Molecular analysis of poplar defense against herbivory: comparison of wound- and insect elicitor-induced gene expression

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Summary

• In order to characterize defense responses of hybrid poplar (*Populus trichocarpa* × *P. deltoides*), we profiled leaf transcript patterns elicited by wounding and by regurgitant from forest tent caterpillar (FTC; *Malacosoma disstria*), a Lepidopteran defoliator of poplars.

• Macroarrays were used to compare transcript profiles. Both FTC-regurgitant (FTC-R) and mechanical wounding with pliers elicited expression of a variety of genes, and for these genes our analysis indicated that these treatments induced qualitatively similar responses.

• Similarily, a comparison of responses of directly treated and systemically induced leaves indicated extensive overlap in the sets of induced genes. FTC-R was found to contain the insect-derived elicitor volicitin.

• The simulated herbivory treatments resulted in the induction of genes involved in poplar defense and secondary metabolism. We also identified wound-responsive genes with roles in primary metabolism, including a putative invertase, lipase, and acyl-activating enzyme; some of these genes may have roles in defense signaling. In addition, we found three unknown genes containing a ZIM motif which may represent novel transcription factors.

Key words: defense gene expression, herbivore defense, hybrid poplar (*Populus*), macroarray, plant defense, systemic response.

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Introduction

Under selective pressure by herbivores, plants have evolved a variety of physical and biochemical defense mechanisms. A common defense strategy is the active deployment of inducible defenses, which can include both proteins and secondary metabolites whose synthesis is triggered by herbivore damage (Walling, 2000; Baldwin *et al.*, 2001; Gatehouse, 2002; Kessler & Baldwin, 2002). Inducible defenses often act as toxins, antifeedants, or antinutrients; for example, many plants synthesize herbivore-induced proteinase inhibitor proteins which inhibit insect digestive enzymes and have directly toxic effects (Ryan, 1990). Other antinutritive defenses include the oxidative enzymes polyphenol oxidase (PPO), peroxidase and lipoxygenase, thought to act by destroying or modifying essential amino acids and fatty acids (Duffey & Felton, 1991). In some species, the induced defense arsenal includes secondary metabolites such as alkaloids, terpenoids, or phenolics (reviewed in Walling, 2000; Kessler & Baldwin, 2002). Herbivory also stimulates the synthesis and release of terpenoid, aromatic and aliphatic volatiles, potential signals which can attract predators and parasitoids of the herbivore and thus contribute to defense (Arimura *et al.*, 2005). It is significant that herbivore defenses are often induced beyond the site of insect feeding, in undamaged leaves of damaged plants. These leaves thus gain considerable resistance to herbivory despite not being directly damaged (Havill & Raffa, 1999). This systemic defense response and its regulation has been especially well

characterized in tomato, where it is now known that jasmonic acid (JA) has a prominent role in systemic signaling (Bergey *et al.*, 1996; Ryan, 2000; Howe, 2004; Schilmiller & Howe, 2005).

A number of insect-derived elicitors that are recognized by plant cells and trigger defense reactions have been discovered in insect regurgitants. They include both proteins, such as β-glucosidase, or fatty acid-amino acid conjugates (FACs) (Tumlinson & Lait, 2005). The first FAC with elicitor activity to be identified was *N*-hydroxylinolenoyl-L-glutamine and named volicitin based on its ability to trigger volatile release (Alborn *et al.*, 1997). Other potent FAC elicitors are *N*-linolenoyl-L-glutamine and *N*-linolenoyl-L-glutamic acid (Tumlinson & Lait, 2005). FACs have since been shown to be widespread among all caterpillar species examined to date, including species of the families Noctuidae (e.g. *Spodoptera* spp.), Geometridae and Sphingidae (e.g. *Manduca* spp.) (Pohnert *et al.*, 1999; Halitschke *et al.*, 2001; Mori *et al.*, 2001, 2003). They induce various defense reactions, including changes in gene expression and volatile release (Alborn *et al.*, 1997; Halitschke *et al.*, 2001; Alborn *et al.*, 2003; Halitschke *et al.*, 2003; Roda *et al.*, 2004).

Transcript profiling using DNA arrays is a powerful tool that is now being applied to investigate plant responses to insect feeding and wound signaling. DNA array studies have compared mechanical wounding and insect feeding, or damage by different insect feeding guilds (Heidel & Baldwin, 2004; Reymond *et al.*, 2004; Voelckel & Baldwin, 2004). In *Arabidopsis*, arrays showed that the vast majority of herbivore-inducible transcripts are also up-regulated by wounding (Reymond *et al.*, 2000; Reymond *et al.*, 2004). Similar experiments revealed that many wound-induced genes are induced by both osmotic stress and heat shock, or have led to the discovery of novel defense genes and biochemical pathways that respond to wounding and herbivory (Reymond *et al.*, 2000; Cheong *et al.*, 2002; Halitschke *et al.*, 2003).

In hybrid poplar, wound-induced gene expression was first described by Gordon and coworkers, who isolated genes encoding an inducible Kunitz trypsin inhibitor (TI), several chitinases, and a vegetative storage protein (Parsons *et al.*, 1989; Davis *et al.*, 1991a, 1993). We subsequently identified PPO as an inducible defense protein in this system (Constabel *et al.*, 2000; Haruta *et al.*, 2001b). Additional studies further characterized poplar-induced defenses at the transcript, protein, and enzyme activity levels (Clarke *et al.*, 1998; Constabel *et al.*, 2000; Haruta *et al.*, 2001a,b; Wang & Constabel, 2003). The efficacy of hybrid poplar-induced defense has been directly demonstrated; poplar saplings previously subjected to herbivory, wounding, or caterpillar regurgitant are subsequently poorer hosts for gypsy moth larvae (Havill & Raffa, 1999). Moreover, the antiherbivore effects of poplar PPO has been directly shown for forest tent caterpillar (FTC; *Malacosoma disstria*) using transgenic poplar overexpressing this protein (Wang & Constabel, 2004). Induced synthesis of poplar terpenoids, which may contribute to indirect defense, has also been described (Arimura *et al.*, 2004). Furthermore, in *Populus tremuloides*, herbivore- and wound-stress induces the expression of flavonoid biosynthesis genes, which is correlated with induced proanthocyanidin accumulation (Peters & Constabel, 2002).

Populus has become a major focus of plant genomics research. The *P. trichocarpa* genome is now fully sequenced (Brunner *et al.*, 2004; Tuskan *et al.*, 2004), and large expressed sequence tag (EST) collections are available. These provide a key resource that can be used for digital analysis of gene expression, gene discovery (Sterky *et al.*, 2004), and to construct DNA microarrays. Poplar cDNA arrays are presently being used to investigate a variety of processes relevant to woody plants and perennials, including autumn senescence and wood development (Andersson *et al.*, 2004; Schrader *et al.*, 2004; Moreau *et al.*, 2005), as well as abiotic and biotic stress resistance (Gu *et al.*, 2004; Smith *et al.*, 2004; Ralph *et al.*, 2006). The large number of naturally occurring poplar insect herbivores make *Populus* a powerful system in which to study plant–herbivore interactions at the molecular and genomic levels. Previously, we undertook a small-scale EST sequencing project in hybrid poplar (*P. trichocarpa* × *P. deltoides*) to provide a set of defense-related genes and to obtain a broader view of the transcriptome in leaves undergoing a defense response (Christopher *et al.*, 2004). A substantial number of ESTs were found to encode proteins involved in defense or secondary metabolism, and many were up-regulated after wounding. Here, we report on the use of macroarrays constructed from this EST set to examine the poplar leaf response to insect-derived cues from FTC. Our analysis revealed that for this collection of wound-induced genes, the responses to FTC-regurgitant and wounding are qualitatively similar, though quantitatively distinct.

Materials and Methods

Plant material

Poplar hybrid H11-11 (*P. trichocarpa* × *P. deltoides*), originating from the University of Washington/Washington State University Poplar Research Program, were propagated from greenwood cuttings in Sunshine Mix #4 (Sungro, Seba Beach, AB, Canada) in 0.25 l propagation containers (RootMaker, Huntsville, AL, USA). After plantlets had rooted and reached a height of approx. 10 cm, they were transplanted into 15-cm-diameter pots containing Sunshine Mix #4 plus slow-release nutrients (8.9 g l^{-1} controlled release 8-6-12 NPK plus micronutrients (Acer, Delta, BC, Canada), 0.458 g l^{-1} superphosphate 0-20-0 (Green Valley, Surrey, BC, Canada), 1.21 g l^{-1} Micromax Micronutrients (Scotts-Sierra, Marysville, OH, USA), and 4.75 g l^{-1} Dolomite lime (IMASCO, Surrey, BC, Canada)). Plants were maintained in the Bev Glover Greenhouse at the University of Victoria. All experiments were conducted between March and May. Supplemental lighting from 600 W high-pressure sodium lamps was used to extend the photoperiod to 16 : 8 h, and the temperature within the

greenhouse was maintained at 25 : 18°C. Plants were watered daily with a solution containing 0.1 g l^{-1} 20-20-20 PlantProd fertilizer (Plant Products, Brampton, ON, Canada). All lateral shoots were pruned as they developed so that each plant consisted of a single main stem, no less than 2 wk before wounding.

FTC-regurgitant collection

Regurgitant was collected from fourth- and fifth-instar FTC larvae found on aspen foliage (*P. tremuloides*) near Drayton Valley (AB, Canada). FTC-R was collected by micropipette, immediately frozen on dry ice, and stored at −80°C. The preparations were incubated at 100°C for 20 min to eliminate potential enzyme activity or enzymatic degradation of elicitors (Mattiacci *et al.*, 1995; Mori *et al.*, 2001; Alborn *et al.*, 2003), and then filter-sterilized to eliminate microbial activity. FTC-R was analyzed by LC-MS for the presence of chemical elicitors by Dr Amy Roda, Dr Bernd Krock and Dr Ian Baldwin (Max Planck Institute for Chemical Ecology, Jena, Germany).

Wounding and FTC-R treatments

Plants were 12 wk old and 1 m tall with approx. 30 leaves when used for experiments. Leaves were mechanically wounded by crushing the margins of leaf blades, while for FTC-R treatments, FTC-R was applied to leaf punctures made with a fabric tracing wheel. A total of 100 μL of FTC-R was applied to 100 punctures (1 μL per puncture) over 10 rows per leaf. We first tested inducing activity of a range of FTC-R-dilutions to establish an appropriate concentration for further experiments. Leaves corresponding to LPI 10 (leaf plastochron index) (Larson & Isebrands, 1971) were treated with sterile ddH_2O or FTC-R diluted 1 : 1, 1 : 5, 1 : 20, 1 : 60, and 1 : 180 (v/v) with water, and harvested after 24 h for analysis. For subsequent experiments, FTC-R was diluted 1 : 5 (v/v) with sterile ddH_2O. FTC-R-treatment was compared between leaves treated with FTC-R and leaves treated with sterile ddH_2O (mock control). For macroarray experiments studying local responses, leaves of LPI 9–17 were treated three times, at 1 h intervals, with either wounding or FTC-R. Leaves LPI 9–11 were harvested 24 h after start of the treatment, frozen in liquid nitrogen, and stored at −80°C until analyzed. For studying systemic responses, leaves of LPI 12–17 were treated three times (1 h intervals), and untreated leaves of LPI 9–11 were harvested 24 h after start of the treatment. This design ensured that leaves designated as systemic were induced equally (see Davis *et al.*, 1991b), and were equivalent in age to directly treated leaves.

RNA extraction and hybridization

Total RNA was isolated from hybrid poplar leaves, quantified by UV absorbance, and quality verified on ethidium bromide-stained agarose gels as previously described (Haruta *et al.*, 2001a). RNA (10 μg per lane) was loaded on to 1.2% (w/v) agarose-formaldehyde gels, and blotted overnight onto Hybond-N^+ nylon membranes (Amersham Biosciences, Baie d'Urfé, PQ, Canada). RNA blots were probed with cDNA clones labeled with [α-^{32}P]dCTP (Rediprime II kit, Amersham). Hybridizations were performed at 65°C and were washed at high stringency according to Church & Gilbert (1984). The blots were detected with a Storm PhosphorImager (Amersham) and signal intensities were quantified using ImageQuant (Amersham). Ethidium bromide staining of RNA or blot hybridization with an actin cDNA probe were used to verify equal loading of lanes.

Macroarray analysis

For macroarray construction, 580 cDNA inserts were amplified by PCR. The majority of the cDNAs (569) were amplified from the unigene set of our EST library (Christopher *et al.*, 2004), generated from systemic leaves of wounded hybrid poplar saplings (Constabel *et al.*, 2000). The remaining 11 cDNAs were derived from a suppression subtractive hybridization (SSH) library generated from systemic leaves of hybrid poplar saplings challenged by FTC (*Malacosoma disstria* Hübner) (J. Patton & C. P. Constabel, unpublished). A total of 10 ng of DNA for each clone was spotted in duplicate onto prewet nylon membranes (Hybond N^+ Amersham) using a handheld multiblot replicator as per the manufacturer's instructions (VP Scientific, San Diego, CA, USA). Care was taken to spot equal amounts of DNA so that we could gain additional information about transcript abundance from our macroarray analysis. Membranes were incubated DNA side up on 0.2 N NaOH for 10 min, followed by 0.5 M Tris-HCl (pH 7.4)/0.5 M NaCl for 5 min, and cross-linked using a low-energy UV cross-linker (Stratagene, La Jolla, CA, USA). Membranes were rinsed in 2 × SSPE/0.1% (w/v) SDS before air drying.

For array analysis, total RNA was isolated from three pooled leaves of each of three independent biological replicates (trees) for all four treatments (local wounding, systemic wounding, local FTC-R, systemic FTC-R) as well as the corresponding controls for each. Each replicate was analyzed on an individual macroarray. For synthesis of ^{33}P-labeled cDNA, poly(A)$^+$ RNA was isolated from 37.5 μg total RNA using Dynabeads Oligo $(dT)_{25}$ (Dynal Biotech, Lake Success, NY, USA), reverse transcribed using Superscript II (Invitrogen, Burlington, ON, Canada) and labeled with ^{33}P using a Rediprime II kit (Amersham). Macroarrays were hybridized at 65°C, washed at high stringency according to Church & Gilbert (1984), and exposed to PhosphoImager screens for 48 h. Images were scanned with a Storm PhosphoImager (Amersham), and the signals were quantified using ArrayVision 7.0 (Imaging Research, St Catherines, ON, Canada). Background intensity surrounding each spot was calculated and subtracted from each spot. The average of duplicate spots was used for all downstream analyses. Signal values < 1% of mean signal intensity were manually raised to avoid extreme expression ratios. Corrected spot

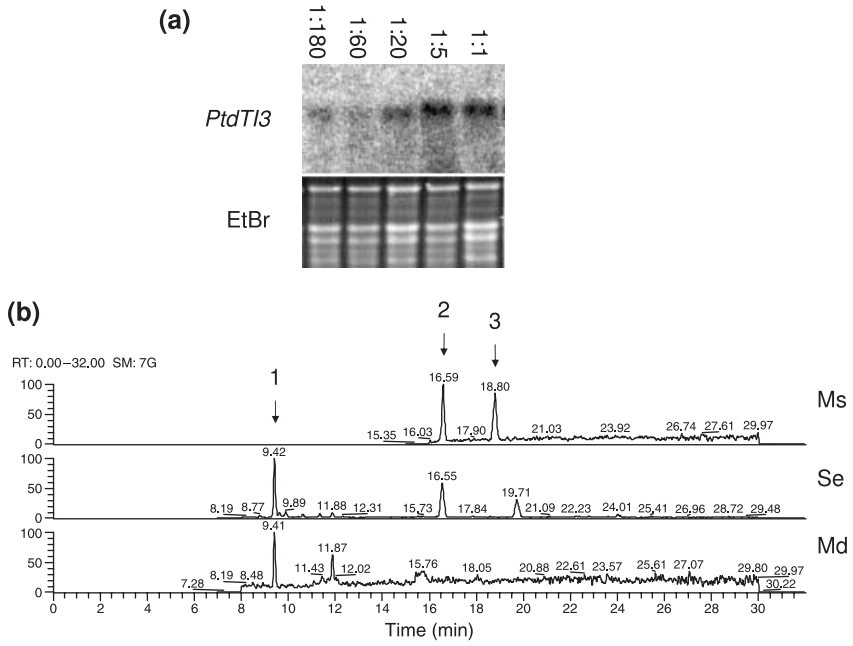


Fig. 1 Analysis of forest tent caterpillar-regurgitant (FTC-R) and FTC-R-induced expression of defense-related Kunitz trypsin inhibitor. (a) Accumulation of *PtdTI3* (Kunitz trypsin inhibitor 3) transcripts in poplar leaves in response to a gradient of FTC-R-dilutions. Dilutions of regurgitant were added to tracing wheel-punctures on leaves of LPI 10. Leaves were harvested at 24 h and analyzed by Northern blot. EtBr, ethidium bromide-stained gel used as a loading control. (b) LC-MS analysis of fatty acid-amino acid conjugates (FACs) from the regurgitant of *Manduca sexta* (Ms), *Spodoptera exigua* (Se), and *Malacosoma disstria* (Md). 1, *N*-hydroxylinolenoyl-L-glutamine (volicitin); 2, *N*-linolenoyl-L-glutamine; 3, *N*-linolenoyl-L-glutamate.

intensities were normalized to the standard deviation of the entire array (Richmond & Somerville, 2000). Relative transcript abundance was calculated directly from intensities of the normalized signals. Test hybridizations comparing control plant mRNAs gave a correlation coefficient of 0.982. Normalized intensities from the three biological replicates were used to calculate average expression ratios.

A Student's *t*-test (paired, one-tailed) on $\log_2$-transformed data was used to determine statistical significance of expression ratios of each treatment and control pair. *Q*-values were calculated using R (http://www.r-project.org/; Storey & Tibshirani, 2003). Hierarchical clustering was performed with EPCLUST (http://ep.ebi.ac.uk/EP/EPCLUST/). Heat maps of *P*-values and transcript abundance were generated with Treeview (http://rana.lbl.gov/EisenSoftware.htm). χ^2 analyses were performed to assess significance of differences in the proportions of functional classes, as carried out in Smith *et al.* (2004).

Sequence analysis and data management were performed with Vector NTI Advance 9.0 (Invitrogen). To confirm the identity of genes, 20 of the most highly induced genes were confirmed by re-sequencing. To obtain full-length sequences for candidate genes, the JGI poplar genome database (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) was queried. Annotations of candidate genes were improved with similarity searches through BLASTP (Altschul *et al.*, 1997) against the NCBI non-redundant protein database (http://www.ncbi.nlm.nih.gov/), the UniProt knowledgebase at EXPASY (http://www.expasy.org/), and the AGI protein database at TAIR (http://www.arabidopsis.org/). Conserved domains and motifs of unknown genes were identified through queries against the conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Predictions of nuclear localization signals were made with Prosite (http://www.expasy.org/prosite/) and PredictNLS (http://cubic.bioc.columbia.edu/predictNLS/).

Results

FTC-regurgitant induces poplar trypsin inhibitor gene expression

We previously demonstrated that mechanical wounding or feeding by FTC triggers the hybrid poplar defense response. To establish if FTC-regurgitant elicits a defense response in poplar leaves and if it could be used as an insect-derived cue for expression profiling, we collected regurgitant from FTC feeding on native *Populus* foliage. We applied a range of FTC-R dilutions to leaves using a tracing wheel, and measured transcript abundance of a Kunitz trypsin inhibitor (*PtdTI3*), a robust marker of the poplar and aspen defense response (Haruta *et al.*, 2001a; Christopher *et al.*, 2004). These experiments showed that *PtdTI3* transcripts accumulated in a concentration-dependent manner in response to FTC-R, reaching maximum induction at a 1 : 5 dilution (Fig. 1a). This concentration was therefore chosen for all subsequent FTC-R-treatments.

The response of poplar leaves to FTC-R suggested that it must contain potent chemical elicitors that trigger defense responses in poplar cells. The regurgitant of other caterpillar species has been found to contain FACs which act as elicitors (Tumlinson & Lait, 2005). FTC-R was therefore tested for the presence of such FACs, together with regurgitant from *Manduca sexta* and *Spodoptera exigua* for comparison. LC-MS analysis revealed that volicitin (*N*-hydroxylinolenoyl-L-glutamine) was the major FAC in FTC-R, although minor peaks may represent additional FACs (Fig. 1b). Consistent with

previous reports, volicitin was also detected in the regurgitant of *S. exigua* (Alborn *et al.*, 1997; Pohnert *et al.*, 1999), but only *N*-linolenoyl-L-glutamic acid and *N*-linolenoyl-L-glutamine were present in *M. sexta* (Halitschke *et al.*, 2001; Alborn *et al.*, 2003). Thus, these experiments revealed that volicitin is a component of FTC-R and could be a key elicitor of the poplar defense response during herbivory by FTC.

Expression profiling reveals that FTC-regurgitant elicits a strong defense response

To study the transcriptional response of hybrid poplar leaves to FTC-R further, we constructed macroarrays containing 580 cDNAs from an EST library, generated from induced hybrid poplar leaves (Constabel *et al.*, 2000; Christopher *et al.*, 2004). In addition to the FTC-R treatment, we also subjected leaves to mechanical damage using a tracing wheel or by crushing leaf margins with pliers (see below). We separately analyzed the response in treated leaves (local response) and untreated leaves on treated saplings (systemic response). Each control and induction treatment was analyzed using three independent biological replicates, and in addition each sample consisted of three pooled mature leaves. To be considered as differentially expressed between treated and control leaves, genes were required to meet two criteria: a ≥ twofold change in expression (either up- or down-regulation), and a significance of $P < 0.05$ as determined by the Student's *t*-test for the three independent replicates. While a significant *P*-value alone has often been used to determine differential expression, a twofold expression threshold provides greater confidence that the expression ratios are meaningful. These relatively stringent criteria, however, may lead to conservative estimates of the extent of differential gene expression.

Compared with unwounded control leaves, FTC-R significantly up-regulated 36 genes from our array. An additional 13 genes were induced by at least twofold, but had *P*-values > 0.05. Some of the induced genes encoded previously identified wound-responsive genes. We therefore tested if the observed changes in gene expression could be the result of the wounding caused by the tracing wheel during application of FTC-R (see Materials and Methods), and analyzed the gene expression ratios in tracing wheel/water-treated leaves relative to unwounded leaves. Only three genes were slightly but significantly up-regulated in this comparison, confirming that the tracing wheel alone had only very minor effects on gene expression. Therefore, we were able to analyze the array data by calculating gene expression ratios of FTC-R treatment relative to the tracing wheel/water control treatment; this should provide an accurate representation of the effects of FTC-R alone and without the potential confounding effects of wounding. This analysis determined that 40 genes met our criteria for significant up-regulation by FTC-R (Fig. 2a; see Table S1 (supplementary material online)). Thus our experiments clearly demonstrated that FTC-R induced a poplar defense response

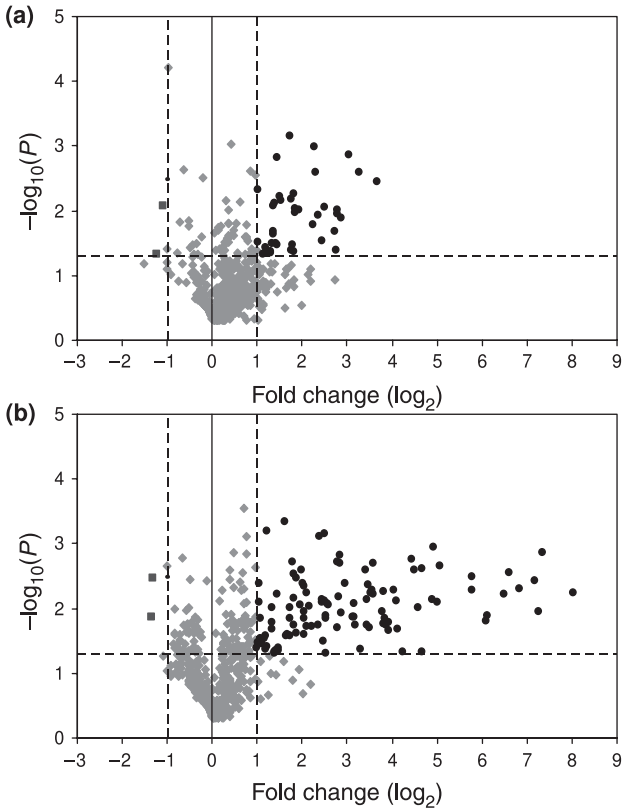


Fig. 2 Summary of changes in gene expression in response to induction treatments as measured by macroarray analysis. Volcano plots with gene expression ratios ($\log_2$ fold change) plotted against the negative $\log_{10}$-transformed *P*-values from a *t*-test calculation. (a) Gene expression ratios of forest tent caterpillar-regurgitant (FTC-R)-treated to water-treated leaves; (b) expression ratios of mechanical wounding to untreated leaves. Vertical dashed lines represent a two-fold change in gene expression threshold (induction or repression). Horizontal dashed line represents a significance level of $P = 0.05$. Black circles and squares represent genes with a statistically significant ($P < 0.05$) fold change of > 2 or < 0.5, respectively. All treatments were performed on leaves of LPI 9–17, and after 24 h leaves of LPI 9–11 were harvested. Experimental treatments and corresponding controls were harvested concurrently.

without significant mechanical wounding, presumably caused by the volicitin and other chemical elicitors present.

One of our objectives was to compare the effects of FTC-R and mechanical wounding on gene expression. Since the tracing wheel caused almost no gene induction, we also performed a more severe wound treatment by crushing the leaf margins with pliers. This method was previously shown to cause a strong defense response in hybrid poplar leaves (Constabel *et al.*, 2000; Christopher *et al.*, 2004). Macroarray analysis of plier-wounded leaves determined that more than 100 genes were induced by this fairly severe wound stress (Fig. 2b, see Table S1). This response was intense, as reflected in expression ratios of as high as 256-fold for defense genes such as chitinases and PPO. In both intensity and number of induced genes, the plier-wounding resulted in a stronger

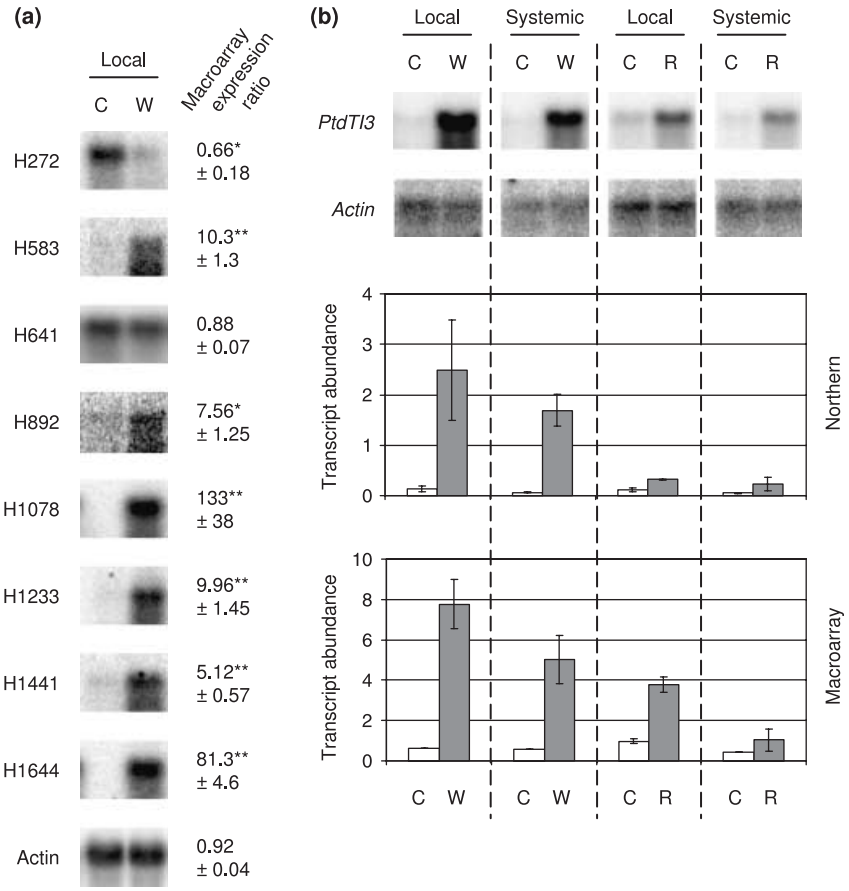


Fig. 3 Validation of macroarray data using Northern blot analysis. (a) Accumulation of transcripts encoding selected genes, 24 h after wounding with pliers (W) or untreated control plants (C). Northern analyses were performed for three biological replicates with similar results; a representative Northern blot is shown. Fold induction ± standard error is shown for macroarray data. Asterisks denote significance of induction calculated from a *t*-test (*, $P < 0.05$; **, $P < 0.01$). Annotations of ESTs used as probes are as follows: H272 (CN193029), carbonic anhydrase; H583 (CN193162), unknown gene, ZIM motif; H641 (CN193209), Rubisco; H892 (CN193384), unknown gene, DUF946 domain; H1078 (CN192556), endochitinase *win6.2C*; H1233 (CN192638), 11S cupin plant seed storage protein; H1441 (CN192724), apyrase; H1644 (CN192786), class 3 lipase. Actin is shown as a loading control. Numbers refer to EST identifiers, with GenBank accessions in parentheses. (b) Accumulation of *PtdTI3* (Kunitz trypsin inhibitor 3) mRNA in leaves of plants wounded with pliers (W) or treated with regurgitant (R). Leaves were harvested after 24 h from treated leaves (local) or untreated leaves on treated plants (systemic). Controls (C) were unwounded plants for wounding treatments, or plants mock treated with water for forest tent caterpillar-regurgitant treatments. The top panel shows representative Northerns from three biological replicates of all four treatments. The lower two panels show quantified transcript abundance from Northerns and macroarrays, respectively. Error bars display standard error.

response than the FTC-R treatment (compare Fig. 2a,b). However, both treatments led to significant up-regulation of many genes, and provided the basis for a more in-depth comparison of these stresses (discussed later). Only two genes were found to be repressed by wounding or FTC-R (Fig. 2a,b, top left quadrants). Other workers have reported a much larger number of genes that are down-regulated by simulated or actual herbivory (Cheong *et al.*, 2002; Halitschke *et al.*, 2003; Roda *et al.*, 2004; Smith *et al.*, 2004; Voelckel & Baldwin, 2004; Ralph *et al.*, 2006). However, Reymond *et al.* (2004) also reported only three out of ~12 000 ESTs to be repressed after feeding by *Pieris rapae*.

Northern blots probed with a series of genes with distinct expression patterns validated our macroarray data; strongly induced (e.g. H1078), unaltered (e.g. H641), and mildly repressed (e.g. H272) expression patterns corresponded well between macroarray and Northern data (Fig. 3a). Macroarray data were likewise validated across the treatments using *PtdTI3* as a probe (Fig. 3b). Further confirmation came from the replication of some genes on the macroarrays, which displayed a highly similar expression profile (data not shown). Because we standardized the quantity of DNA spotted on the macroarrays, we were also able to use the arrays to obtain a measure of total transcript abundance. Using this measure, macroarray- and Northern-derived expression patterns were found to correlate well for *PtdTI3* and other genes (Fig. 3b). Thus we are confident our macroarrays provided a reliable measure of gene expression, and that they accurately

captured the induction of genes by both mechanical wounding and FTC-R.

Comparative analysis of simulated herbivory treatments reveals that FTC-R induces many wound-induced genes

We compared our macroarray data from the four treatments (local wounding, systemic wounding, local FTC-R, systemic FTC-R) by cluster analysis for all genes whose expression was significantly regulated ($P < 0.05$, irrespective of fold change) in at least one treatment (Fig. 4); this set of genes encompassed approximately one-half of the ESTs spotted on our macroarrays. We generated color-coded 'heat maps' for gene expression ratios (degree of induction, left panel), *t*-test significance (*P*-value, middle panel) and transcript abundance (right panel). The heat maps provide a graphical comparison of the expression data; differences in color intensity are observable between the four treatments. All induced genes clustered together at the top of the panel, as seen by the most intense color pattern in this zone. Inspection of this set of genes revealed that plier wound-treatments showed more intense color, indicating stronger induction, greater significance, and higher transcript abundance than FTC-R-treatments (compare color intensities of two leftmost lanes and two rightmost lanes in each panel). Thus the plier wound-treatments stimulated a stronger response than FTC-R-treatments, as already observed in Figs 2 and 3. The group of genes at the top of the cluster contained the known poplar defense-related genes, including *win6.2C* and *win8* endochitinases (Davis *et al.*, 1991a), *PtdPPO1* (Constabel *et al.*, 2000), vegetative storage proteins (VSPs) *win4.5* and *pni288* (Davis *et al.*, 1993; Lawrence *et al.*, 2001), and trypsin inhibitors (TIs) (Christopher *et al.*, 2004) (discussed later). Other genes found in this cluster included those for jasmonate biosynthesis (allene oxide synthase, AOS; 13-lipoxygenase, 13-LOX) and genes likely involved in secondary metabolism (phenylalanine ammonia lyase, PAL; cytochromes P450) (Fig. 4). We previously identified some of these genes in the poplar wound-response (Christopher *et al.*, 2004). In addition, the genes in this group were both the most strongly FTC-R- and wound-induced genes; thus there was an excellent correlation between both treatments for individual genes (discussed later).

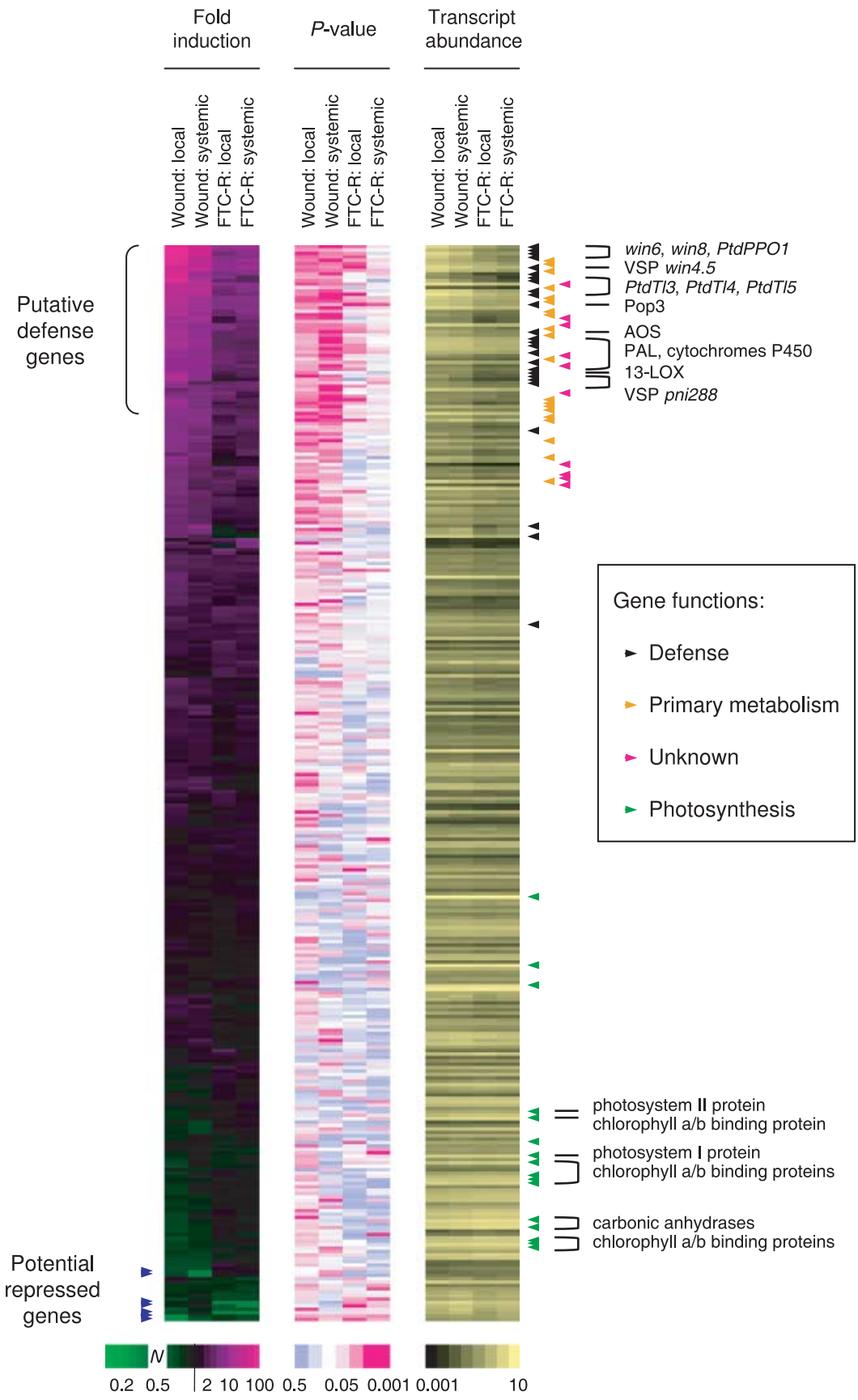
Below the highly induced genes seen in Fig. 4, the majority of genes showed variable degrees of expression (right panel) and nonsignificant *P*-values (left and center panels), as well as apparent down-regulation. However, only seven genes showed significant repression in any of the treatments at $P < 0.05$. Among these were genes encoding peroxidase, catalase, ferredoxin, and a putative thiamine biosynthetic enzyme (Table S1), but these need to be corroborated. Interestingly, genes with putative roles in photosynthesis were clustered at the bottom with the repressed genes, although none of these were significant (Fig. 4, arrows). The repression of photosynthetic genes as a component of the defense response has been reported by expression profiling studies (Hermsmeier *et al.*, 2001; Halitschke *et al.*, 2003; Heidel & Baldwin, 2004; Qu *et al.*, 2004; Smith *et al.*, 2004; Voelckel & Baldwin, 2004; Ralph *et al.*, 2006). This may be related to accumulation of JA during the defense response, since JA has been shown to down-regulate expression of photosynthetic genes (Creelman & Mullet, 1997).

The pattern of transcript abundance confirmed that both plier wounding and FTC-R had strong effects on the leaf transcriptome; the transcript abundance of several wound-inducible genes was among the highest of any transcript in the leaves (Fig. 4, right panel). For example, transcripts encoding chitinase *win6.2C*, VSP *win4.5*, *PtdTI3*, stress-related protein Pop3/SP1-like, and several unknown proteins were among the 95th percentile in transcript abundance of all genes present on our macroarrays. These were comparable in strength to signals obtained for photosynthetic genes such as chlorophyll a/b binding proteins, Rubisco, carbonic anhydrase, Rubisco activase, and photosystem I subunits.

Poplar responses to severe wounding and FTC-R are similar

Previous studies in other species have suggested that some plant defense genes may be responsive to insect feeding but not to physical damage alone (Hermsmeier *et al.*, 2001; Reymond *et al.*, 2004), and that such responses can often be mimicked with insect-derived cues found in regurgitant (Schittko *et al.*, 2001; Halitschke *et al.*, 2003; Roda *et al.*, 2004). Thus, we hypothesized that FTC-R may elicit an insect-specific defense response in poplar leaves. To further dissect differences between wound- and FTC-R-induced responses, we directly plotted the intersection of all genes that our criteria defined as induced by mechanical wounding or FTC-R using Venn diagrams. For this analysis, only expression data from local responses were used. We found that the transcriptional response following FTC-R was a subset of the response induced by mechanical wounding, as this analysis showed that 38 of 40 (95%) FTC-R-inducible genes were also wound-inducible (Fig. 5a). The remaining two FTC-R-inducible genes (6%) were tentatively considered to be FTC-R-specific. In addition, the majority of wound-inducible genes (70 of 108, 65%) were not classified as induced by FTC-R and thus appeared to be wound-specific; however, this may reflect our threshold for defining induction (discussed later).

We examined the expression of those genes tentatively classified as wound- or FTC-R-specific further by hierarchical clustering (Fig. 5b). The generation of heat maps was carried out as before. The top panels represent those genes classified as wound-specific while the bottom panels represent potential FTC-R-specific genes. Comparison of right and left lanes of each panel indicated that the majority of wound-specific genes also appeared to be induced by FTC-R but with



lower or non-significant inductions (< twofold, or $P > 0.05$); they thus did not meet the threshold for differential expression (Fig. 5b, top panels). Moreover, the pattern of transcript abundance of these genes as seen by the overall banding pattern was roughly similar in both treatments (Fig. 5b, top right panel). Two subgroups of genes appeared to exhibit strong wound-inducibility with no apparent response to FTC-R (Fig. 5b, asterisks). Subsequent Northern analysis, however, showed that these genes exhibited a very low degree of FTC-R-inducibility (data not shown). Thus, these genes should be considered to be preferentially expressed by wounding, perhaps with differences in induction kinetics that masked FTC-R-induced gene expression on the array. More detailed time course experiments will have to be carried out to test this.

Two genes, encoding mannose pyrophosphorylase and a Pop3/SP1-like protein, showed stronger induction by FTC-R than by wounding, and are thus potential FTC-R-specific genes (Fig. 5b, bottom panels). Northern analysis again showed that wounding did in fact weakly induce these genes (data not shown). Thus our analysis failed to detect any exclusively wound-induced or FTC-R-induced genes, although several genes appeared to be preferentially induced by either stress. Overall, we conclude that the responses to FTC-R and wounding, as monitored by our suite of genes, differ quantitatively but are qualitatively similar.

Induced gene expression patterns in local and systemic leaves show extensive overlap

Upon insect challenge, hybrid poplar saplings induce defense genes such as PPO and chitinases systemically (Parsons *et al.*, 1989; Constabel *et al.*, 2000). Therefore we investigated induced gene expression patterns in systemically induced leaves. Much like the directly treated leaves, systemic leaves of wounded saplings showed a strong transcriptional response, and we identified 91 genes as significantly induced in these leaves. Fewer genes (22) met our criteria for induction in systemic leaves of FTC-R-treated saplings (see Tables S1 and S2), and thus we did not include FTC-R treatments in this analysis. To compare the local and systemic wound-responses of poplar, we plotted the intersection of genes found to be induced in both local and systemic leaves, and found an extensive overlap between local and systemic responses: 85 genes, comprising 79% of locally inducible genes and 93% of systemically inducible genes, were induced in both types of leaves (Fig. 6a). Inspection by cluster analysis of the subsets of potential local-specific and systemic-specific genes emphasized the similarity of both responses (Fig. 6b). It also revealed that no genes were uniquely induced in local or systemic leaves, although some may be preferentially expressed in either group. Therefore, we conclude that for our set of genes there are no major qualitative differences in the defense response of local and systemic leaves.

The most highly FTC-R- and wound-induced genes are involved in defense, secondary metabolism and primary metabolism

A major objective of this study was the identification of novel-induced defense genes from poplar leaves, as part of our long-term aim of a global characterization of poplar defense. Expression profiling studies in other plants have successfully identified many wound- or herbivore-induced genes and pathways, particularly during early events in the defense response. By studying the defense response at a 24 h time point, we aimed to focus on later events and to identify genes directly responsible for insect resistance.

A list of combined FTC-R- and wound-induced genes with the highest fold induction demonstrated that known defense-related poplar genes, including endochitinases, PPO, TIs and the VSP *win4.5*, are the most strongly FTC-R-induced genes (Table 1). Genes encoding enzymes for octadecanoid synthesis, phenylpropanoid metabolism, or cytochrome P450 were also strongly induced and comprised functional categories which were well represented in our EST set (Christopher *et al.*, 2004). In addition, we identified a number of very responsive genes with putative roles in primary metabolism and not previously known to be involved in defense, although many were also identified by Ralph *et al.* (2006) in their study of FTC-induced gene expression. These include genes encoding enzymes of carbohydrate, lipid, and phosphate metabolism, as well as genes of unknown function (Table 1). Comparison of the FTC-R- and wound-induced rankings revealed that they are generally similar. For example, the seven top genes are ranked within 1–10 for both FTC-R and wound inductions. This supports our earlier conclusion that the FTC-R and

Fig. 4 Heat map representing expression of all genes responding to wounding or forest tent caterpillar-regurgitant (FTC-R). Hierarchical clustering was performed for all genes significantly regulated ($P < 0.05$) for at least one treatment. Heat maps illustrate fold induction, *P*-values, and absolute expression levels. Genes clustered at the top are strongly induced (magenta) and putative defense genes, while those clustered at the bottom (green) are potential repressed genes. A discontinuous discretization was introduced for the repressed genes to highlight the genes repressed twofold. Black arrowheads denote known or suspected poplar defense genes; orange and magenta arrowheads denote highly induced genes annotated for primary metabolism and unknown function, respectively (see Tables 2 and 3); green arrowheads denote genes with predicted functions in photosynthesis; and blue arrowheads denote genes that were repressed twofold by one of the treatments. Poplar defense genes are labeled with brackets, indicating the position of the genes on the heat map. *win6*, endochitinase *win6.2C*; *win8*, endochitinase *win8*; *PtdPPO1*, polyphenol oxidase 1; *VSP win4.5*, vegetative storage protein *win4.5*; *PtdTI3*, Kunitz trypsin inhibitor 3; *PtdTI4*, Kunitz trypsin inhibitor 4; *PtdTI5*, Kunitz trypsin inhibitor 5; *AOS*, allene oxide synthase; *PAL*, phenylalanine ammonia lyase; *13-LOX*, 13-lipoxygenase; *VSP pni288*, vegetative storage protein *pni288*.

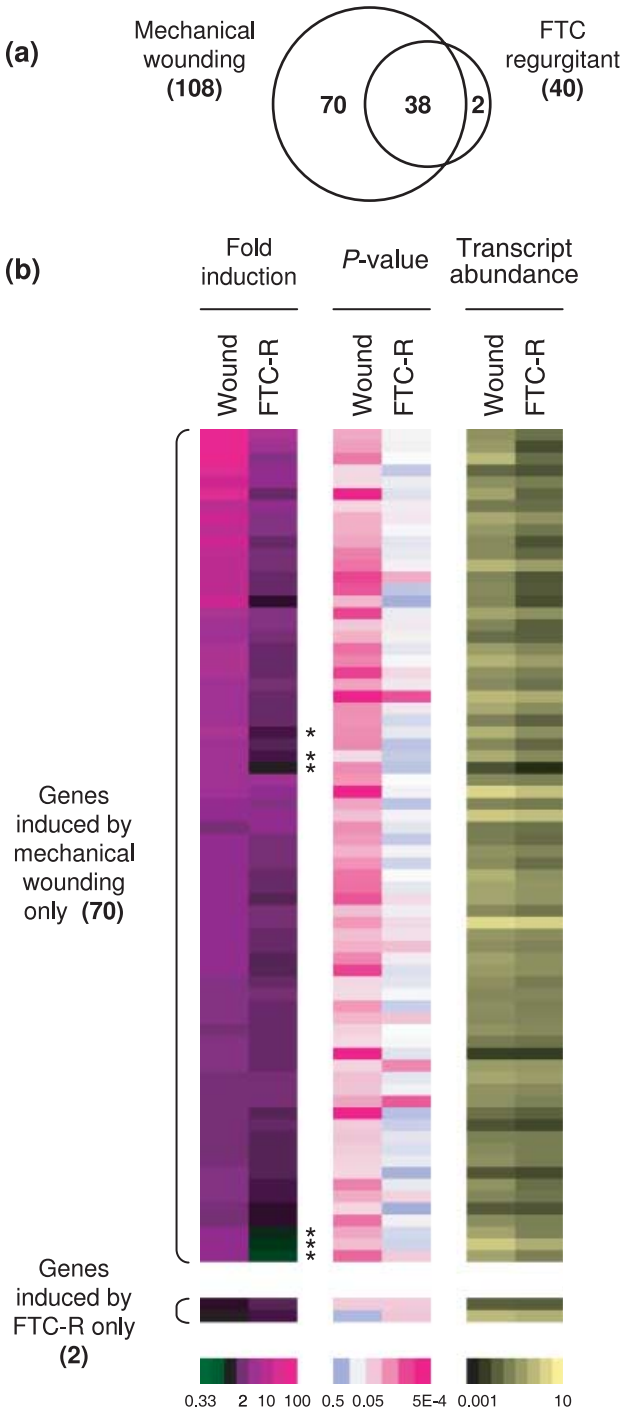


Fig. 5 Comparison of gene expression after wounding or forest tent caterpillar-regurgitant (FTC-R) treatment. (a) Venn diagram representing distribution of transcripts significantly induced by plier wounding or FTC-R on macroarrays; for clarity, only the analysis of gene expression in local leaves is shown. (b) Heat map of hierarchical clustering performed for genes classified as induced by wounding only or regurgitant only. Representation of average fold induction, *P*-values, and transcript abundance for local and systemic data are as described in Fig. 4. Asterisks represent genes apparently preferentially induced by wounding.

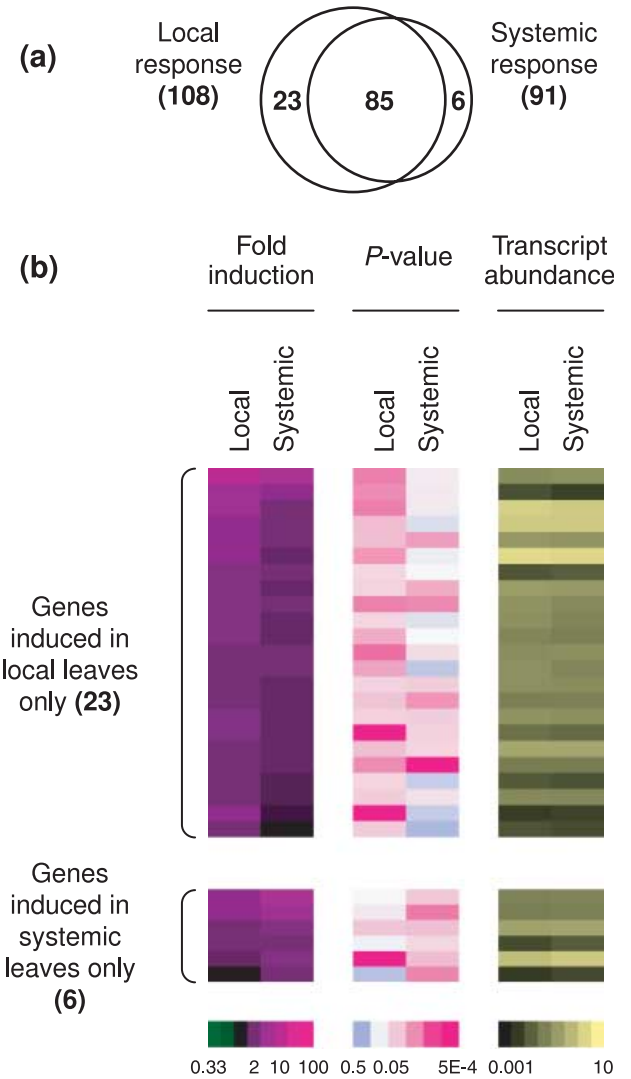


Fig. 6 Comparison of local and systemic gene expression. (a) Venn diagram representing the distribution of significantly induced transcripts in treated (local) or untreated (systemic) leaves on macroarrays; for clarity, only the analysis of gene expression from mechanical wounding is shown. (b) Heat map of hierarchical clustering performed for genes classified as induced locally only or systemically only. Representation of average fold induction, *P*-values, and transcript abundance for wounding and forest tent caterpillar-regurgitant (FTC-R) are as described in Fig. 4.

wound responses are qualitatively similar for this set of genes. A significant exception appeared to be the TI genes (*PtdTI3* and *PtdTI4*) and the *pni288* VSP, which were both strongly induced by wounding but less so by FTC-R (Table 1). While FTC-R did induce *PtdTI4* and *pni288*, these inductions were not significant; if the *P*-values for *PtdTI4* and *pni288* are disregarded, their fold inductions by FTC-R rank as 20 and 29, respectively. These genes may thus be preferentially induced by wounding or their induction blocked by FTC-R. This will require more detailed experiments to be corroborated.

Table 1 Comparison of most strongly forest tent caterpillar-regurgitant (FTC-R)- and wound-induced genes from macroarray analyses[a]

Putative function	GenBank accession	JGI gene model[b]	AGI accession[c]	*E*-value[d]	FTC-R rank[e]	Wound rank[e]
Endochitinase *win6.2C*	CN192741	grail3.0001024001	At3g12500	10^{-100}	**1**	**1**
Lipase, class 3	CN192786	estExt_Genewise1_v1.C_LG_IV2794	At4g18550	10^{-105}	**2**	**5**
Endochitinase *win8*	CN192595	estExt_fgenesh1_pg_v1.C_LG_IV1440	At3g12500	5×10^{-87}	**6**	**2**
Apyrase	xxxxxxxx	eugene3.00190357	At5g18280	10^{-116}	**4**	**6**
Vegetative storage protein *win4.5*	CN192930	eugene3.00130800	At4g24340	3×10^{-49}	**3**	**8**
Polyphenol oxidase *PtdPPO1*	CN193334	eugene3.00110805	At2g20590	0.14*	**10**	**3**
Unknown	CN192936	eugene3.00870012	At3g03150	2×10^{-26}	**5**	**9**
Kunitz trypsin inhibitor *PtdTI5*	CN192805	eugene3.00190800	At1g73325	1×10^{-9}	**–[8]**	**7**
Acid phosphatase, class B	CN193016	estExt_Genewise1_v1.C_LG_I0437	At4g25150	2×10^{-67}	**7**	**14**
Pop3/SP1	xxxxxxxx	estExt_fgenesh1_pm_v1.C_LG_X0481	At3g17210	4×10^{-21}	**11**	**13**
Pop3-/SP1-like	xxxxxxxx	estExt_Genewise1_v1.C_LG_X0703	At3g17210	6×10^{-18}	**9**	**17**
Acyl-activating enzyme	CN192663	eugene3.00040736	At1g65890	0	**–[15]**	**12**
Kunitz trypsin inhibitor *PtdTI3*	CN192549	estExt_Genewise1_v1.C_LG_XIX2762	At1g73325	2×10^{-9}	**17**	**11**
Kunitz trypsin inhibitor *PtdTI4*	CN193330	eugene3.00040289	At1g17860	4×10^{-28}	**–[26]**	**4**
β-glucosidase	CN192799	estExt_fgenesh1_pm_v1.C_LG_X0568	At5g36890	0	**8**	**22**
β-amylase	CN192760	grail3.0064001202	At4g15210	0	**19**	**15**
Unknown	CN193014	estExt_fgenesh1_pg_v1.C_LG_XII0482	At2g37010	0.76*	**12**	**26**
Allene oxide cyclase	CN193019	eugene3.00040854	At1g13280	2×10^{-69}	**21**	**18**
Cytochrome P450	CN193274	eugene3.00030238	At5g07990	10^{-102}	**28**	**16**
Cytochrome P450	CN193412	eugene3.00030238	At5g07990	10^{-102}	**25**	**19**
18S rRNA gene	CN192944	xxxxxxxxxxxxxxxxx	At3g41768	10^{-125}	**20**	**24**
Cytochrome P450	CN193236	eugene3.00030242	At5g07990	10^{-104}	**16**	**29**
Unknown protein (MOSC domain)	CN193222	grail3.0047000902	At1g30910	10^{-128}	**15**	**31**
Phenylalanine ammonia lyase	CN192894	estExt_Genewise1_v1.C_280661	At2g37040	0	**13**	**34**
Cytochrome P450	CN193273	eugene3.00280025	At5g07990	10^{-100}	**26**	**23**
Lipolytic enzyme, G-D-S-L	CN193295	eugene3.00121141	At5g45670	3×10^{-79}	**22**	**27**
Cinnamyl alcohol dehydrogenase	CN192800	estExt_fgenesh1_pm_v1.C_LG_II1065	At4g39330	10^{-112}	**14**	**39**
Esterase/Lipase	CN192875	eugene3.00090620	At3g48690	2×10^{-59}	**–[33]**	**20**
Unknown (DUF946 domain)	CN193384	eugene3.00180760	At2g44260	0	**24**	**37**
Cytochrome P450	CN193433	estExt_Genewise1_v1.C_LG_IV4159	At3g25180	10^{-144}	**27**	**35**
Pop3-/SP1-like	CN192903	estExt_fgenesh1_pm_v1.C_LG_X0482	At3g17210	4×10^{-20}	**29**	**41**
HVA22-related protein	CN192744	estExt_Genewise1_v1.C_LG_XII0452	At1g74520	7×10^{-71}	**18**	**52**
13-Lipoxygenase	CN192531	xxxxxxxxxxxxxxxxx	At3g45140	5×10^{-5}	**–[49]**	**28**
Vegetative storage protein *pni288*	CN193425	eugene3.00190336	At4g24340	2×10^{-39}	**–**	**10**
Anthocyanidin synthase	CN192891	estExt_fgenesh1_pm_v1.C_LG_XVI0363	At5g05600	10^{-137}	**–**	**21**
ADP-glucose pyrophosphorylase	CN192812	eugene3.00120693	At1g74910	0	**23**	**–**
Neutral invertase	CN193364	estExt_Genewise1_v1.C_LG_VIII2120	At1g56560	0	**–**	**25**

[a]Genes significantly induced (twofold induction, $P < 0.05$) by FTC-R or wounding were ranked by induction; the 30 most strongly FTC-R-induced genes and the 30 most strongly wound-induced genes were combined in a dually ranked list and shown.
[b]JGI gene model from the *P. trichocarpa* genome (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) that corresponds to the EST.
[c]AGI (Arabidopsis Genome Initiative) code for best match of poplar gene (JGI gene model) to *Arabidopsis thaliana* determined by BLASTX of TAIR (http://www.arabidopsis.org/). For 18S rRNA gene (CN192944), the *A. thaliana* gene that is most similar to the EST determined by BLASTN is shown.
[d]Expect value of best match of poplar gene to *A. thaliana* from BLASTX (BLASTN for rRNA gene). Asterisks indicate genes for which significant similarity were not found in the *A. thaliana* genome.
[e]Genes were ranked by level of induction. Genes with no ranking were not significantly induced. Superscript numbers denote ranking if the *P*-value is ignored; these genes were induced, but with a non-significant *P*-value.

Further inspection of Table 1 indicated that genes classified into defense or secondary metabolism, including several cytochromes P450, were over-represented among genes induced by both simulated herbivory treatments. These differences in representation were significant, except for the FTC-R-response in systemic tissues (because of small sample size, $n = 22$). While the macroarrays contained only approx. 4% each of defense or secondary metabolic genes, defense-related genes represented 9–15% of induced genes (χ^2 analysis, $P < 0.01$) and secondary metabolism represented 11–21% (χ^2 analysis, $P < 0.01$) of induced genes. Ralph *et al.* (2006) also observed that secondary metabolism genes are activated in FTC-treated poplar leaves. Thus it appears that secondary compounds are likely important for poplar defense.

FTC-R and wounding strongly induced several genes annotated for roles in primary metabolism as well as genes of unknown function

Several of the most strongly wound- and FTC-R-inducible genes had predicted functions in primary metabolism, and others encoded proteins with no known functions. To confirm our annotation and gain additional information on possible functions, we searched the recently sequenced genome of *P. trichocarpa* (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) to obtain full-length sequences. Public databases (NCBI, UniProt, AGI) and databases for conserved domains (CDD, Interpro, ProSite) were then re-queried with these full-length genes in order to refine the earlier annotation and to ensure that no potential motifs or functions had been overlooked. As a functional group, primary metabolism encompassed 17.6% of the genes represented on the macroarrays, but up to 29% of the wound- and FTC-R-inducible gene set (not significant, χ^2 analysis, $P > 0.1$). This set of genes thus represents a substantial component of the herbivory-induced transcriptome. We found 24 ESTs corresponding to 17 unique genes with putative functions in primary metabolism that were strongly wound-induced (fivefold induction, $P < 0.05$; Table 2). Previous expression profiling of plant defense has suggested that the induction of primary metabolic genes is required for resource or nutrient reallocation during the defense response (Reymond *et al.*, 2004). However, considering their strong induction by wounding and FTC-R, these primary metabolic genes could play more direct roles in defense (see Discussion). Among these genes was a group of carbohydrate-related enzymes, including a β-amylase, β-glucosidase, and neutral invertase. A second group of FTC-R- and wound-induced genes has putative functions in lipid metabolism, including several lipases and a gene belonging to the acyl-activating enzyme (AAE) superfamily, recently described for *Arabidopsis* (Shockey *et al.*, 2003). Smith *et al.* (2004) had identified both of these types of lipid metabolism-related genes in a microarray analysis of abrasion wound-induced genes of poplar leaves, which supports the idea they may be of general importance. The gene represented by H1644 encodes a class 3 lipase and was extremely responsive to our simulated herbivory treatments, ranking second for FTC-R induction and fifth for wound induction (Table 2). Lipases belonging to the class 3 family have been previously identified with roles in defense signaling (see Discussion). We also identified genes encoding storage proteins that were FTC-R- and wound-induced. The wound inducibility of two of these VSPs, *win4.5* and *pni288*, has been described previously (Davis *et al.*, 1993; Lawrence *et al.*, 2001). Our analysis also identified two wound-inducible genes encoding novel storage proteins annotated as 11S cupin and embryo-specific 3. The roles of wound-inducible storage proteins may be related to allocation of resources for defense, but their exact role is unknown (Christopher *et al.*, 2004).

Seven highly wound- and FTC-R-inducible genes (fivefold induction, $P < 0.05$; Table 3) encoded proteins of unknown function. Two such unknowns (H241, H1958) are among the most strongly induced genes and showed the most abundant cellular transcripts in response to wounding or FTC-R (95th percentile in transcript abundance). Database searches for conserved domains revealed that three unknowns (H81, H522, H583; Table 3) contained the ZIM motif; this is a short motif found in many GATA-type Zn-finger plant transcription factors. All three genes are significantly induced by wounding, and preliminary analysis of the kinetics of H583 induction indicated that expression is induced as early as 1 h (data not shown). This would be consistent with a role as a novel transcription factor involved in defense signaling (see Discussion).

Discussion

In order to characterize the hybrid poplar response to insect herbivory, we used macroarrays to investigate gene expression after treatment with FTC-R and mechanical wounding. Our experiments demonstrated that FTC-R-application induces a strong defense response in poplar leaves. Comparative macroarray analyses showed that severe mechanical wounding with pliers could elicit even higher amounts of expression and larger numbers of significantly induced genes. Moreover, our analyses showed that the response induced by FTC-R is a substantive subset of the wound-induced response, and that while the gene expression patterns between wound and FTC-R induction differed in intensity, they were qualitatively similar. In addition, we found extensively overlapping patterns of gene expression in local and systemic leaves, indicating a broad systemic response in poplar.

Induction of defenses by FTC-R and wounding

Forest tent caterpillar-regurgitant is a potent inducer of herbivore defenses of poplar leaves, and it contains the FAC volicitin (*N*-hydroxylinolenoyl-L-glutamine; Fig. 1). The presence of volicitin in FTC-R suggests that it contributes to the elicitor activity of FTC-R. However, it is possible that FTC-R contains additional elicitors, and the efficacy of volicitin in poplar will have to be verified directly. In other plants, regurgitant from a variety of caterpillar species has been shown to induce many plant responses elicited by herbivory, including defense gene expression in tobacco (Halitschke *et al.*, 2001, 2003; Schittko *et al.*, 2001; Roda *et al.*, 2004) and volatile release in corn, cotton, maize, tobacco, and alfalfa (Alborn *et al.*, 1997, 2003; Halitschke *et al.*, 2001; Roda *et al.*, 2004). Thus, caterpillar regurgitant appears to be an excellent proxy for herbivory.

Both FTC-R and wounding induced strong defense responses, and, in general, inducible genes present on the array were up-regulated by both treatments. Moreover, we did not find any genes which were exclusively induced by either treatment (Fig. 5). It is possible that our array was simply too small to

Table 2 Macroarray data for selected induced genes with putative functions in primary metabolism

Putative function	GenBank accession	JGI gene model[a]	AGI accession[b]	*E*-value[c]	Induction factor[d]	
					Wound	FTC-R
Amino acid transport and metabolism						
Prephenate dehydratase	CN193183	eugene3.00660027	At1g08250	10^{-172}	**7.77**** ± 1.53	**1.58** ± 0.38
Carbohydrate transport and metabolism						
β-Amylase	CN192760	grail3.0064001202	At4g15210	0	**21.68**** ± 4.15	**3.40*** ± 1.02
β-Glucosidase	CN192799	estExt_fgenesh1_pm_v1.C_LG_X0568	At5g36890	0	**14.16**** ± 3.49	**5.67**** ± 1.17
Neutral invertase	CN193364	estExt_Genewise1_v1.C_LG_VIII2120	At1g56560	0	**12.00**** ± 1.73	**1.70*** ± 0.16
Glyoxalase	CN192888	estExt_Genewise1_v1.C_LG_IV1582	At1g11840	10^{-139}	**5.68**** ± 0.37	**1.78**** ± 0.07
UDP-Glucoronosyl/UDP-glucosyl transferase	CN192670	eugene3.00160092	At1g07250	10^{-106}	**7.05**** ± 1.70	**1.84** ± 0.43
Lipid transport and metabolism						
Lipase, class 3	CN192786	estExt_Genewise1_v1.C_LG_IV2794	At4g18550	10^{-105}	**97.79**** ± 26.44	**9.57**** ± 1.43
Acyl-activating enzyme	CN192663	eugene3.00040736	At1g65890	0	**25.52*** ± 20.08	**3.93** ± 2.46
Esterase/Lipase	CN192875	eugene3.00090620	At3g48690	2×10^{-59}	**14.56*** ± 7.22	**2.60** ± 0.77
Lipolytic enzyme, G-D-S-L	CN193295	eugene3.00121141	At5g45670	3×10^{-79}	**11.81**** ± 3.02	**2.74**** ± 0.16
Nucleotide transport and metabolism						
Apyrase	CN193208	eugene3.00190357	At5g18280	10^{-116}	**55.00**** ± 18.66	**6.84**** ± 1.76
Phosphatase						
Acid phosphatase, class B	CN193016	estExt_Genewise1_v1.C_LG_I0437	At4g25150	2×10^{-67}	**22.72**** ± 4.76	**6.66*** ± 2.56
Plant storage proteins						
Vegetative storage protein win4.5	CN192930	eugene3.00130800	At4g24340	3×10^{-49}	**55.41**** ± 19.13	**8.21**** ± 0.94
Vegetative storage protein pni288	CN193425	eugene3.00190336	At4g24340	2×10^{-39}	**30.36**** ± 4.77	**1.77** ± 0.67
11S Cupin plant seed storage protein	CN192638	eugene3.01070077	At1g07750	2×10^{-88}	**11.10**** ± 2.61	**1.97** ± 0.56
Embryo-specific 3 seed protein	CN192906	eugene3.00151111	At5g62200	3×10^{-37}	**9.87*** ± 6.03	**3.38** ± 1.12
Posttranslational modification, protein turnover, chaperones						
Glutathione S-transferase	CN192910	eugene3.00020134	At3g03190	2×10^{-49}	**8.83**** ± 2.08	**1.20** ± 0.15

[a]JGI gene model from the *P. trichocarpa* genome (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) that corresponds to the EST.
[b]AGI (Arabidopsis Genome Initiative) code for best match of poplar gene (JGI gene model) to *Arabidopsis thaliana* determined by BLASTX of TAIR (http://www.arabidopsis.org/).
[c]Expect value of best match of poplar gene to *A. thaliana* from BLASTX.
[d]Mean expression ratios (± SE) in wounded or forest tent caterpillar-regurgitant (FTC-R)-treated leaves. Only selected genes with putative functions in primary metabolism and significant expression ratios of at least fivefold ($P < 0.05$) after plier wounding are shown. Asterisks denote the significance levels of treated plants compared with control plants (**, $P < 0.01$; *, $P < 0.05$).

Table 3 Macroarray data for selected induced genes with novel or unknown functions

Clone ID	GenBank accession	JGI gene model[a]	AGI accession[b]	*E*-value[c]	Conserved domain/motif[d]	Induction factor[e]	
						Wound	FTC-R
H1958	CN192936	eugene3.00870012	At3g03150	2×10^{-26}		**33.40**** ± 7.23	**6.86*** ± 2.08
H241	CN193014	estExt_fgenesh1_pg_v1.C_LG_XII0482	At2g37010	0.76		**11.93**** ± 3.28	**4.74*** ± 1.39
H66	CN193222	grail3.0047000902	At1g30910	1×10^{-28}	MOSC	**10.89**** ± 2.66	**3.55*** ± 1.18
H583	CN193162	estExt_Genewise1_v1.C_280164	At1g19180	2×10^{-48}	ZIM motif	**10.64**** ± 1.71	**1.58** ± 0.70
H892	CN193384	eugene3.00180760	At2g44260	0	DUF946	**8.68*** ± 2.39	**2.69*** ± 0.59
H1007	CN192515	fgenesh1_pg.C_LG_IX000909	CN192515	1×10^{-118}		**5.42**** ± 1.23	**0.98** ± 0.48
H604.12	CN193182	fgenesh1_pg.C_scaffold_166000056	At4g22290	7×10^{-96}		**5.13*** ± 1.59	**2.19** ± 0.67
H81	CN193314	grail3.0037000501	At1g19180	2×10^{-31}	ZIM motif	**4.48**** ± 0.83	**4.34** ± 2.27
H522	CN193114	estExt_fgenesh1_pg_v1.C_LG_VI0512	At5g20900	7×10^{-29}	ZIM motif	**4.27*** ± 1.20	**1.87** ± 0.62
H764	CN193276	estExt_fgenesh1_pg_v1.C_LG_I2037	At1g07050	4×10^{-37}	CCT motif	**4.09*** ± 0.81	**2.17** ± 1.21

[a]JGI gene model from the *P. trichocarpa* genome (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) that corresponds to the EST.
[b]AGI (Arabidopsis Genome Initiative) code for best match of poplar gene (JGI gene model) to *Arabidopsis thaliana* determined by BLASTX of TAIR (http://www.arabidopsis.org/).
[c]Expect value of best match of poplar gene to *A. thaliana* from BLASTX.
[d]Conserved domain/motif predicted by the conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Domain/motif accession numbers (pfam, InterPro): DUF946: PF06101, IPR009291; MOSC: PF03473, IPR005302; ZIM motif: pfam06200, IPR010399; CCT motif: pfam06203, IPR010402.
[e]Mean expression ratios (± SE) in wounded or forest tent caterpillar regurgitant (FTC-R)-treated leaves. Only selected genes with unknown functions and significant expression ratios of at least fourfold ($P < 0.05$) after plier wounding are shown. Asterisks denote the significance levels of treated plants compared with control plants (**, $P < 0.01$; *, $P < 0.05$).

identify such elements; however, based on our EST analysis, it should include the most abundant wound-inducible genes. Thus, we tentatively conclude that the major elements of the wound response were to a large extent also inducible by FTC-R. This was initially surprising, since previous studies of gene expression and volatile release had suggested there are major differences between herbivore- and wound-induced responses (Alborn *et al.*, 1997; McCloud & Baldwin, 1997; Halitschke *et al.*, 2001, 2003; Reymond *et al.*, 2004; Roda *et al.*, 2004). However, how plants are wounded appears to have a major effect in demonstrating such differences, making it difficult to compare studies. Our wounding treatment was relatively severe and involved the crushing of leaf margins with pliers, repeated three times at 1 h intervals (see Materials and Methods). Previous studies have also damaged leaves by crushing (Reymond *et al.*, 2000), but many use more subtle methods such as puncturing with fabric tracing wheels (Halitschke *et al.*, 2003; Hui *et al.*, 2003; Roda *et al.*, 2004) and light abrasion (Smith *et al.*, 2004). In addition to physically damaging leaf blades, some workers have mimicked feeding damage by removing leaf tissue using cork borers, scissors or even larval mandibles (McCloud & Baldwin, 1997; Schittko *et al.*, 2001; Reymond *et al.*, 2004). Significantly, these treatments all have different effects on gene expression. McCloud & Baldwin (1997) demonstrated that damage to tobacco plants caused by removing leaf tissue designed to mimic herbivory induces a lighter response than wounding with a tracing wheel. Moreover, a comparison of *Arabidopsis* transcript profiles comparing wounding of different intensities demonstrates that some genes induced by leaf crushing do not respond to leaf tissue removal (Reymond *et al.*, 2000, 2004). Finally, a recent study in lima bean using a 'mechanical caterpillar' engineered to closely mimic insect feeding damage over an extended period found that both the spatial and temporal extents of physical damage affect the composition of released volatiles (Mithofer *et al.*, 2005). Thus, the volatile profile previously thought to require live insects can be reproduced by accurately mimicking insect feeding behavior (Mithofer *et al.*, 2005), which suggests that continuous damage is a key component of damage perception by the plant. The repeated plier-wounding used for our study may therefore be sufficiently sustained and severe to be perceived as insect feeding, which is reflected by our inability to find significant differences in wound and FTC-R responses. By contrast, in our system, light wounding with a tracing wheel had essentially no effect on up-regulation of our genes in both macroarray and Northern analyses (Figs 4 and 5; data not shown). This treatment was therefore not useful for direct comparisons with FTC-R, and emphasizes that wounding intensity should be carefully controlled and may have different effects in different species.

We note that our comparisons were only carried out at a single (24 h) time point, so that rapid and early differences in the kinetics of induction in response to FTC-R and wounding would have escaped detection. We chose the 24 h time point since our previous studies indicated the induced expression of most poplar defense genes is sustained and maximal at 24 h after damage (Constabel *et al.*, 2000; Christopher *et al.*,

2004). Other expression profiling studies have shown more transient defense gene expression (Reymond *et al.*, 2000; Cheong *et al.*, 2002; Halitschke *et al.*, 2003; Qu *et al.*, 2004). Thus our inference that FTC-R and wounding by pliers cause qualitatively similar responses is preliminary; however, our experiments would have detected large-scale differences in gene expression profiles between FTC-R and wounding.

Our inability to find major differences between FTC-R and wounding in poplar leaves under our conditions suggests that FTC-R elicits a defense response at least in part by generating or mimicking an endogenous wound signal. How volicitin and insect elicitors interact with cellular signaling pathways to induce defense responses is still unclear. Volicitin from regurgitant was shown to enter leaves during feeding by *S. exigua* (Truitt & Pare, 2004), and a putative receptor for volicitin has been identified in plasma membrane fractions of *Zea mays* (Truitt *et al.*, 2004). Volicitin and other FACs may interact with JA signaling, as regurgitant from *M. sexta* amplifies wound-induced increases in JA in tobacco and maize (McCloud & Baldwin, 1997; Halitschke *et al.*, 2001; Schmelz *et al.*, 2003; Roda *et al.*, 2004). Given the central role of JA in wound responses, increased amounts of JA could explain the elevated systemic responses elicited by insect regurgitants. How FACs interact with endogenous jasmonates and other signals will become more apparent as the complexity of these stress signals is dissected further (Howe, 2004).

A secondary aim of our study was to determine if the defense response differed in systemically induced and directly damaged or treated leaves. Some reports have found distinct differences in local vs systemically induced gene expression; for example, in *Arabidopsis*, preferential expression of some genes in either local or systemic tissues is detected (Titarenko *et al.*, 1997; Rojo *et al.*, 1999). In tomato, expression of locally induced octadecanoid pathway genes, and subsequent accumulation of JA, are barely detected in systemic tissues (Strassner *et al.*, 2002). In contrast, an extensive overlap was demonstrated between local and systemic responses of *Arabidopsis* challenged by *P. rapae* (Reymond *et al.*, 2004). Our current macroarray experiments failed to detect significant qualitative differences between damaged and systemic leaves, although there were quantitative differences (Fig. 6). Again, because of the modest size of the array, some differentially expressed genes might have been missed. However, the similarity in expression does suggest that the poplar defense response is very similar in systemic and wounded leaves, although in previous work we also found some differences in these responses (Christopher *et al.*, 2004). This discrepancy could be the result of different plants modulating systemic defenses differently, possibly because of age effects in systemic leaves. In this study, we minimized differences caused by leaf age and development by selecting three comparable leaves, which were pooled for analysis to minimize individual leaf variability. As mentioned, we only investigated responses at a single time point of 24 h after treatment, and discrepancies with our previous data may be attributable to differences in kinetics. Nevertheless, our result is consistent with current ideas of systemic defense signaling, where wounding activates a signal cascade that is suggested to amplify a local defense signal that then undergoes long-distance transport to systemic tissues and induces the defense response (Ryan & Moura, 2002; Howe, 2004). This would predict little or no attenuation of the wound signal; the strong systemic response following wounding we have detected is likely the result of such a signal cascade linking damaged and systemic leaves. This defense system would ensure induced resistance in undamaged leaves and thereby reduce overall damage by feeding insects.

Many induced poplar genes encode enzymes of secondary and primary metabolism

Transcript profiling studies in *Arabidopsis* indicate that dozens to hundreds of genes can be induced by physical and insect damage (Cheong *et al.*, 2002; Reymond *et al.*, 2004), and estimates from tobacco indicate that at least 500 genes are affected by herbivory (Hermsmeier *et al.*, 2001). In hybrid poplar, 9% of genes on a 10 000 gene array were found to be induced 14 d after abrasion damage (Smith *et al.*, 2004). Recently, Ralph *et al.* (2006) reported that 1191 genes on a 15 500 gene array showed up-regulation 24 h after FTC-herbivory of *P. trichocarpa* × *P. deltoides*. Our more specialized and smaller macroarray analysis identified FTC-R and wound induction of approx. 100 unique genes, with both known and unknown functions, which together constituted approx. 20% of the genes on our macroarrays (Fig. 2). As expected, among the inducible genes we found a significant representation of genes known to be involved in defense, including Kunitz trypsin inhibitors, polyphenol oxidase, chitinase, as well as cytochromes P450 and enzymes of secondary metabolism. However, we also found that a substantial number of genes predicted to encode enzymes of primary metabolism are strongly induced.

Our analysis indicated that genes implicated in carbohydrate and lipid metabolism, and those encoding VSPs, are prevalent among the highly induced genes (Tables 1 and 2). Previous workers have noted that herbivory stress can result in suppression of genes involved in important metabolic processes such as photosynthesis. This has led to the suggestion that altered expression of primary metabolic genes is a reflection of a shift of resources to defense (Reymond *et al.*, 2004; Voelckel & Baldwin, 2004). The systemic up-regulation of cell wall invertase during herbivore defense in poplar has been associated with increased sink strength and a concomitant enhancement of carbohydrate import and condensed tannin accumulation (Arnold & Schultz, 2002). Such an increase in sink strength could be indicative of resource allocation for defense in young leaves. Interestingly, it would also have implications for systemic signaling, since systemic signaling

in poplar is dependent on phloem transport and moves preferentially from source to sink (Davis *et al.*, 1991b; Arnold & Schultz, 2002). Whether the neutral invertase identified by our array analysis has a similar function is unclear; however, this provides a starting point for future studies on the reallocation of resources during defense.

Other enzymes identified by our analysis, such as a putative class 3 lipase, esterase, and AAE are involved in lipid metabolism (Tables 1 and 2). Significantly, class 3 lipases were previously identified in *Arabidopsis* with roles in pathogen and insect defense. For example, the *PAD4* and *EDS1* genes encode class 3 lipase proteins that are involved in salicylic acid accumulation during pathogen defense (Falk *et al.*, 1999; Jirage *et al.*, 1999). A distinct class 3 lipase from *Arabidopsis*, *DAD1* (defective in anther dehiscence), has phospholipase A1 activity and catalyzes the initial step of JA biosynthesis by releasing linolenic acid from chloroplast membrane phospholipids (Ishiguro *et al.*, 2001). However, DAD1-catalyzed JA accumulation is required for floral development in *Arabidopsis*, but not for herbivore defense (Schaller *et al.*, 2004). Instead, another of the 12 *Arabidopsis DAD1*-related genes, which includes the putative ortholog of our lipase, appears to be involved in JA-based defense signaling (Beisson *et al.*, 2003). JA metabolism could also provide a rationale for the induction of a putative AAE, which belongs to a larger superfamily of AAEs (Shockey *et al.*, 2003). *JAR1*, which conjugates isoleucine to JA, is a member of this family that is required for many JA responses (Staswick *et al.*, 2002; Staswick & Tiryaki, 2004). While we do not know if our class 3 lipase and the AAE genes are directly involved in JA metabolism, they provide potential links between fatty acid metabolism and herbivore defense to be investigated further.

Several unknown genes containing the ZIM motif are induced during poplar defense

Detailed bioinformatic analysis identified three unknown genes with a ZIM motif. The function of the ZIM motif is unknown, but it has been suggested to be involved in DNA binding (CDD, pfam06200; InterPro, IPR010399). The ZIM (Zn-finger protein expressed in Inflorescence Meristem) protein is an *Arabidopsis* transcription factor with a putative role in development, and has discrete domains responsible for DNA binding, transcriptional activation, putative protein–protein interactions, and nuclear localization (Nishii *et al.*, 2000; Shikata *et al.*, 2003, 2004). All three of our genes with the ZIM motif lack the DNA binding GATA-type Zn-finger domain, but it is possible that they activate transcription through an interacting partner. Shikata *et al.* (2003) suggested that *Arabidopsis* ZIM requires an interacting partner for full-strength transcriptional activation. Our ZIM motif genes also each contain an N-terminus rich in acidic residues, which has transcriptional activation activity in *Arabidopsis* (Shikata *et al.*, 2003). Finally, these genes contain a region rich in basic residues with several conserved basic residues to the probable nuclear localization signal (NLS) of *Arabidopsis* ZIM (Nishii *et al.*, 2000). A predicted NLS was not detected from any of our genes using available search tools (PredictNLS, Prosite), but these tools also failed to detect an NLS from *Arabidopsis* ZIM. Additional experiments are required to establish whether our ZIM motif genes are localized to the nucleus or active as transcriptional activators.

Conclusion

The expression profiling approach we have described in this report has allowed us to get a first glimpse of poplar defense responses induced by an insect elicitor compared with mechanical wounding. We have shown that FTC-R is a potent elicitor of defense responses in poplar, and that for our suite of genes, the FTC-R- and wound-induced responses were qualitatively similar while quantitatively distinct. The availability of larger poplar microarrays will help test this result; if corroborated, this may reflect a general defense strategy which evolved in perennial plants, or the effect of a response elicited by a polyphagous generalist. The large number of natural poplar pests will facilitate comparative experiments with herbivores having differing host ranges and allow a unique molecular understanding of plant defense. Poplars are keystone species of the boreal forest, and both *Populus* and *Malacosoma* species are found throughout the northern hemisphere. With the recent completion of the poplar genome sequence, the poplar-FTC system presents an excellent opportunity for molecular and ecological studies of a plant–herbivore interaction that is relevant on a global scale.

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Supplemental Material

The following supplementary material is available for this article online:

Table S1 Mean expression ratios for all significantly induced or repressed genes.

Table S2 Mean expression ratios for all genes represented on macroarray.

Table S3 Raw and normalized signal intensities for all macroarray replicates and treatments.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/asp/10.1111/j.1469-8137.2006.01877.x
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