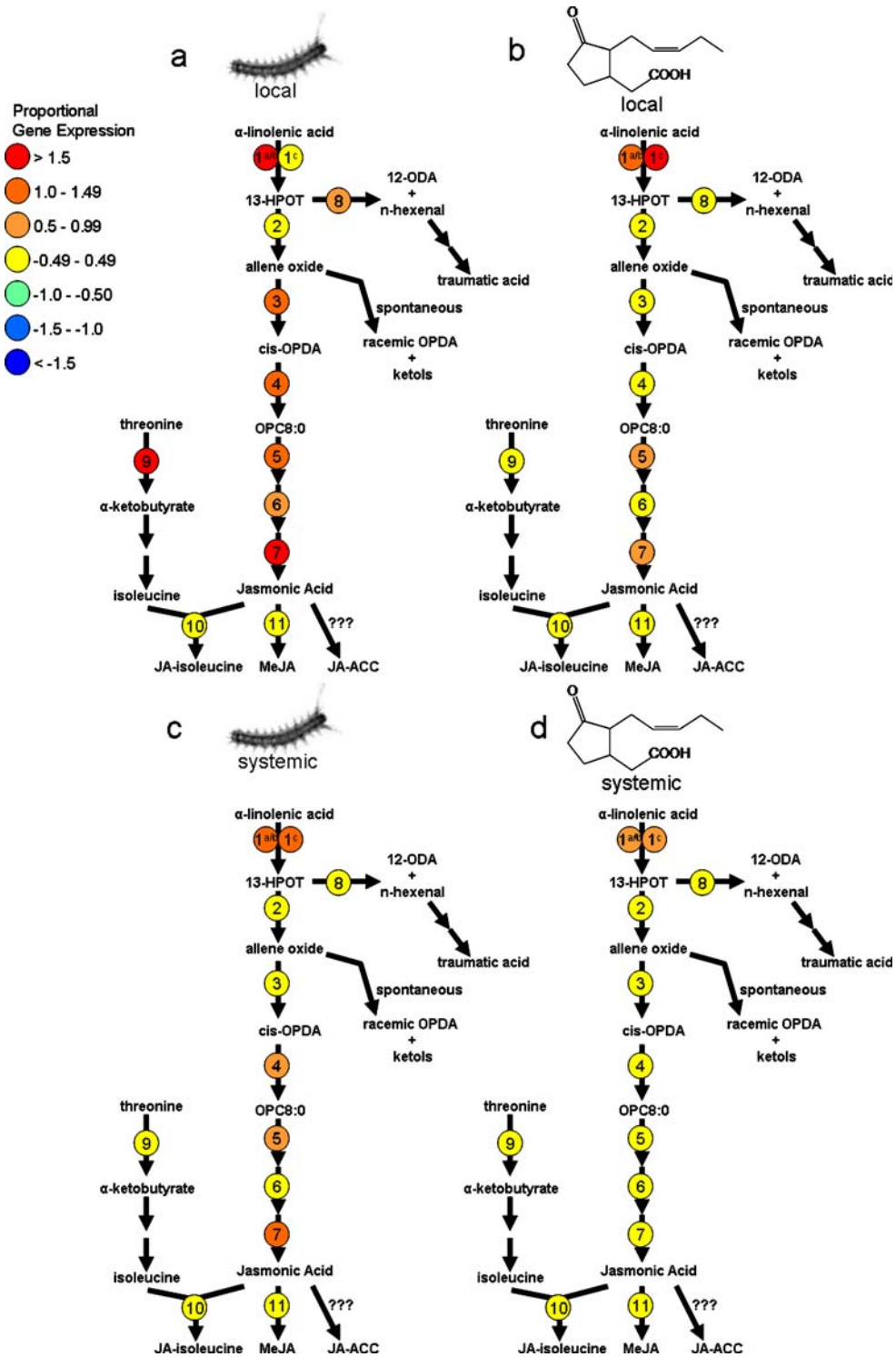

Fig. 3 Transcriptional changes locally and systemically in the jasmonic acid biosynthetic pathway following **a**, **c** herbivory by third instar gypsy moth larvae, or **b**, **d** treatment with exogenous JA. Changes in gene expression are indicated by coloring within *circles*. *Numbers* represent the following genes: *1 LOX2* lipoxygenase 2, *2 AOS* allene oxide synthase, *3 AOC* allene oxide cyclase, *4 OPR2* 12-OPDA reductase, *5 ACX1* acyl-CoA oxidase, *6 MFP2* multifunctional protein (3-hydroxyacyl-CoA dehydrogenase; enoyl-CoA hydratase/isomerase family protein: 3-hydroxyacyl-CoA dehydrogenase), *7 KAT2* 3-ketoacyl-CoA-thiolase, *8 HPL* hydroperoxide lyase, *9 TD* threonine dehydratase/deaminase, *10 JAR1* jasmonic acid responsive gene 1, *11 JMT* jasmonic acid carboxyl methyltransferase. *13-HPOT* (9Z, 11E, 15Z, 13S)-13-hydroperoxy-9, 11, 15 octadecatrienoic acid, *12-ODA* 12-oxo-9 (Z)-dodecenoic acid, *OPDA* 12-oxo-10, 15(Z)-octadecatrienoic acid, *OPC8:0* 3-oxo-2(2′(Z)-pentenyl)-cyclopentane-1-octanoic acid, *MeJA* methyl jasmonate, *JA-ACC* jasmonic acid–1-aminocyclopropane-1-carboxylic acid conjugate. JA biosynthetic pathway was constructed based on the review by (Schaller et al. 2005)

flavonoid biosynthetic pathways, cinnamate-4-hydroxylase 2 (using *Populus* annotation from Tsai et al. 2006), 4-coumarate/CoA ligase 3 (4CL3), and a flavonol/cinnamoyl CoA reductase. The flavonoid metabolism category was upregulated by GM, but not JA_{trt}, although JA_{trt} upregulated several flavonoid biosynthetic genes (Fig. 4). The key regulatory point and first committed step of flavonoid biosynthesis, chalcone synthase, was upregulated only by GM, but chalcone isomerase, the second step, was upregulated by both GM and JA_{trt}. Further downstream, GM also upregulated a series of genes with putative functions leading to proanthocyanidin precursors (i.e., epicatechin), including dihydroflavonol reductase 1, anthocyanidin synthase 2 (or leucoanthocyanidin dioxygenase)

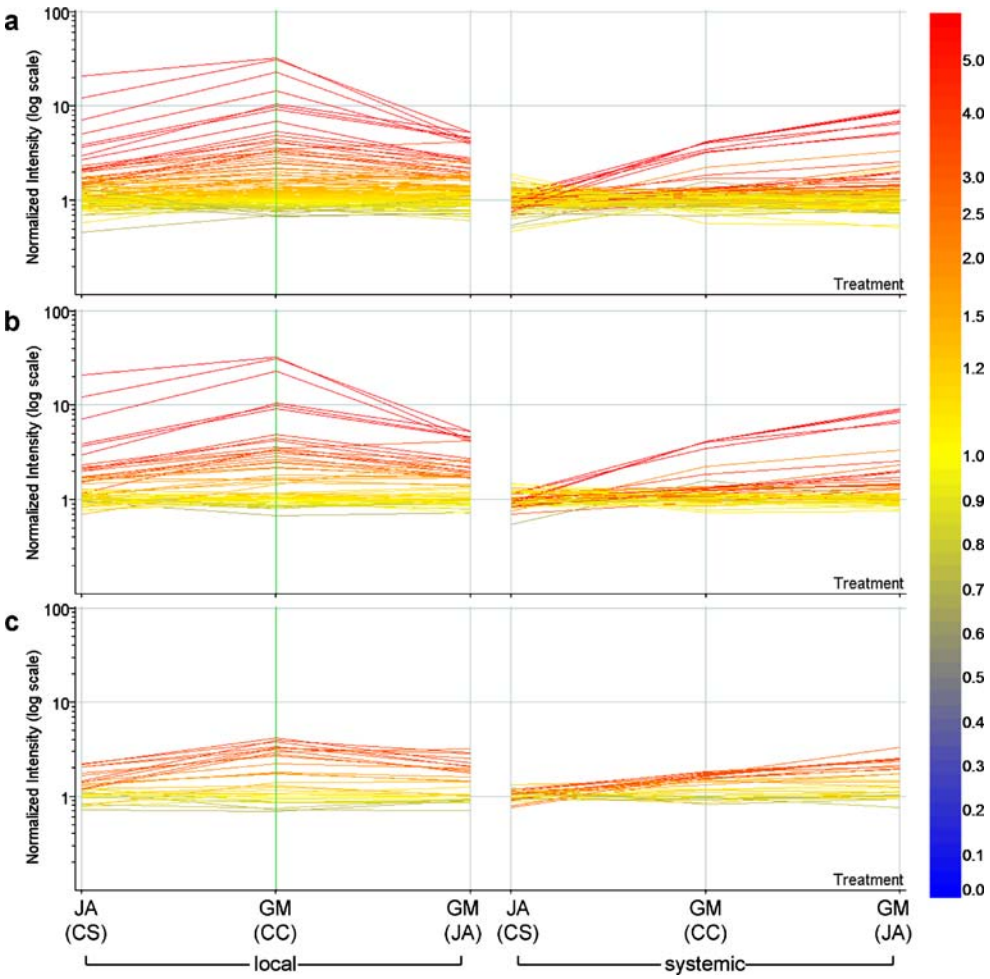

Fig. 4 Expression of genes in **a** phenylpropanoid biosynthesis, **b** flavonoid biosynthesis, and **c** the shikimate pathway of aromatic amino acid biosynthesis Gene Ontology categories in treated plants referenced against control plants. Local responses are shown on the *left* and systemic responses on the *right*. The *y*-axis represents the ratio of treated to reference gene expression, such that a 1 indicates no difference and 10 indicates ten times higher expression. Within each leaf type, the points on the *x*-axis indicate the treatment (*top*) and the reference (*bottom in parentheses*), with abbreviations as described in Fig. 1 above. Each *line* represents the average of three biological replicates for a single EST and is colored based on local leaf expression in the herbivory treatment relative to the clip cage control as reference

and anthocyanidin reductase 1 (or BANYULS), but not leucoanthocyanidin reductase.

Terpenoid metabolism and isoprenoid biosynthesis GO categories were upregulated by GM treatment, but not JA_{trt}. GM affected genes involved in isopentenyl diphosphate biosynthesis (e.g., mevalonate kinase), terpenoid biosynthesis (e.g., geranylgeranyl pyrophosphate synthase), and carotenoid/xanthophyll biosynthesis (e.g., β-carotene hydroxylase).

Although protein-based defenses, such as proteinase inhibitors (PIs), do not have specific GO categories, our gene lists revealed upregulation of several putative defensive genes. Three putative polyphenol oxidase (PPO) genes were upregulated by GM, but not by JA_{trt}. However, for PIs, JA_{trt} strongly—and GM to a lesser extent—upregulated putative Kunitz type PIs (Fig. 5), similar to those reported by Haruta et al. (2001). Additionally, GM upregulated threonine deaminase (TD) which may serve a direct defensive role, in addition to its role in isoleucine biosynthesis (Chen et al. 2005).

GM also altered transcription of genes involved in primary metabolism Perhaps the most striking difference between the GM and JA_{trt} treatments were the effects of GM on multiple primary metabolic pathways (e.g., carbohydrate metabolism, organic acid metabolism, fatty acid metabolism). Changes in primary metabolism elicited by JA_{trt} were much fewer. Expression of several photosynthetic genes was altered, but the photosynthesis GO category was not significantly affected by GM or JA_{trt}.

The most numerous effects of GM and JA_{trt} were downstream of the photosynthetic reactions. GO analysis indicated significant upregulation of carbohydrate and organic acid metabolism, but downregulation of glycolysis, hexose metabolism, and carbohydrate transport in response to GM, but not JA_{trt} (Table 1). For example, phosphofructokinase and enolase were upregulated by GM, and fructose-1,6-bisphosphate phosphatase was downregulated (Fig. 6a), which would favor increased flux of pentose phosphate intermediates into other pathways, for example phosphoenolpyruvate and erythrose 4-phosphate into the shikimate pathway. There were few GO carbohydrate-related pathways significantly affected by JA_{trt}, but these included the downregulation of carboxylic acid transport and the upregulation of cell wall organization and biogenesis, which were not affected by GM.

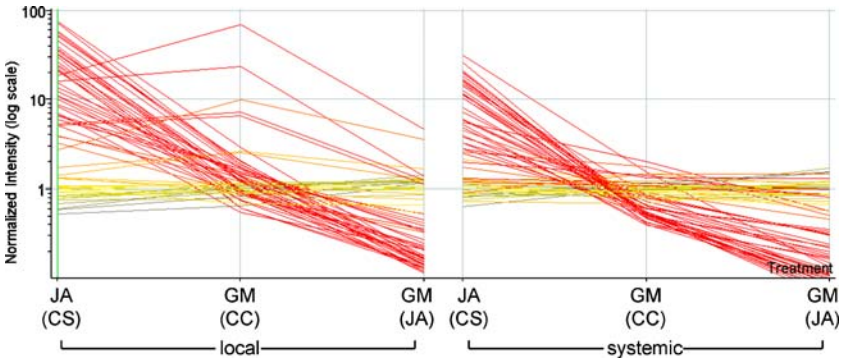

Fig. 5 Expression of proteinase inhibitor genes. Graph layout is similar to Fig. 4, with local responses on the *left* and systemic responses on the *right*. The *y*-axis represents the ratio of treated to reference gene expression, such that a 1 indicates no difference and 10 indicates ten times higher expression. On the *x*-axis the treatment (*top*) and the reference (*bottom in parentheses*) are indicated. Each *line* represents the average of three biological replicates for a single clone on the microarray and is colored based on expression in the JA treatment relative to the control spray as reference (*red* indicating local upregulation by JA)

GM, but not JA_{trt}, upregulated the aromatic amino acid biosynthetic pathway (i.e., shikimate pathway). GM and JA_{trt} both upregulated the first shikimate pathway gene, 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase (Fig. 6a, b). But GM also upregulated most of the other genes in the pathway (Fig. 7a), including chorismate mutase and prephenate dehydratase, the first and second steps committing chorismate to phenylalanine and tyrosine biosynthesis. Outside of the shikimate pathway, JA_{trt} downregulated the L-serine metabolism and protein folding categories, which were not affected by GM. GM also upregulated genes specific to leucine, lysine, and tryptophan biosynthesis.

The nitrogen compound biosynthesis category was upregulated by GM but not JA_{trt}. Nitrogen transport and assimilation transcripts were affected, seeming to favor a net leaf nitrogen decrease. GM upregulated a putative nitrate transporter and an amino acid transporter (AAT)-like gene, similar to a *Pinus* AAT (de la Torre et al. 2006). JA_{trt} not only downregulated nitrate reductase transcripts and several AATs, including a gene similar to *Arabidopsis* LHT1, necessary for uptake of transport amino acids from the xylem (Hirner et al. 2006) but also very strongly upregulated an AAT2-like aspartate aminotransferase, which is essential for normal phloem export of nitrogen as aspartate or asparagine (Schultz et al. 1998). Several other genes specific to nitrogen assimilation via glutamine biosynthesis and sulfate assimilation via cysteine biosynthesis were downregulated by both GM and JA_{trt}.

There were a number of transcriptional changes that were categorized under GO lipid metabolism, including fatty acid catabolism, fatty acid biosynthesis, and myoinositol metabolism, as well as ascorbic acid and carotenoid biosynthesis. Not only five genes involved in peroxisomal fatty acid β-oxidation were upregulated locally by GM but also several genes that may be involved in lipid biosynthesis, including storage lipids and waxes, were upregulated by both GM and JA_{trt} treatments (e.g., fatty acid desaturase 6, a fatty acid desaturase, and malic enzyme). GM and JA_{trt} upregulated two separate genes that both have high sequence similarity to *Arabidopsis* CER1, which is essential for normal levels of waxy cuticle formation (Aarts et al. 1995).

Systemic responses were less pronounced than local There was a high percentage overlap between systemic and local responses to GM (Fig. 2c), but only moderate overlap between systemic and local responses to JA_{trt} (Fig. 2d). Within systemic leaves, there was little similarity in the gene expression changes caused by GM and JA_{trt} (Fig. 2b). GOstats analysis showed that a small subset of the signaling, secondary metabolism, and primary metabolism categories responsive to GM in local leaves also responded in systemic leaves, plus several additional categories (Table 1). Relatively few GO categories were affected by JA_{trt} systemically, including several categories and effects unique to systemic JA_{trt}. For example, secondary metabolism and phenylpropanoid biosynthesis, which were upregulated locally by both JA and GM, were downregulated systemically by JA_{trt}. Categories unique to systemic JA_{trt} included photosynthetic light harvesting, negative regulation of signal transduction, snRNA transcription, and lignin biosynthesis, which were downregulated (Table 1).

Any response in untreated (i.e., systemic) leaves is dependent on the long distance movement of some signal from directly treated (i.e., local) leaves, which triggers

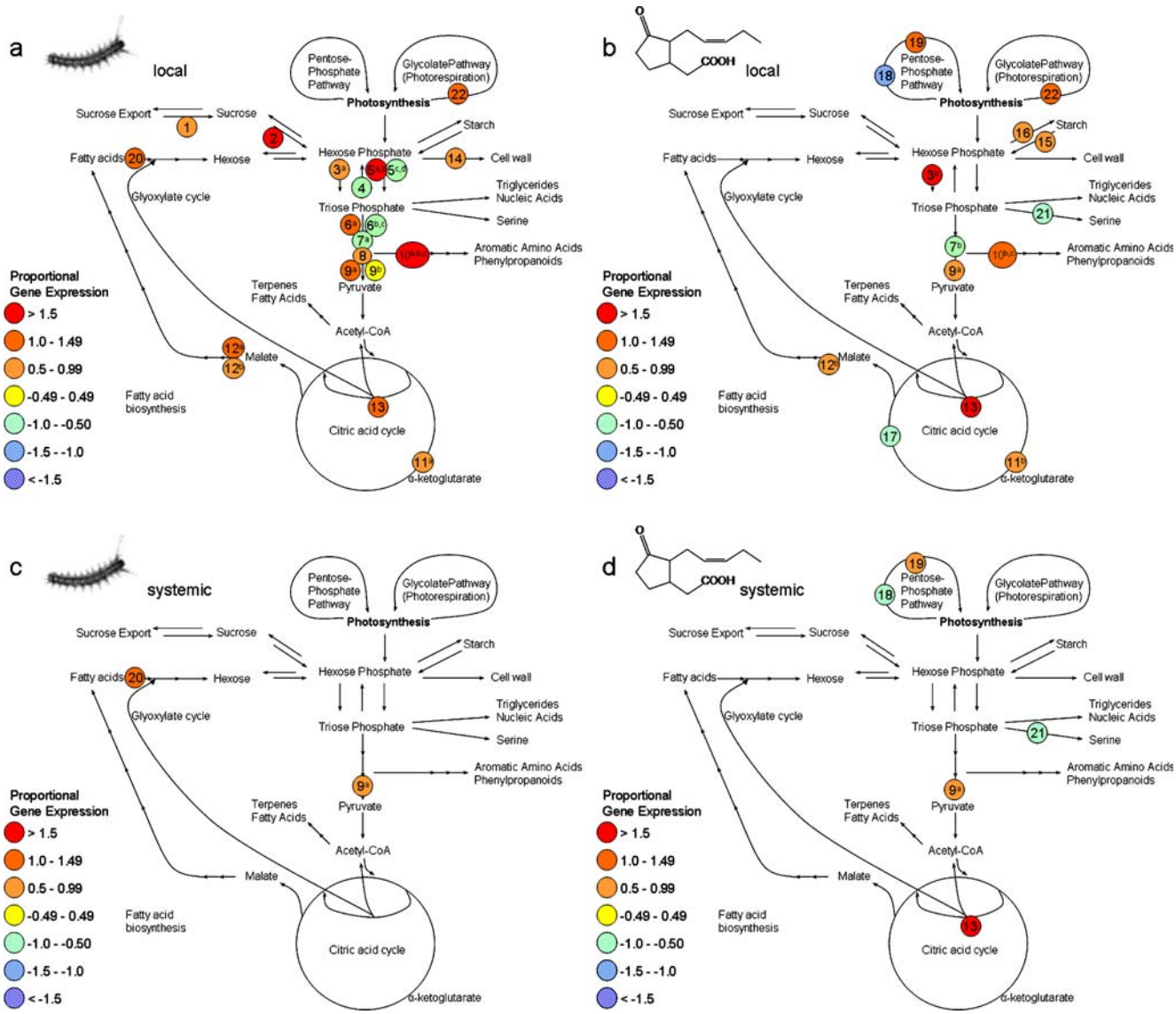

Fig. 6 Gene expression changes in carbohydrate metabolism in local leaves in response to **a** gypsy moth herbivory and **b** jasmonic acid and in systemic leaves in response to **c** herbivory and **d** JA. Changes in transcription compared to controls are indicated by *color*, according to the color key. For easier viewing, genes are not shown where there was no significant treatment effect. Where there are *multiple circles*, the enzyme is encoded by a multigene family, with members differentially affected by treatments. Otherwise, multiple isozymes are indicated by *superscript letters*. Numbers represent the following genes: *1* SUC3 sucrose transporter, *2* invertase, *3* phosphofructokinase, *4* fructose-1,6-bisphosphatase, *5* fructose-bisphosphate aldolase, *6* glyceraldehyde 3-phosphate dehydrogenase, *7* phosphoglycerate kinase, *8* enolase, *9* pyruvate kinase, *10* deoxy-D-*arabino*-heptulosonate-7-phosphate synthase, *11* isocitrate dehydrogenase, *12* malic enzyme, *13* isocitrate lyase, *14* UDP-xylose epimerase, *15* starch phosphorylase, *16* starch branching enzyme, *17* fumarate hydratase, *18* glucose-6-phosphate dehydrogenase, *19* 6-phosphogluconolactonase, *20* acyl-activating enzyme 12 (fatty acid catabolism), *21* D-3-phosphoglycerate dehydrogenase, and *22* glycolate oxidase. Carbohydrate metabolic pathways diagram modified from Buchanan et al. (2000)

another signaling cascade in systemic tissues, which then leads to a response. GM upregulated systemically the GO categories for JA biosynthesis, response to ABA stimulus and response to wounding, but not the response to pathogen category (Table 1; Fig. 3c). Exogenous JA_{trt} did not have systemic effects on JA, ABA, or auxin metabolism categories, but upregulated the response to ABA stimulus category and downregulated the negative regulation of signal transduction category (Table 1). Within our list of protein modifying genes and transcription factors, changes were the strongest for genes also affected locally, with a few exceptions (S3).

The systemic induction of defense-related pathways by GM again appeared to be a weaker version of the local

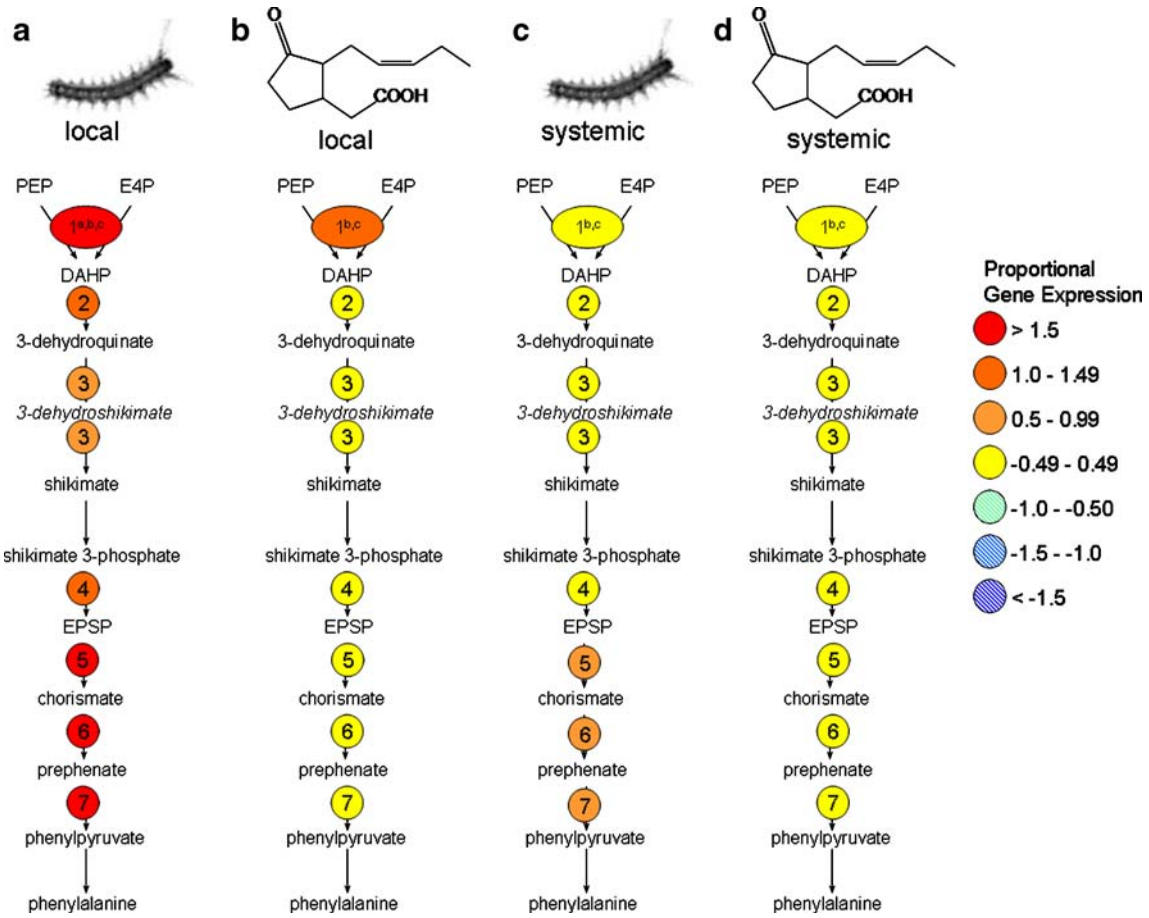

Fig. 7 Shikimate pathway transcriptional changes in response to **a**, **c** herbivory and **b**, **d** JA locally and systemically, respectively. Changes in transcription compared to controls are indicated by *color*, according to the color key. Multiple isozymes are indicated by *superscript letters*. *Numbers* represent the following genes: *1*, deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (*DAHPS*), *2* dehydroquinate synthase, *3* dehydroquinate dehydratase/shikimate dehydrogenase, *4* *enol*pyruvylshikimate-3-phosphate (*EPSP*) synthase, *5* chorismate synthase, *6* chorismate mutase, and *7* prephenate dehydratase. 3-Dehydroshikimate is *italicized* since it is an intermediate of a bifunctional enzyme, dehydroquinate dehydratase/shikimate dehydrogenase

response, mainly consisting of phenylpropanoid metabolism and response to wounding categories (Table 1). JA_{trt} did not alter phenylpropanoid metabolism or response to wounding categories systemically, but affected categories of genes associated with, "responses to", various other stimuli, including water, hormone stress, and heat. At the individual gene level, we saw both smaller changes in expression of genes and fewer genes affected, as exemplified by phenylpropanoid biosynthesis, flavonoid biosynthesis, and the shikimate pathway genes (Fig. 4). The systemic upregulation of several PIs was also much weaker than local induction by GM, whereas several other genes encoding defense-related proteins that were upregulated locally by GM (i.e., PPO and TD) were not upregulated systemically. However, JA_{trt} strongly upregulated systemically most of the PIs that were upregulated locally by JA_{trt} and nearly to the same extent (Fig. 5).

GM and JA_{trt} had little effect on primary metabolism GO categories in systemic leaves (Table 1). For example, photosystem II photosynthetic light harvesting was downregulated systemically by JA_{trt}. The fatty acid metabolism GO category was upregulated systemically by GM. This was mostly due to upregulation of genes involved in β-oxidation, which may be related to both membrane composition and JA biosynthesis (Fig. 3c).Within pathways affected locally, there were very few and only weak systemic effects on individual genes relating to carbohydrate and organic acid metabolism (Fig. 6c, d). However, pyruvate kinase was upregulated by both GM and JA_{trt} systemically, as it was in local leaves (Fig. 6). JA_{trt} also altered expression of several pentose phosphate pathway genes, similar to the response in local leaves. The latter half of the shikimate pathway was upregulated systemically by GM, but not JA_{trt} (Fig. 7). However, JA_{trt} did downregulate

Table 2 Selected *Populus* genes exhibiting altered expression by both GM here and FTC in a previous microarray study (Ralph et al. 2006a)

Reporter ID	Annotation	M	*T* test	*B* stat	Closest *Arabidopsis thaliana* hit
	Defense related				
PU12110	Trypsin proteinase inhibitor	5.0	$P<0.001$	30.7	At1g73325
PU03006	Peroxisomal membrane protein	1.1	$P<0.01$	0.7	At2g39970
PU20375	Peroxidase	0.6	$P<0.05$	0.2	At5g05340
PU09263	LOX2	2.0	$P<0.001$	16.1	At3g45140
PU29697	Jacalin lectin	8.7	$P<0.001$	28.3	At1g19715
PU25511	Glutathione *S*-transferase	0.9	$P<0.01$	0.6	At3g03190
PU06017	Wound inducible proteins (*Arabidopsis*)	4.2	$P<0.001$	6.2	At4g24220
PU25107	Dehydration-induced protein (ERD15)	0.9	$P<0.001$	3.1	At2g41430
PU21905	Cytochrome P450	3.0	$P<0.001$	14.8	At3g25180
PU26508	Apyrase (APY2)	4.0	$P<0.001$	13.9	At5g18280
PU13396	4CL3	1.0	$P<0.001$	5.4	At1g65060
PU26210	"Beta 1,3-glucanase (GH family 17)"	0.6	$P<0.01$	0.8	At3g57270
PU10405	UDP-glycosyltransferase	0.7	$P<0.01$	1.4	At3g16520
	Primary metabolism				
PU01407	DAHP synthase	2.0	$P<0.001$	7.9	At1g22410
PU26549	DAHP synthase 1	1.7	$P<0.001$	16.7	At4g39980
PU00151	Chorismate synthase	2.0	$P<0.001$	11.4	At1g48850
PU06861	Prephenate dehydratase	2.1	$P<0.001$	9.4	At1g08250
PU25397	Serine carboxypeptidase	1.4	$P<0.001$	5.1	At5g22860
PU08833	Methionine gamma lyase	2.4	$P<0.001$	8.1	At1g64660
PU09309	Nitrate transporter (NTP3)	0.7	$P<0.001$	4.0	At3g21670
PU12762	Pyruvate kinase	1.4	$P<0.001$	8.6	At3g52990
PU09110	Oxoglutarate/malate transporter	−0.5	$P<0.01$	1.0	At5g12860
PU13124	Malic enzyme	1.1	$P<0.001$	3.6	At1g79750
PU01907	ATP-citrate (pro-S-)-lyase	1.2	$P<0.001$	3.3	At5g49460
PU30654	Fructose bisphosphate aldolase	0.7	$P<0.001$	5.6	At2g01140
PU21563	Omega-3 fatty acid desaturase (FAD8)	1.3	$P<0.001$	9.2	At5g05580
PU26639	GDSL-motif lipase	0.8	$P<0.01$	0.5	At5g45670
PU06552	Triacylglycerol lipase	1.0	$P<0.01$	1.7	At5g22460
PU02110	Dehydroascorbate reductase	1.1	$P<0.001$	6.5	At1g75270
PU27584	Calcium transporter (plasma membrane)	0.6	$P<0.01$	1.3	At3g21180

Gene lists from the two studies were matched based on the *Arabidopsis thaliana* gene with closest sequence similarity

M log2 fold change in response to GM

D-3-phosphoglycerate dehydrogenase, an enzyme diverting triose phosphates for serine biosynthesis, as in local leaves (Fig. 6b, d).

Common responses of Populus to GM and FTC There were many differences in the overall transcriptional responses of *Populus* to GM and FTC, which could be due to numerous differences between the two experiments. However, the induced genes common to both *Populus* species are likely to be highly important conserved components of the defensive response. For example, LOX2, which is involved in biosynthesis of JA from fatty acids, was upregulated by both GM and FTC herbivory. The GM- and FTC-induced genes include well known defense-related genes (Table 2). Some of the putative defense genes upregulated by both herbivores have not been characterized (e.g., wound inducible proteins), but several have been well studied; for example, a phenylpropanoid gene (4CL) and PIs, which are thought to reduce digestibility of plant protein in the herbivore gut, were upregulated by both herbivores.

Adjustments to primary metabolism appear to be another important aspect of plant defense against herbivory. For example, GM and FTC both upregulated DAHP synthase

and prephenate dehydratase, key genes in phenylalanine biosynthesis, which may be geared toward increasing the flux of primary metabolites into the phenylpropanoid biosynthetic pathway. But there were also genes involved in carbohydrate metabolism (e.g., pyruvate kinase and fructose bisphosphate aldolase) and lipid metabolism (e.g., lipases and fatty acid desaturase 6). Further, genes at the interface between carbohydrate and lipid metabolism were upregulated by both GM and FTC, such as ATP citrate lyase and malic enzyme.

Discussion

Interactions between plants and their antagonists (pathogens and herbivores) are very complex and traits relating to biotic interactions are under strong selection in plants. Studies in different plant–herbivore systems not only have provided many insights into general plant responses but also have revealed a huge amount of variation between plant species and between antagonists attacking the same species. Thus, the many studies of annual herbaceous plants cannot be generalized to understand the defense responses of trees and other perennials, neither for mechanisms to reduce nor to tolerate herbivory (Haukioja and Koricheva 2000). In *Populus*, there are remarkable genotypic differences in induced resistance (Havill and Raffa 1999; Osier and Lindroth 2001; Robison and Raffa 1997; Stevens and Lindroth 2005). The *P. nigra* clone NC5271 chosen for our study is highly responsive to herbivory, reducing palatability and suitability of leaf tissue in response to herbivore feeding (Robison and Raffa 1997), making it a suitable genotype for exploring induced defense and identifying candidate genes that may play critical roles in enhancing the level of plant resistance.

GM herbivory induced dramatic changes in the expression of genes involved in multiple signaling pathways, secondary metabolism, and primary metabolism in *P. nigra*, and the response to herbivory was propagated systemically, but tended to be a narrower and weaker version of the local response. Possibly certain localized responses serve particularly in wound healing, in coping with localized stresses associated with wounds (e.g., water loss, oxidative stress) and defending against pathogens that enter through wounds, while the systemic responses may serve to reduce damage by detering or killing the herbivores. Recognizing that gene transcription does not always match perfectly with plant biochemistry, transcriptional responses give a fair portrait of the responses that have been selected through evolution and by focusing on pathway responses revealed by GOStats, some solid patterns emerge.

Any plant response to an environmental disturbance will begin with translation into signal transduction events. Our data show multiple possible points at which selection could act to alter phenotypic plasticity, as related to herbivory, for example by biosynthesis of signal compounds such as JA and ABA. In the case of herbivory, JA signaling has long been thought to have a central role (Creelman and Mullet 1997; Wasternack and Parthier 1997), and in *P. nigra*, the JA biosynthetic pathway was upregulated by GM. However, it is clear from our comparison of GM and JA_{trt} that responses to herbivory are mediated by a complex network of signaling pathways. Recent evidence suggests ABA may also be important in herbivore signaling (Cheong et al. 2002; Park et al. 2006), interacting positively with JA signaling (Thaler and Bostock 2004). In *P. nigra*, ABA biosynthetic genes were upregulated by GM locally, while genes associated with the response to ABA were upregulated systemically, suggesting that ABA might be a mobile signal propagating a systemic response (but see also Frost et al. 2007). To what extent other changes we recorded among hormone pathways are biologically significant will require more detailed studies using hormone treatments and/or mutants, but this illustrates at least the potential for complex interactions of plant hormonal pathways to modulate plant growth, defense, and maintenance processes.

Many plants exhibit inducible defenses when attacked by herbivores, reducing nutritive value of plant tissues, while increasing chemical and structural deterrents and toxic agents, as well as indirect defenses, such as volatile compounds that attract natural enemies of herbivores. A previous study including this *P. nigra* clone found changes in deterrent qualities of leaf extracts in response to FTC, but the nature of those changes was not characterized (Robison and Raffa 1997). In *Populus*, biochemicals possibly important to direct defense include salicylate-containing phenolic glycosides, phenylpropanoid compounds, such as condensed tannins, and protein-based defense compounds (Haruta et al. 2001; Hwang and Lindroth 1997; Osier and Lindroth 2001). Although genes coding for salicylate biosynthetic enzymes have not yet been identified, GM upregulated biosynthesis of certain phenylpropanoid transcripts. Most of the known genes leading to the biosynthesis of condensed tannins were upregulated locally by GM. Putative protein-based defenses were also upregulated, including storage proteins/proteinase inhibitors and amino acid deaminases. In addition to these direct defenses, terpenoid biosynthetic genes were upregulated locally in damaged leaves, not in systemic leaves or by JA_{trt}, in contrast to a previous study where FTC herbivory increased terpenoid gene expression and emissions both locally and systemically in a *Populus* hybrid (Arimura et al. 2004). Increased emissions of volatile terpenoids may serve as indirect defenses, attracting natural enemies (e.g., predators and parasitoids) of herbivores to damaged plants (Schnee et al. 2005; Schröder et al. 2005).

These diverse defenses that are induced by herbivory require greater than normal inputs of energy and carbon. Therefore, we would expect modifications to primary metabolism that provide support for induced secondary metabolism. Indeed, in response to GM, we found upregulation of several phenylalanine biosynthetic genes and most of the shikimate pathway genes. There were also some changes in carbohydrate metabolism that favor biosynthesis of shikimate pathway precursors. This suggests that induction of phenylpropanoids in this *P. nigra* clone was not due to one or two key genes, but a broad swath of genes being upregulated.

Not all herbivore-induced changes in primary metabolism have necessarily evolved to support secondary metabolism. Some changes may play a direct role in coping with or tolerating lost and damaged tissues and the stresses associated with damage, such as increased water loss (Tang et al. 2006). The distinction between "resistance" and "tolerance" genes is not always clear-cut, particularly since some genes may be dual purpose, serving directly in defense against herbivores but also in tolerating damage. Further, the plant must tolerate not only the direct effects of the damage incurred but also must cope with the metabolic changes associated with defense induction, which may lead to suboptimal performance in the absence of herbivory. As an example, pyruvate kinase was upregulated locally and systemically by GM and JA_{trt} and was also upregulated by FTC (Ralph et al. 2006a). This is unlikely to aid in phenylpropanoid induction, since pyruvate kinase is downstream of the branchpoint where carbohydrate intermediates become precursors for the shikimate pathway. Upregulation of pyruvate kinase might maintain the flux into the citric acid cycle at a time when more carbon is being appropriated for the biosynthesis of secondary metabolites. Alternatively, upregulation of pyruvate kinase could be important for altering the biosynthesis of fatty acids, terpenoids, and certain amino acids, which require carbon backbone intermediates downstream of pyruvate kinase (e.g., pyruvate, acetyl-CoA, 2-oxoglutarate).

Several recent reports indicate that decreased photosynthetic gene expression may be a common, but not ubiquitous, plant response to herbivory (e.g., Hermsmeier et al. 2001; Ralph et al. 2006a; Voelckel and Baldwin 2004), and photosynthetic rates may decrease following herbivory (Tang et al. 2006), or even oviposition (Schröder et al. 2005). Very few transcriptional changes in photosynthetic genes were observed here, but did include upregulation of a RUBISCO small subunit. In a separate study, using the same *P. nigra* clone, GM herbivory resulted in a small increase in photosynthesis (Babst 2006). It is tempting to speculate that a small boost in photosynthesis might be an adaptive mechanism to maintain carbon acquisition despite damage to photosynthetic leaf area, but regulation of photosynthesis is complex (e.g., Sjödin et al. 2008), and further studies will be needed to determine if there is any realized impact on plant function. There were also changes in nitrogen metabolism genes, which could possibly be related to tolerance. In a previous study, hybrid poplars (*P. nigra* Muench × *Populus maximowiczii* A. Henry) exhibited altered nitrogen partitioning in response to exogenous methyl jasmonate application, favoring nitrogen accumulation in stems (Beardmore et al. 2000). Future studies should address whether partitioning or metabolism of nitrogen is in fact altered by herbivory in *Populus* to reduce the nutritive value of leaves for the herbivores, or to increase nitrogen uptake to help replace nitrogen that is lost during defoliation.

Defense induction often extends to undamaged leaves due to specific mobile signaling molecules (Orians 2005). But also, the gross metabolic effects of defense induction may extend to the entire plant, as evidenced by increases in basipetal carbon partitioning from mature leaves to stems and roots in both *Populus* and *Nicotiana* (Babst et al. 2005, 2008; Schwachtje et al. 2006). Although the consequences have not been fully explored yet, increased basipetal carbon partitioning improved tolerance of herbivory in *Nicotiana* (Schwachtje et al. 2006). Unlike *Nicotiana* (Schwachtje et al. 2006), there were no changes in expression of sucrose nonfermenting-1-related kinases due to either GM or JA_{trt} treatment in *P. nigra*, suggesting that at least one additional mechanism of control over carbon transport remains to be discovered. Carbon partitioning can be influenced by rates of phloem unloading in sink tissue, for example by cell wall invertases (Arnold and Schultz 2002), but previous partitioning studies with this *P. nigra* clone strongly suggested a mechanism within the exporting leaf (Babst et al. 2005, 2008). We did not find substantial changes in genes involved in the biosynthesis or transport of sucrose, the major transport sugar in *Populus*. However, there were changes in expression of chloroplast–cytosol transporters, which might play an important supporting role, increasing the sucrose available for vascular transport (Lundmark et al. 2006).

To conclude, it is known that plants generally do not put forward a "silver bullet" defense, but utilize multifaceted defenses against herbivores. These defenses serve to reduce nutritive value of the plant tissue, increase the repellent properties of the tissue, increase concentrations of toxic chemicals, and attract natural enemies of herbivores using volatiles. Plants must cope with the metabolic changes incidental to the induction of these defenses, autotoxicity of secondary metabolites and defensive proteins, and they must tolerate incidental stresses associated with physical damage to tissues. We have identified a large number of candidate genes with possible roles in each of these aspects of the response of *Populus* to herbivory. These candidate

genes include many unstudied genes that, once characterized, may reveal novel weapons in the antiherbivore arsenal. Further, we suggest that, since traits that confer tolerance are likely to be more conserved than strictly defensive traits, which tend toward diversification, the primary metabolic genes affected by both FTC and GM in separate *Populus* hybrids may be good initial targets for studying possible roles in tolerance to understand how trees cope with tissue damage and its incidental stresses and ultimately to provide the means to genetically manipulate or select for variants in these tolerance-related traits.

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

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