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Gene expression profiling of systemically wound-induced defenses in hybrid poplar

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Abstract As part of an ongoing effort to identify genes involved in poplar defense responses, and to provide a resource for comparative analysis of woody and non-woody plant defense, we generated expressed sequence tags (ESTs) from a library constructed from systemically wounded leaves of hybrid poplar (*Populus trichocarpa* × *P. deltoides*). Partial sequences were obtained from the 5′ ends of 928 individual cDNAs, which could be grouped into 565 non-overlapping sequences. Of these, 447 sequences were singletons, while the remainder fell into 118 clusters containing up to 17 partially overlapping ESTs. Approximately 81% of the EST sequences showed similarity to previously described sequences in public databases. Of these, the distribution of gene functions within the EST set indicated that approximately 11% of the ESTs encode proteins potentially involved in defense or secondary metabolism, while photosynthesis and primary metabolism accounted for 45% of the expressed genes. Two types of defense proteins, Kunitz trypsin inhibitors and chitinases, were found among the ten most abundant ESTs, indicating the significant impact of wounding on the leaf transcriptome and suggesting that these functions are important for hybrid poplar defense. In the course of this work, three new wound-inducible Kunitz trypsin inhibitor-like genes and two new chitinase-like genes were characterized. A suite of other systemically wound-induced genes were identified using northern and macroarray analysis, indicating diversity and multiplicity in the induced defense response. Overall, we demonstrate that defense-related genes of hybrid poplar have a variety of functions, and show remarkably diverse expression patterns upon wounding.

Keywords Expressed sequence tag · Herbivory · Macroarray · Plant defense · Poplar · Trypsin inhibitor

Abbreviations EST: Expressed sequence tag · PPO: Polyphenol oxidase · TI: Trypsin inhibitor

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Introduction

Plants are exposed to many potential pests and herbivores, and have evolved a variety of physical and biochemical mechanisms to defend themselves. In particular, many plants mount an induced defense response that involves major shifts in gene expression, ultimately leading to the accumulation of toxic or anti-nutritive proteins. The first induced herbivore defense proteins to be discovered were wound-induced protease inhibitors in potato (Green and Ryan 1972). Since then, stress-regulated protease inhibitors of a variety of protein families have been discovered in different plant species. Protease inhibitors block the activity of digestive enzymes, and many are active against insect herbivores and pathogens (Ryan 1990; Richardson 1991). Their efficacy as defense proteins against insect herbivores has been clearly demonstrated in many experiments, and their overexpression in transgenic plants reduces the performance of insects feeding on those plants (reviewed by Ryan 1990; Schuler et al. 1998). In addition, plants make use of a variety of other induced defenses, including the oxidative enzymes peroxidase, lipoxygenase, and polyphenol oxidase (PPO). These enzymes act as anti-nutrients, destroying or modifying essential amino acids and fatty acids, which has negative consequences for herbivores (Duffey and Felton 1991). Other induced defense proteins include enzymes required for synthesis of toxic secondary metabolites such as

nicotine, or reinforcement of the cell wall (reviewed in Constabel 1999; Walling 2000).

The availability of DNA array technology and new techniques for identifying differentially expressed genes has led to the realization that herbivore damage and wounding leads to the transcriptional activation of hundreds of genes. For example, in *Nicotiana attenuata*, differential display and specialized microarrays were used to identify a suite of genes that respond to herbivory by *Manduca sexta*; at least 73 differentially regulated genes were identified, including genes involved directly in defense, defense signaling, or production of defensive compounds (Hermsmeier et al. 2001). Cheong et al. (2002) found that in *Arabidopsis*, approximately 8% of transcribed genes are up- or down-regulated by wounding. Many of the induced genes do not have any obvious defensive role and some genes are repressed by wounding, suggesting that the plant is not only activating defenses but is also partially reorganizing its metabolism to deal with the stress. Therefore, many of the transcriptional changes observed reflect adjustment of metabolism, signal transduction, and other cellular processes (Cheong et al. 2002).

Single-pass sequencing of anonymous cDNA clones (expressed sequence tags, or ESTs) has become a rapid and cost-effective means of gaining information about gene expression and coding capacity in plants, in particular for non-model species where whole-genome sequencing has not been achieved. This approach has been successfully applied to identifying genes expressed in specialized tissues and organs, and to assist in the identification of the genes involved in specialized processes, such as monoterpene and phenylpropene synthesis in glandular trichomes (Lange et al. 2000; Gang et al. 2001), fragrance in rose flowers (Guterman et al. 2002), and nitrogen-fixing nodule formation (Györyey et al. 2000; Poulsen and Pødenphant 2002). EST sequencing has been used to study wood formation in poplar and loblolly pine (Sterky et al. 1998; Allona et al. 1998), as well as for investigating responses to pathogen attack in soybean and canola (Fristensky et al. 1999; Qutob et al. 2000). In addition to the identification of specific genes, EST sets derived from non-normalized libraries can be used for 'digital northern' analyses, which provide quantitative estimates of gene expression, since the number of EST clones can be expected to be proportional to the abundance of the mRNA used to construct the library (Audic and Claverie 1997; Mekhedov et al. 2000; Ohlrogge and Benning 2000). Mahalingam et al. (2003) used digital northerns to characterize the abundance of stress-related transcripts in healthy *Arabidopsis*, but found that a significant fraction of stress-modulated genes is not represented in existing EST collections. This emphasizes the need of genertating EST from challenged tissues in order to identify defense-related genes.

We are interested in the induced defense response of hybrid poplar (*Populus trichocarpa* × *P. deltoides*), which is a useful experimental model and is known to express a variety of defense proteins and genes following herbivore damage. To date, induced genes encoding trypsin inhibitors, endochitinases, vegetative storage proteins and PPO have been identified (Bradshaw et al. 1989; Davis et al. 1991, 1993; Constabel et al. 2000). These genes are all upregulated in unwounded leaves on wounded plants, indicating a systemic response. To obtain a broader view of the induced defense response in hybrid poplar leaves and to pursue the identification of novel defense genes, we undertook a small-scale EST sequencing project of cDNAs obtained from systemically wounded poplar leaves. To our knowledge, EST sequencing has not yet been applied to herbivore and wound defense responses of plants. These ESTs provide a snapshot of gene expression during hybrid poplar defense, and provide a bank of clones for more detailed investigations.

Materials and methods

Plant material and treatment

Poplar hybrid H11-11 (*Populus trichocarpa* × *P. deltoides*), originating from the University of Washington/Washington State University Poplar Research Program, were propagated and grown as previously described (Constabel et al. 2000). For general wounding experiments, leaves were mechanically wounded with pliers at LPI 12–14 (leaf plastochron index; Larson and Isebrands 1971; Constabel et al. 2000). Systemic leaves corresponded to LPI of 9–11 on wounded plants. Samples were harvested at 24 h after wounding unless otherwise indicated.

EST sequencing

A cDNA library (Lambda Zap II, Stratagene, La Jolla, USA) constructed from systemically wounded hybrid poplar leaves (Constabel et al. 2000) was subjected to mass excision to release the plasmid (pBluescript SK +). T3 and T7 primers corresponding to the region flanking the multiple cloning site of pBluescript SK + were used to amplify the entire insert from the bacterial colony. DNA for sequencing was purified directly from the PCR reaction (PCR purification kit, Qiagen, Mississauga, Canada), or if more than one PCR product was generated, each band was individually cut out of the gel and purified (QIAEX II Gel Extraction Kit, Qiagen). Sequencing reactions were performed from the 5′ end of the cDNA insert using either the T3 primer or a nested primer that overlapped the 3′ end of the T3 primer sequence extending into the multiple cloning site (5′-AGGGAACAAAAGCTGGAGC-3′), using the CEQ DTCS sequencing kit (Beckman Coulter, Fullerton, USA). Reactions were carried out for 30 cycles (96°C 20 s, 50°C 20 s, 60°C 4 min), and samples precipitated with ethanol, resuspended in 40 µl formamide and analyzed on a Beckman CEQ 2000XL capillary sequencer as per the manufacturer's instructions.

Sequence analysis

Sequences were evaluated for the quality parameters indicative of reliable sequence, as specified by the CEQ 2000XL manual (Beckman Coulter). Removal of vector sequence and trimming of low quality and short sequences (<150 bp) was performed with the EMBOSS package (Rice et al. 2000). Trimmed ESTs were submitted for similarity searches through a standalone BLASTX program (Altschul et al. 1997) against the non-redundant protein database at NCBI (http://www.ncbi.nlm.nih.gov). BLASTX results were classified according to their expectation values (E-values). Matches with an E-value $\leq 10^{-15}$ (Yao et al. 2002; Kirst et al. 2003) were assigned to cellular functional categories, based on a slightly modified annotation system of the Munich Information Center for Protein Sequences (MIPS; http://www.mips.biochem.mpg.de/). Assembly of overlapping sequences was performed using PHRAP (http://www.phrap.org) with default parameters.

RNA blot hybridization and macroarray analysis

Total RNA was isolated from hybrid poplar leaves as previously described (Haruta et al. 2001). RNA (15 μg per lane) was loaded onto 1.2% (w/v) agarose-formaldehyde gels, blotted and hybridized as previously described (Constabel et al. 2000). RNA blots were probed with gel-purified and ^{32}P-labeled cDNA inserts. Hybridization, washes and quantification of signal intensities were as previously described (Constabel et al. 2000). Ethidium bromide staining of RNA was used to verify equal loading of lanes.

For macroarray construction, cDNA inserts corresponding to 569 ESTs were amplified by PCR, and each clone was spotted in duplicate onto nylon membranes (Amersham Biosciences, Little Chalfont, UK) using a handheld multi-blot replicator (VP Scientific, San Diego, USA). Membranes were denatured, neutralized and cross-linked using a low energy UV source (Stratagene). For array analysis, total RNA was isolated from three independent biological replicates for both systemically wounded or control leaves. For each probe, poly(A+) was isolated from 37.5 μg total RNA using Dynabeads Oligo (dT) 25 (Dynal Biotech, Oslo, Norway), reverse transcribed using Superscript II (Gibco/BRL, Rockville, USA), and cDNA labeled with ^{33}P using a Rediprime II Kit (Amersham Biosciences). Macroarrays were hybridized using the method of Church and Gilbert (1984) at 65°C overnight, washed at high stringency, and exposed to Phosphoimager screens for 48 h. Images were scanned with a Storm Phosphoimager (Amersham Biosciences), and the spots were quantified using ArrayVision 7.0 (Imaging Research, St. Catherines, Ontario, Canada). Spot intensities were corrected for local background, and normalized to the standard deviation of the entire array. Normalized intensities from the three biological replicates were used to measure the average ratio of expression and to determine statistical significance using a one-tailed *t*-test.

Results

Sequencing and functional classification of hybrid poplar ESTs

A cDNA library made from unwounded leaves on mechanically wounded hybrid poplar saplings (Constabel et al. 2000) was used to study gene expression of inducible systemic wound-responses. Clones were randomly selected and single-pass sequenced with a vector primer upstream of the 5′-end of the inserts. A total of 928 ESTs were obtained after trimming off vector and removing poor quality and short (<150 bp) sequences. The average sequence length of the inserts was 364 bp. To gain an idea of the redundancy of this EST set, overlapping sequences were assembled into clusters using PHRAP (http://www.phrap.org). Of the 928 ESTs, 447 (48.2%) were singletons, and did not overlap with any other EST. The remaining 481 (51.8%) sequences were assembled into 118 clusters that contained up to 17 ESTs. This EST collection therefore comprises a total of 565 non-redundant sequences.

In order to assign putative gene functions, all ESTs were queried against the GenBank protein database at NCBI (http://www.ncbi.nlm.nih.gov) using the BLASTX algorithm (Altschul et al. 1997). The BLASTX outputs were sorted for significance of the match using E-values. Database hits with E-values equal to or lower than 1×10^{-15} were considered significant and likely to represent true sequence similarity (Yao et al. 2002). Using this cut-off, 592 hits (63.8%) were found to be significant matches, and 287 hits (30.9%) were classified as not significant. The remaining 49 sequences (5.3%) returned no BLAST hits. This lack of detectable similarity with GenBank sequences could be the result of short query sequences, especially from the 5′-UTR region of a cDNA (Kirst et al. 2003). Therefore, the 336 sequences that yielded E-value $>1\times10^{-15}$ or returned no hits were re-analyzed using standalone BLASTN against EST collections from *P. trichocarpa*, *P. tremula*and *P. tremula* × *P. tremuloides*. These contain more than 80,000 nucleotide sequences and are publicly available in GenBank, but are not functionally annotated (hereafter referred to as *Populus* ESTs). Our previous comparative analyses showed that the transcriptome of different *Populus* species and hybrids share a high degree of similarity, with a mean nucleotide identity of at least 95%, facilitating homology analyses across *Populus* species (Miranda et al. 2004). Therefore, those *Populus* ESTs that matched our ESTs with a BLASTN E-value of $<1\times10^{-35}$ and were 90% identical over >100 bp were considered probable orthologs; 209 of 336 queries matched these criteria. Orthologous *Populus* ESTs were then searched against the GenBank protein database using BLASTX, and 161 sequences yielded significant

hits (E-value $< 1\times10^{-15}$). The publicly available *Populus* ESTs therefore facilitated the annotation and functional classification of additional ESTs, so that the number of ESTs in our collection without a significant hit was reduced to 175 (18.9%).

The ESTs with annotated database matches (753 ESTs in total) were classified according to their likely biological functions. This analysis indicated that a large proportion of these ESTs represent genes with roles in primary metabolism (179 ESTs, 23.8%), especially in photosynthesis and energy generation (156 ESTs, 20.8%) (Fig. 1). Other functional categories were represented at lower frequencies, for example DNA synthesis and transcription (4.9%), protein synthesis and modification (10.7%), and cellular biogenesis and organization (4.0%). A total of 42 ESTs (5.6%) identified gene products directly connected with plant defense, such as protease inhibitors and other anti-nutritive proteins, or proteins showing strong similarity to defense-related genes from other plants (Fig. 1). Other stress response-related genes were represented by 18 ESTs (2.4%). In addition, 39 ESTs (5.2%) fell into the secondary metabolism class, which includes enzymes of the phenylpropanoid pathway and cytochromes P450, many of which are involved in the synthesis of secondary plant metabolites (see below).

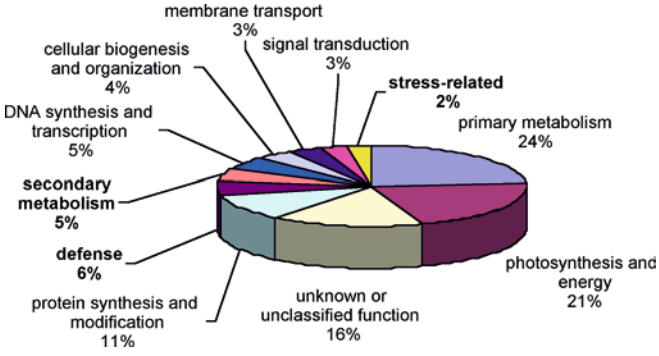

Fig. 1 Classification of poplar expressed sequence tags (ESTs) identified in a systemically wounded leaf cDNA library. ESTs with significant matches to known sequences (BLAST E-values $\leq 10^{-15}$) were assigned cellular roles and classified into functional categories using a modified Munich Information Center for Protein Sequences (MIPS) (http://www.mips.biochem.mpg.de/) annotation system. The percentage of ESTs in each of the categories is shown

We obtained a more detailed view of the EST set by determining which predicted proteins were represented by the greatest number of ESTs, and generated a ranking of gene products with more than five hits in the library (Table 1). The most abundant transcripts encode one or more chlorophyll a/b binding proteins (CAB), with 57 ESTs, while ribulose bisphosphate carboxylase (Rubisco) homologs were the second-most abundant transcripts, with 29 ESTs. Other photosynthesis-related genes that are highly represented in our EST set include Rubisco activase and carbonic anhydrase. As expected, transcripts encoding defense-related genes were also found to be very common in our EST set, in particular those encoding chitinases (16 ESTs) and trypsin inhibitors (TIs, 10 ESTs). Cysteine proteases were highly represented (16 hits); while these can have diverse biological functions, some members of this protease family from *Zea mays* have been shown to be directly toxic to insect larvae by disrupting the peritrophic membrane (Pechan et al. 2002). The remaining abundant genes appear to be involved in other aspects of primary metabolism and cellular functions, with no obvious direct defensive roles (Table 1). Overall, we conclude that this non-normalized leaf library from systemically

Table 1 Most abundant gene products in hybrid poplar leaves identified by expressed sequence tag (EST) sequencing of a systemically wound-induced leaf cDNA library

Predicted gene product	Number of occurences in EST set[a]	Functional category[b]
Chlorophyll a/b binding protein	57	Photosynthesis/energy
Ribulose bisphosphate carboxylase (Rubisco)	29	Photosynthesis/energy
Cysteine proteinase	16	Protein synthesis/modification
Chitinase	16	Defense
Cytochrome P450	14	Secondary metabolism
Rubisco activase	12	Photosynthesis/energy
Fructose bisphosphate aldolase	11	Photosynthesis/energy
Trypsin inhibitor	10	Defense
Elongation factor 1-alpha	10	Protein synthesis/modification
Acid phosphatase	9	Primary metabolism
Ubiquitin	8	Protein synthesis/modification
Carbonic anhydrase	7	Photosynthesis/energy
Lipase-like protein	7	Primary metabolism
14.3.3-like protein	7	Protein synthesis/modification
Glyceraldehyde-3-P dehydrogenase	6	Primary metabolism
S-Adenosylmethionine synthetase	6	Primary metabolism
Alanine:glyoxylate aminotransferase	5	Photosynthesis/energy
Photosystem I reaction center subunit III	5	Photosynthesis/energy
Glutamate synthetase	5	Primary metabolism
Heat shock protein 70 kDa	5	Stress-related
Galactinol synthase	5	Primary metabolism
Glycolate oxidase	5	Photosynthesis/energy

[a]Number of occurences of each gene product (BLAST E-values $\leq 10^{-15}$)

[b]Functional classification adapted from Munich Information Center for Protein Sequences (MIPS) database (see legend to Fig. 1)

wound-induced plants includes a significant number of defense-related genes. Nevertheless, gene products that play roles in photosynthesis and primary metabolism still comprise the largest functional category.

Identification and analysis of Kunitz TI-like genes

Since a major objective of this work was to gain insight into the defense response of hybrid poplar, we analyzed the EST set for genes with products likely to play a direct role in plant defense (Table 2). This category included anti-nutritive or toxic proteins, hydrolytic enzymes, and oxidative enzymes. Based on the BLASTX results, 10 ESTs were identified as similar to Kunitz TIs; these assembled into three clusters. Pairwise comparisons of the contigs showed that they had low similarity to one another, suggesting they are all divergent members of the Kunitz TI family. The longest clones from each of the TI clusters were fully sequenced and named *PtdTI3*, *PtdTI4*, and *PtdTI5*. Comparison of the full-length sequences with protein databases confirmed that they all belong to the soybean Kunitz TI family (PFAM PF00197; http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF00197), and pairwise sequence comparisons confirmed the low sequence similarity (16–25% amino acid identity) of these TI-like sequences among themselves (Miranda et al. 2004). Surprisingly, none of these TI-like genes corresponded to *win3*, the previously characterized poplar Kunitz TI (Bradshaw et al. 1989). The respective top BLASTX hits of *PtdTI3*, *PtdTI4*, and *PtdTI5* were the wound-induced *P. tremuloides PtTI3* (86% amino acid identity; AAK32691), a *Theobroma cacao* storage protein/TI (33%; GenBank accession P32765), and a *Sesbania rostrata*nodule-expressed Kunitz TI (46%; AJ441323), respectively (Table 2). The proteins predicted by these newly identified cDNAs contained the conserved elements of Kunitz TIs, including the N-terminal signature sequence of the soybean Kunitz TI family (L/I/V/M)-X-D-X-(E/D/N/T/Y)-(D/G)-(R/K/H/D/E/N/Q)-X-(L/I/V/M)-X(5)-Y-X-(L/I/V/M) (Brenner et al. 1998). However, in the case of *PtdTI5*, only six of eight consensus residues are present (data not shown). Furthermore, *PtdTI5* contained only two of the four conserved Cys residues, which form two disulfide bridges in typical Kunitz TIs. However, a recently characterized Kunitz TI from *Swartzia pickellii* also contains only one pair of Cys residues and a single disulfide bridge, yet is a fully functional TI (Cavalcanti et al. 2002).

We performed northern analyses to determine if transcription of these three TI-like genes is upregulated by wounding, and to compare their expression with the previously characterized *win3* gene (Bradshaw et al. 1989). Time-course experiments were carried out in which plants were wounded on lower leaves, and both the wounded leaves and the upper unwounded (systemic) leaves were harvested for RNA extraction. While leaves harvested prior to wounding did not accumulate transcripts of the TI-like genes, all four were clearly induced upon wounding (Fig. 2). Interestingly, the induction kinetics and regulation patterns of the four TIs were distinct; specifically, *PtdTI3* was the most rapidly induced, with a strong signal at 4 h after wounding, and clearly had the highest systemic induction. The other three TIs showed very similar induction in the wounded leaves, but of these, only *PtdTI4* showed appreciable expression in systemic leaves (Fig. 2). Overall, the observation that all four known hybrid poplar Kunitz TIs are induced by mechanical wounding supports the hypothesis that these proteins function in defense against chewing insects.

Table 2 Probable herbivore defense genes identified in a systemically wound-induced hybrid poplar leaf cDNA library[a]

Gene	Number of occurrences in EST set	Best database match[b]	E-value of best match	GenBank accession[c]
Protease inhibitors				
Kunitz trypsin inhibitor (*PtdTI3*)	6	*Populus tremuloides* trypsin inhibitor	7×10^{-25}	CN193385
Kunitz trypsin inhibitor (*PtdTI4*)	3	*Theobroma cacao* storage protein/inhibitor	2×10^{-15}	CN193094
Kunitz trypsin inhibitor (*PtdTI5*)	1	*Sesbania rostrata* trypsin inhibitor	3×10^{-13}	CN192805
Chitinases				
Acidic endochitinase (*win6*)	6	*P. trichocarpa* × *P. deltoides win6*	5×10^{-31}	CN193306
Acidic endochitinase (*win8*)	6	*P. trichocarpa* × *P. deltoides win8*	2×10^{-88}	CN192717
Acidic chitinase	1	*Glycine max* chitinase	1×10^{-29}	CN193068
Basic chitinase	3	*Arabidopsis thaliana* basic chitinase	2×10^{-44}	CN192845
Oxidative enzymes				
Polyphenol oxidase (PPO)	1	*P. tremuloides* PtPPO	5×10^{-42}	CN193334
Peroxidase	4	*Gossypium hirsutum* peroxidase	7×10^{-51}	CN192809
Lipoxygenase	3	*Solanum tuberosum* 13-lipoxygenase	1×10^{-22}	CN193283
Other				
Cysteine proteinase	14	*Zea mays* mir3 cysteine protease	1×10^{-60}	CN192935

[a]Genes encoding proteins with high similarity to proteins with potential anti-herbivore activity
[b]Most similar sequence in GenBank as calculated using BLASTX (Altschul et al. 1997)
[c]GenBank accession of poplar EST with best match to database hit

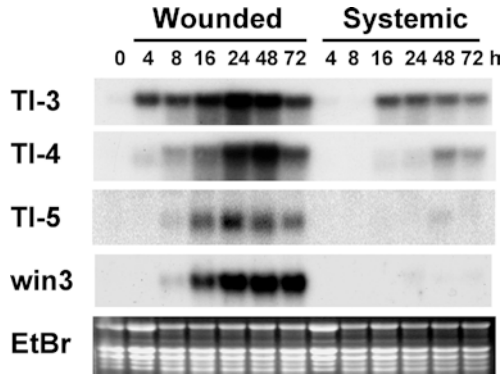

Fig. 2 Northern analysis of trypsin inhibitor (TI) mRNA accumulation following wounding of hybrid poplar. Wounded and unwounded (systemic) leaves on wounded plants were harvested for RNA extraction at the times indicated. Northern blots were hybridized with labeled cDNA probes encoding four members of the Kunitz family of serine TIs. *TI-3*, *TI-4*, and *TI-5* are novel TIs (*PtdTI3*, *PtdTI4* and *PtdTI5*) identified in this work. *Win3* (Bradshaw et al. 1989) was included for comparison. EtBr, Ethidium bromide-stained gel used as a loading control

Analysis and expression of other defense-related genes

Clones homologous to chitinases formed another abundant set of defense-related gene products among our ESTs (Tables 1, 2), with 16 significant chitinase hits. Chitinases are commonly pathogen stress-induced, and are known to function in defense against fungal pathogens (Collinge et al. 1993; Neuhaus 1999). We identified two clusters of ESTs that were highly similar to the previously identified *win6* and *win8*wound-induced chitinase genes of hybrid poplar (Parsons et al. 1989; Davis et al. 1991); these were present in our EST set six times each (Table 2). *Win6*and *win8* share only ~50% amino acid identity, and are unusual in that they have an acidic isoelectric point but otherwise have all the features of wound-induced, vacuolar, basic chitinases (Neuhaus 1999). In addition, two other clusters of chitinase-like genes clearly distinct from *win6* and *win8* were identified; one of these clusters has greatest similarity to a basic family 19 glucosyl hydrolase (chitinase) from *Arabidopsis*, and the other is most similar to a distinct acidic chitinase from soybean (Table 2). Therefore, our data show that at least four distinct types of chitinase-like genes are expressed in systemically wounded poplar leaves.

Additional abundant ESTs with potential direct defense roles identified based on primary sequence analysis included the oxidative enzymes PPO, peroxidase and lipoxygenase (Table 2). These enzymes are considered anti-nutritive defenses and can act by destroying or alkylating essential nutrients, including essential amino acids and fatty acids (Duffey and Felton 1991). PPO is known as a systemically inducible defense in tomato and poplar (Constabel et al. 1995; 2000). It is thought to act against herbivores by producing reactive quinones, which alkylate and modify essential dietary amino acids (Felton et al. 1992). Peroxidases use H_2O_2 to oxidize phenolics, and thus are proposed to act in a fashion similar to that of PPO. Lipoxygenase activity destroys linolenic acid, an essential fatty acid for insects, and thus acts as an antinutrient (Duffey and Felton 1991); it is also the first step of the octadecanoid pathway leading to the synthesis of the defense signal jasmonic acid (Farmer and Ryan 1990). The oxidative enzymes PPO, peroxidase, and lipoxygenase have all been shown to contribute to plant defense against herbivores in transgenic experiments, and thus are likely to be functionally important for defense in hybrid poplar (Dowd and Lagrimini 1997; Royo et al. 1999; Wang and Constabel, 2004).

Our EST set included a number of genes with high similarity to genes that are likely involved in defense or whose products could have indirect defensive functions, but which are not directly anti-nutritive themselves (Table 3). These included ESTs with significant similarity to proteins required for the synthesis of the defense signals ethylene and jasmonate, for example ACC oxidase and allene oxide cyclase. Also considered likely to have functions in the hybrid poplar defense response are the phenylpropanoid enzymes that were represented in our EST collection; these may be important for cell wall reinforcement via deposition of lignin-like polymers, as well as for the synthesis of other phenylpropanoids (Table 3). *Populus* as a genus is known for its diversity of phenolics, many of which have biological activity and are known to have negative effects on insects (Palo 1984; Lindroth and Hwang 1996). Finally, ESTs with similarity to wound-inducible genes considered likely to have defensive functions in other plants were considered part of this set, based on their induction by wounding in several different plant species.

We selected a suite of candidate defense genes of particular interest for northern analyses in time-course experiments. Transcripts that showed consistent wound-induction are shown in Fig. 3. In the wounded leaves, the strongest inductions were shown by a gene encoding a cytochrome P450 (highest similarity to a hydroxylase of terpenoid phytoalexin synthesis; Ralston et al. 2001) and hydroperoxide lyase, an enzyme of lipid metabolism leading to short-chain volatile alcohol and aldehyde formation in plant tissues (Hatanaka 1993). The rapid induction of hydroperoxide lyase could indicate a signaling function in mediating the defense response (see below). Lipoxygenase, the *Populus* awi31 homolog, and a protein belonging to the short-chain dehydrogenase/reductase superfamily and with high similarity to secoisolariciniol dehydrogenase, (Gang et al. 1998), were also rapidly induced. In contrast, the vegetative storage protein *win4.5* was among the slowest to be upregulated (Fig. 3); the biochemical function of these genes in defense is not yet known. As seen for the TIs, not all genes highly induced in wounded leaves showed equally strong systemic induction (Fig. 3). For comparative purposes, the blots were also probed with a CAB gene; in both local and systemic wounded leaves no induction of CAB mRNA was observed. Circadian decreases in CAB

Table 3 Genes with potential defensive functions identified in a systemically wound-induced hybrid poplar leaf cDNA library[a]

Gene	Number of occurrences in EST set	GenBank accession[b]
Enzymes for signaling pathways		
ACC oxidase	4	CN193268
S-Adenosylmethionine synthetase	6	CN193074
Fatty acid desaturase	2	CN193101
13-Lipoxygenase	3	CN193283
Allene oxide cyclase	1	CN193019
Hydroperoxide lyase	1	CN192806
Phenylpropanoid pathway		
Phenylalanine ammonia lyase	2	CN192894
Caffeic acid O-methyltransferase	1	CN193252
Caffeoyl-coA-3-O-methyltransferase	1	CN193027
O-methyltransferase	1	CN193109
4-Coumarate-coA ligase	1	CN193104
Cinnamyl alcohol dehydrogenase	2	CN192800
Flavonol 3-O-glucosyltransferase	3	CN192917
Dihydroflavonol reductase	1	CN193226
Anthocyanidin synthase	1	CN192891
Phenylcoumaran benzylic ether reductase	3	CN193406
Short-chain dehydrogenase/reductase-like	2	CN193146
Inducible genes		
Acid phosphatase	9	CN193027
AWI31,*Arabidopsis* wound-induced protein	4	CN193163
Ethylene-inducible protein HEVER	3	CN192991
Glutathione S-transferase	4	CN193144
Thaumatin homolog	1	CN192577

[a]Genes encoding proteins with possible defense roles based on their predicted biochemical activity in signaling, phenylpropanoid metabolism, or wound-induced expression in other species

[b]GenBank accession of poplar EST with best match to database hit

transcripts were observed at 16 h after wounding, a timepoint that corresponded to the dark period. Overall, our northern analyses demonstrate the wound-induction of a suite of genes, suggesting they are important in defense. Furthermore, they also demonstrate the wide variety of genes induced and the distinct induction kinetics observed.

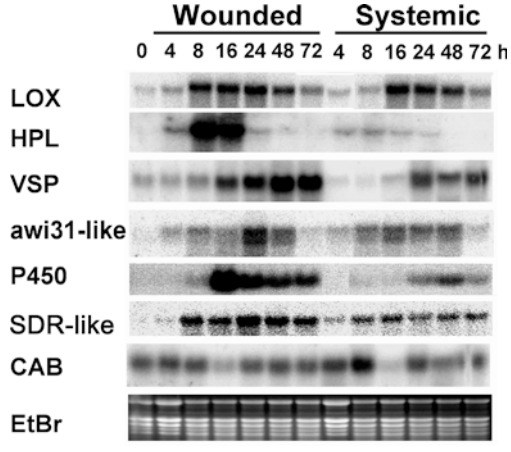

Fig. 3 Northern analysis of defense-related mRNA accumulation following wounding of hybrid poplar. Wounded and unwounded (systemic) leaves on wounded plants were harvested for RNA extraction at the times indicated. Blots were hybridized with probes for 13-lipoxygenase (*LOX*), hydroperoxide lyase (*HPL*), *win 4.5* vegetative storage protein (*VSP*), *awi31*-like gene, cytochrome P450 (*P450*), short-chain dehydrogenase/reductase-like (SDR), and chlorophyll a/b binding protein (*CAB*). EtBr, Ethidium bromide-stained gel used as a loading control

Analysis of wound-induced gene expression using macroarrays

In addition to northern analysis of candidate genes, we tested our EST genes for wound-inducibility using nylon macroarrays. A cDNA macroarray of 569 cDNA clones was prepared and hybridized with RNA from control and systemically wounded leaves. The analysis consisted of three complete biological replicates, and used a one-tailed *t*-test to determine if control leaf gene expression was significantly different from induced leaf expression. At a significance threshold of $P < 0.05$, 85 of 569 cDNAs on the array (15%) showed a change of 2-fold or greater. These genes were ranked by degree of induction (normalized induced vs. control leaf expression), and those with an induction of greater than 10-fold are listed in Table 4. A more detailed analysis of the macroarrays will be published separately (I. Major and P. Constabel, MS in preparation). Significantly, all three newly identified wound-induced trypsin inhibitors *PtdTI3*, *PtdTI4*, and *PtdTI5*, and three previously discussed wound-induced genes (chitinases *win6* and *win8*, and PPO), as well as a wound-induced vegetative storage protein *win4.5* (Davis et al. 1993), were among the most strongly upregulated genes. This confirms that the macroarray analysis clearly identified those genes known to be wound-inducible, and that many of our likely candidate defense genes from Table 2are in fact strongly upregulated by wounding. Other strongly induced genes had high similarity to apyrase, lipase, acid phosphatase, anthocyanidin synthase, cytochrome P450, and β-amylase sequences (Table 4). Several genes of unknown

Table 4 Most strongly systemically wound-induced genes as assayed using macroarrays[a]

Clone ID	Annotated gene	Induction factor[b]	E-Value of best match	GenBank accession[c]
H1078	Chitinase (*win6*)	60	0.00	CN192556
H1506	Chitinase (*win6*)	58	0.00	CN192741
H1141	Chitinase (*win8*)	53	1×10^{-43}	CN192595
H1949	Vegetative storage protein-like	40	2×10^{-6}	CN192930
H831	PPO	33	1×10^{-27}	CN193334
H64	Apyrase	33	6×10^{-49}	CN193208
H1644	Lipase-like	31	2×10^{-6}	CN192786
H1059	Kunitz trypsin inhibitor (*PtdTI3*)	29	1×10^{-27}	CN192549
H1496	Kunitz trypsin inhibitor (*PtdTI3*)	26	8×10^{-23}	CN192736
H1958	Unknown protein	24	1×10^{-26}	CN192936
H828	Kunitz trypsin inhibitor (*PtdTI4*)	13	1×10^{-27}	CN193330
H583	Unknown protein	14	1×10^{-13}	CN193162
H244	Acid phosphatase-like	13	2×10^{-13}	CN193016
H1685	Kunitz trypsin inhibitor (*PtdTI5*)	13	3×10^{-13}	CN192805
H1286	No hits	12		CN192663
H1863	Anthocyanidin synthase	11	5×10^{-29}	CN192891
H762	Cytochrome P450	11	2×10^{-30}	CN193274
H760	Cytochrome P450	11	2×10^{-47}	CN193273
H930	Cytochrome P450	11	2×10^{-45}	CN193412
H1594	β-Amylase	11	3×10^{-37}	CN192760

[a]Genes significantly induced (one-tailed *t*-test, $P<0.05$) with a mean induction of 10-fold or greater in three independent biological replicates
[b]Fold-induction of mRNA in response to systemic wounding, calculated as the ratio of mean wounded/control normalized hybridization intensities
[c]GenBank accession of poplar EST with best match to database hit

function were also identified as upregulated. While the anthocyanidin synthase and cytochromes P450 may be potentially involved in the biosynthesis of defensive secondary metabolites, the potential role of the genes annotated for primary metabolism is unknown. On the basis of our macroarray experiments, we conclude that, in addition to the induction of genes with obvious defensive roles, wounding influences a variety of metabolic processes. One should note that similarity of our ESTs to annotated genes in the databases does not necessarily imply an identical function, and that such annotations need to be viewed with caution until the appropriate biochemical roles of the gene products can be verified. Transcription of photosynthesis-related and other abundant primary metabolism genes was generally not affected by wounding, although for two photosynthesis-related genes we observed some down-regulation (see below).

Discussion

Small-scale EST projects have been used to identify genes relevant to specific tissues and processes (Gang et al. 2001; Lange et al. 2000), as well as for genome-wide gene discovery. To our knowledge, this project is one of the first to use EST sequencing to characterize the wound-induced transcriptome in plants. We were interested both in discovering novel wound-induced defense genes from poplar leaves (''marker discovery''), as well as in gaining insight into global patterns of gene expression of the defense response (''biology discovery'') (Richmond and Sommerville 2000).

We obtained 928 informative ESTs from a non-normalized library made from systemically induced leaves, and thus the profile of expressed genes can provide a snapshot of gene expression and resource allocation during the systemic wound response. While the wound response may differ somewhat from the response to actual herbivory, in other species such differences in gene expression have been found to be largely quantitative, with only some genes expressed exclusively in response to one or the other of the treatments (Hermsmeier et al. 2001; Reymond et al. 2000; Schittko et al. 2001). Thus, we are confident that the overall pattern of wound-induced gene expression we have observed provides an informative preliminary picture of induced defense in poplar.

The functional classification of ESTs showed that nearly one-half (45%) of expressed genes are involved in photosynthesis, carbon fixation, and primary metabolism, indicating that the leaf is actively engaged in these processes despite the imposed stress. Previous studies have shown that herbivory can reduce the abundance of photosynthesis-related transcripts (Hermsmeier et al. 2001); in our experiments, only carbonic anhydrase and Rubisco appeared to be somewhat downregulated (data not shown). Defense-related and phenylpropanoid/secondary metabolism functions each accounted for over 5% of the EST set (Fig. 1). Many of these defense-related genes are presumably highly represented in the cDNA library because the wound treatment has stimulated their transcription and accumulation. For example, chitinases and TIs were not only among the most abundant transcripts in our library (Table 1), they were also among the most inducible genes in our EST set (Table 4). Other fairly abundant ESTs encoded acid phosphatase-, apyrase-, and lipase-like proteins, and these were also highly induced in systemic leaves (Tables 1, 4). The presence of strongly inducible genes among the most abundant type of gene products indicates that the wound stress has had a significant impact on the leaf transcriptome as a whole. The strong effect of wounding on leaf gene expression was confirmed by

macroarray analysis of our EST set, which indicated that 85 genes (15%) are clearly wound-induced by 2-fold or greater, with a P-value < 0.05. The macroarray also identified 7 additional genes as probably upregulated by wounding, for which greater experimental variability led to higher *P* values (data not shown). Overall, these numbers are roughly consistent with the proportion of genes induced in other plants; expression profiling in wounded *Arabidopsis* leaves found that approximately 8% of genes of the 8,200 genes analyzed showed altered expression in response to wounding (Cheong et al. 2002). Some of the major induced defense genes of poplar, such as chitinases, trypsin inhibitors, hydroperoxide lyase, and cytochromes P450 have also been identified as wound-induced genes in *Arabidopsis* (Cheong et al. 2002; Reymond et al. 2000); other upregulated genes identified here including storage protein-, lipase- and acid phosphatase-like genes, however, appear not to be wound-induced in that species. Our data thus corroborate the idea that herbivore defense responses may be quite species-specific, as was suggested previously in a study of herbivore-induced transcripts of *N. attenuata* (Hermsmeier et al. 2001).

This analysis expands the list of known wound-induced and defense-related genes in hybrid poplar. First, we identified three new hybrid poplar Kunitz TI-like genes, in addition to the previously identified *win3* TI (Bradshaw et al. 1989). One of these (*PtdTI3*) is the likely hybrid poplar ortholog of the previously described *P. tremuloides PtTI3* (Haruta et al. 2001), while *PtdTI4* and *PtdTI5*represent newly identified genes. The four TI-like sequences are very distinct, with less than 50% pairwise identity among them, and represent rather diverse members of the Kunitz TI family in the *Populus* genome. Such multiplicity and sequence diversity of Kunitz TIs may be a general phenomenon; a recent analysis of potato ESTs also found that potato tubers express at least 21 distinct Kunitz TI genes (Heibges et al. 2003a). Kunitz TIs are one of several families of serine protease inhibitors, typical of legume seeds but also found in other plant species (Richardson 1991). While at present we do not have direct evidence that these new TI-like gene products are functional as trypsin inhibitors, the necessary structural elements, such as the N-terminal consensus and essential Cys residues, are present. Moreover, northern analyses indicated that all four TIs are wound-induced in poplar leaves, supporting the idea that they act in herbivore defense.

The multiplicity represented by four very distinct wound-induced Kunitz TIs was surprising, particularly because no other types of protease inhibitors were detected. In contrast, in tomato leaves the set of systemically induced defense proteins includes inhibitors of cysteine, metallo, and aspartic proteases, which belong to different structural families and which may be active against different pests or pathogens (Bergey et al. 1996). We speculate that some of our newly identified TIs could be active against other types of proteolytic enzymes, and may not be specific for trypsin or serine proteases. For example, other workers have demonstrated that Kunitz-type TIs can be active against the cysteine protease papain in addition to trypsin (Franco et al. 2002; Heibges et al. 2003b). The inhibition of proteases other than trypsin by the new TIs will be tested experimentally using recombinant proteins. For herbivore-stressed poplar, the availability of different TIs with distinct specificities could be advantageous, since at least some insect pests have been shown to produce a variety of proteases and to alter gut protease expression in the presence of plant protease inhibitors (Jongsma et al. 1995; Broadway 1996).

Diversity in defense was also demonstrated by the presence of four distinct chitinase genes in our EST set. In addition to the known *win6* and *win8*chitinases, we identified one new acidic and one basic chitinase (Table 2).*Win6* and *win8* were previously shown to be induced by wounding of poplar leaves (Parsons et al. 1989), and our EST and array data confirm that chitinases represent a significant proportion of the wound-induced defense response of poplar (Tables 1, 4). Chitinases are generally induced by pathogens and some other stresses, and accumulate as PR (pathogenesis-related) proteins during systemic acquired resistance against pathogens (Collinge et al. 1993; Neuhaus 1999); their expression during the wound response has sometimes been thought to reflect a function in inhibiting opportunistic fungal pathogens in wound sites (Clarke et al. 1998). However, their very strong induction in wounded poplar leaves also suggests a more direct role in poplar herbivore defense. One function of chitinases against insects may be the disruption of the glycoproteins of the peritrophic membrane, a structure that protects underlying gut cells from damage by digestive enzymes (Barbehenn 2001). Direct toxicity against insects has been shown for microbial chitinases, and a purified chitinase from the soil bacterium *Streptomyces albidoflavus*was shown to be active against a range of herbivorous insects (Broadway et al. 1998). Transgenic tobacco plants overexpressing an insect chitinase show elevated resistance against *Heliothis virescens* (Ding et al. 1998).

The identification of 14 cytochrome P450 hits in the EST set indicates that this is a highly expressed gene family. These proteins are heme-containing mixed-function oxidases that can be involved in many biological processes. Many have been found to function in secondary metabolism as hydroxylases, but many other types of reactions may also be catalyzed (Feldmann 2001). The fact that several P450s are highly induced suggests that synthesis of secondary metabolites could be induced, but there are also many other possible functions.

In northern analyses of defense-related genes, we observed a surprising diversity of expression profiles and kinetics (Figs. 2, 3). The trypsin inhibitors showed substantial differences in the degree of systemic induction, since only *PtdTI3*, and to a much lesser extent *PtdTI4*, were expressed strongly in systemically wounded leaves

(Fig. 2). The almost undetectable level of *win3* systemic expression may explain why we did not find any ESTs corresponding to this gene in the library, which was constructed from systemically-induced leaf tissue. Differences in kinetics of gene expression were also very distinct for the defense-related genes shown in Fig. 3, where there are clear early- and late-induced genes. The significance of such differences in induction profiles is not clear, but could represent outputs of different signaling pathways or differences in function. For example, the rapid induction of 13-lipoxygenase is consistent with its presumed role in generating jasmonate signals, which are required for a later induction of antinutritive proteins. In tomato, genes encoding lipoxygenase and allene oxide synthase are also more rapidly induced by wounding than those encoding proteinase inhibitors (Bergey et al. 1996; Ryan 2000). Hydroperoxide lyase (HPL) is not required for jasmonate but leads to the synthesis of common plant volatiles, hexenals and hexenols; these are known to induce at least some defense genes in *Arabidopsis* (Bate and Rothstein 1998). Perhaps this enzyme is important for the release of these plant volatiles in a signaling context.

In summary, we have carried out an EST analysis of gene expression during the herbivore defense response as induced by wounding of hybrid poplar leaves. We have discovered novel Kunitz TI and chitinase genes in poplar, as well as a number of induced genes that could be important for defense and which will be characterized further. In addition, our EST set gives some insight into the relative importance of defense-related expression relative to primary processes, and provides evidence that wound-induced transcripts make up a significant part of the poplar leaf transcript profile.

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