# Shoot–root defense signaling and activation of root defense by leaf damage in poplar[1]

**Ian T. Major and C. Peter Constabel**

**Abstract:** Shoot–root systemic defense signaling of hybrid poplar (*Populus trichocarpa* Torr. & A. Gray × *Populus deltoides* Bartr. ex Marsh.) was investigated with molecular techniques to extend existing knowledge of poplar defense. Treatment of roots with methyl jasmonate demonstrated that transcripts of *PtdTI3,* a poplar trypsin inhibitor and marker of poplar defense responses, can be induced in poplar roots as well as leaves. Moreover, simulated herbivory of poplar leaves with methyl jasmonate treatment or wounding with pliers also induced *PtdTI3* mRNA in roots, which implies downward, or basipetal, systemic signaling from shoots to roots. In addition, the inducible root-defense response comprised both increased PtdTI3 protein levels and trypsin-inhibitor activity. The inducible systemic response was further investigated with comparative macroarray analyses which indicated that in addition to *PtdTI3*, other genes respond in roots after wounding and methyl jasmonate treatment of leaves. The majority of the 17 genes encode previously identified leaf herbivory defense genes; however, some genes strongly up-regulated in leaves were not induced in roots. The identification of multiple defense genes that are inducible in roots following leaf damage is clear evidence of a systemic defense response in roots and the presence of basipetal shoot–root defense signaling.

*Key words:* belowground defense response, herbivory, plant–insect interaction, signals, systemic tree defense.

**Résumé :** Afin d'étendre la connaissance actuelle sur les mécanismes de défense du peuplier, les auteurs ont examiné, à l'aide de techniques moléculaires. la signalisation de défense tige–racine systémique, chez un peuplier hybride, le *Populus trichocarpa* Torr. & Gray × *P. deltoides* Bartr. ex Marsh. Un traitement des racines avec du jasmonate de méthyl (MJ) démontre que les transcriptions du *PtdT13*, un inhibiteur de la trypsine chez le peuplier et un marqueur des réactions de défense du peuplier, peuvent être induites dans les racines aussi bien que dans les feuilles de l'arbre. De plus, la simulation de l'herbivorie, par un traitement MJ ou des blessures au sécateur, peut aussi induire le *PtdT13* mARN dans les racines, ce qui implique un mouvement systémique basipète de la signalisation, de la tige vers les racines. La réaction de défense inductible des racines implique à la fois une augmentation des teneurs en protéine PtdT13 et l'activité inhibitrice de la trypsine. Les auteurs ont continué à examiner la réaction systémique inductible à l'aide d'analyses macroarrays comparatives, qui indiquent qu'en plus du *PtdT13*, d'autres gènes réagissent dans les racines, après une blessure foliaire ou un traitement avec le MJ. La majorité des 17 gènes codent des gènes de défense liés à l'herbivorie foliaire, déjà identifiés; cependant, certains gènes qui augmentent fortement dans les feuilles, ne sont pas induits dans les racines. L'identification de multiples gènes de défense inductibles dans les racines, suite à un dommage foliaire, constitue une preuve nette de l'existence d'une réaction systémique dans les racines et la présence d'une signalisation de défense basipète tige-racine systémique.

*Mots-clés :* réaction de défense hypogée, herbivorie, interaction plante–insecte, signaux, défense systémique de l'arbre.

[Traduit par la Rédaction]

## Introduction

Plants respond to challenge by herbivores with inducible defenses that have been extensively documented in aerial tissues such as shoots and leaves and their effects on aboveground pests studied. By contrast, the effects of belowground pests and inducible root defenses are often ignored, even though roots constitute a significant component of plant biomass and are a food source for a substantial number of herbivores (Blossey and Hunt-Joshi 2003; Schoonhoven et al. 2005). Root herbivores can affect a variety of physiological processes, including uptake of water, nutrients, and minerals, carbohydrate storage, and production of hormones and phytochemicals. Studies of above- and below-ground herbivore feeding have shown that feeding on either shoots or roots can enhance herbivore resistance in both organs. For example, rice defoliation by the fall armyworm (*Spodoptera frugiperda* J.E. Smith) reduces growth rates of the rice water weevil (*Lissorhoptrus oryzophilus* Kuschel), and likewise, root feeding by the rice water weevil reduces growth rates of fall armyworm (Tindall and Stout 2001). However, only recently have shoot–root defense interactions been investigated in the context of reciprocal plant resistance between above- and below-ground herbivores, as these negative effects are often interpreted as a reduction of plant biomass and nutritional quality.

Received 3 January 2007. Published on the NRC Research Press Web site at canjbot.nrc.ca on 9 January 2008.

**I.T. Major[2] and C.P. Constabel.[3]** Centre for Forest Biology and Biology Department, University of Victoria, P.O. Box 3020, Stn. CSC, Victoria, BC V8W 3N5, Canada.

[1]This article is one of a selection of papers published in the Special Issue on Poplar Research in Canada.

[2]Present address: Natural Resources Canada, Canadian Forest Service, Laurentian Forestry Centre, P.O. Box 10380, Stn. Sainte-Foy, Québec, QC G1V 4C7, Canada.
[3]Corresponding author (e-mail: cpc@uvic.ca).

 doi:10.1139/B07-090 

Inducible defenses against herbivory include toxins, antifeedants, and antinutrients. For example, many plants synthesize antinutritive proteinase inhibitor proteins, which inhibit insect digestive enzymes, in response to herbivory (Ryan 1990). Plant proteinase inhibitors are often present as multigene families of several nonhomologous types that inhibit all four mechanistic classes of proteinases, thereby conferring resistance against a broad range of phytophagous pests. In some species, the defense arsenal includes secondary metabolites such as alkaloids, terpenoids, glucosinolates, and phenolics (reviewed by Kessler and Baldwin 2002). These types of defenses can also be induced in roots. For example, *Brassica nigra* (L.) W.D.J. Koch challenge by cabbage maggot (*Delia radicum* (L.)) induces antifeedant glucosinolate levels in roots (van Dam and Raaijmakers 2006). In cotton, root feeding by the lined click beetle or wireworm (*Agriotes lineatus* (L.)) increases terpenoid levels in roots (Bezemer et al. 2004). Interestingly, in both cases root herbivory also induces a systemic increase in the leaves, which illustrates a root–shoot systemic defense response. Recently, indirect inducible defense was shown for maize roots, which, in response to feeding by the western corn rootworm (*Diabrotica virgifera* J.L. LeConte), release the volatile compound (*E*)-$\beta$-caryophyllene, which diffuses through the soil and attracts the entomopathogenic nematode *Heterorhabditis megidis* Poinar, Jackson, and Klein (Rasmann et al. 2005).

Similarly, induction of root defenses by leaf treatment has been observed. For example, in *Brassica rapa* L., glucosinolate levels in roots also increase after foliar methyl jasmonate (MeJa) treatment (Loivamaki et al. 2004). In *Nicotiana attenuata* Torr. ex S. Wats., simulated herbivory of leaves increases nicotine levels and proteinase inhibitor activity in roots (van Dam et al. 2001; Kessler and Baldwin 2002). This induction of defenses in roots implies the transport of a systemic defense signal from the shoot to the roots. Downward, or basipetal, systemic responses have been reported in only a few systems such as potato (Peña-Cortes et al. 1988), tobacco (Schittko and Baldwin 2003), and poplar (Jones et al. 1993). Current understanding of systemic signaling suggests that a jasmonic acid (JA) or JA derivative signal generated in response to herbivory is transported via phloem and is responsible for inducing systemic defense responses (Schilmiller and Howe 2005). However, basipetal signaling is often ignored in investigations of the systemic defense signal, which focus on upward, or acropetal, signaling. The basipetal systemic signal responsible for inducing root responses may also be JA, since leaf damage increases root JA pools and JA is directly transported from leaves to roots of tobacco (Baldwin et al. 1994; Zhang and Baldwin 1997). Moreover, foliar application of JA has been shown to increase root resistance to herbivory. For example, JA applied to leaves increases resistance against the root-feeding grape phylloxera (*Daktulosphaira vitifoliae* (Fitch)) by *Vitis vinifera* L. (Omer et al. 2000) and the root-knot nematode *Meloidogyne incognita* (Kofoid and White) Chitwood by tomato (Cooper et al. 2005).

Few studies have investigated inducible root-defense responses from a molecular perspective, although Baldwin and co-workers showed that folivore feeding generates a systemic signal that induces the expression of putrescine *N*-methyltransferase mRNA in roots of *N. attenuata* (Winz and Baldwin 2001). This gene encodes a key regulatory enzyme for nicotine biosynthesis, and its induction leads to production of nicotine in roots, which is subsequently transported to leaves. This demonstrates that plants can respond to attack by aboveground pests with active regulation of gene expression in roots, but it is not clear whether the increased nicotine levels in roots enhance their resistance to belowground pests. However, protein-based defenses have been shown to have negative effects on root pests. For example, transgenic overexpression of sporamin (a serine proteinase inhibitor) in sugar beet and of oryzacystatin (a cysteine proteinase inhibitor) in *Arabidopsis thaliana* (L.) Heynh. increases its resistance to nematodes (Urwin et al. 2000; Cai et al. 2003).

In poplars, established inducible defense genes include Kunitz-type trypsin inhibitors, chitinases, and polyphenol oxidase (Parsons et al. 1989; Davis et al. 1991*a*; Constabel et al. 2000; Lawrence and Novak 2001; Wang and Constabel 2004; Lawrence and Novak 2006). The list of known herbivory-inducible poplar genes has expanded rapidly as a result of transcript profiling studies (Christopher et al. 2004; Lawrence et al. 2006; Major and Constabel 2006; Ralph et al. 2006), which highlight the application of genomics to the analysis of inducible defense responses. We recently employed macroarrays to profile the transcriptional changes in hybrid poplar (*Populus trichocarpa* Torr. & A. Gray × *Populus deltoides* Bartr. ex Marsh.) triggered by wounding or by an insect-elicitor in regurgitant of forest tent caterpillar (*Malacosoma disstria* Hubner) (Major and Constabel 2006). Comparison of local and systemic leaf responses indicated extensive overlap, and we suggested that a strong systemic response would ensure induced resistance in undamaged leaves and reduce overall damage by feeding insects. Here we extend the study of systemic defense induction in poplar with an investigation of shoot–root systemic signaling. We present evidence for a basipetal systemic defense signal and a systemically induced root response, and compare and contrast the responses in leaves and roots.

## 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, was propagated from greenwood cuttings in Sunshine Mix No. 4 (Sungro®, Seba Beach, Alta.) in 0.25 L propagation containers (Root-Maker®, Huntsville, Ala.). After plantlets had rooted and reached a height of approximately 10 cm, they were transplanted to 15 cm diameter pots containing Sunshine Mix No. 4 plus slow-release nutrients (8.9 g·L$^{-1}$ controlled-release N–P–K 8–6–12 plus micronutrients (Acer, Delta, B.C.), 0.458 g·L$^{-1}$ superphosphate 0–20–0 (Green Valley, Surrey, B.C.), 1.21 g·L$^{-1}$ Micromax Micronutrients (Scotts-Sierra, Marysville, Ohio), and 4.75 g·L$^{-1}$ dolomite lime (IMASCO, Surrey, B.C.)). Plants were maintained in the Bev Glover Greenhouse at the University of Victoria. Supplemental lighting from 600 W high-pressure sodium lamps (P.L. Light Systems Inc, Beamsville, Ont.) was used to ex-

tend the photoperiod to 16 h light : 8 h dark, and the temperature within the greenhouse was maintained at 25°C (day) or 18 °C (night). Plants were watered daily with a solution containing 0.1 g·L$^{-1}$ 20–20–20 PlantProd® fertilizer (Plant Products, Brampton, Ont.). All lateral shoots were pruned as they developed so that each plant consisted of a single main stem no less than 2 weeks prior to experiments.

### MeJa and wounding treatments

Plants were 12 weeks old and 1 m tall with approximately 30 leaves when used for experiments. MeJa (Bedoukian Research, Danbury, Conn.) was diluted 1:10 with 95% (*v*/*v*) ethanol and then rediluted 1:500 with water – 0.1% (*v*/*v*) Triton X-100 for leaf treatment (final MeJa concentration 820 μmol·L$^{-1}$). For these treatments, untreated and mock-treated (solution without MeJa) saplings were both compared with MeJa-treated saplings. Shoots were treated by spraying leaves to the point of runoff three times at 1 h intervals. For root treatments, the MeJa solution without surfactant was added to standard fertilizer solution in a 1:5000 dilution (82 μmol·L$^{-1}$ final concentration). Saplings watered with this solution were compared with controls plants watered with fertilizer solution without MeJa. In other plants, similar JA treatment of shoots and roots is reported to induce responses in roots (Baldwin et al. 1994; van Dam et al. 2004; Cooper et al. 2005). For mechanical wounding, leaf margins were wounded by crushing with pliers three times at 1 h intervals. We have shown that this wounding method elicits a strong defense response (Major and Constabel 2006). Leaves were numbered using the leaf plastochron index (LPI) (Larson and Isebrands 1971); the index leaf (LPI 0) is defined as the first developing leaf with a lamina length ≥20 mm. For studying transcriptional changes, leaves of LPI 9–17 were wounded and tissue was collected 24 h after the start of treatment. For studying changes in protein levels and activity, all unfolded leaves (LPI >0) were wounded and tissue was collected 4 d after the start of treatment. Unless otherwise indicated, root samples consisted of the root crown (main root, sampled <10 cm from the soil surface). Immediately after harvesting, tissues were frozen in liquid nitrogen and stored at –80 °C until analyzed.

### RNA extraction and hybridization

Total RNA was isolated from hybrid poplar leaves and quantified by UV absorbance, and quality was verified on ethidium bromide (EtBr)-stained agarose gels as previously described (Haruta et al. 2001). RNA (10 μg per lane) was loaded onto 1.2% (*w*/*v*) agarose-formaldehyde gels, and blotted overnight onto Hybond-N$^+$ nylon membranes (GE Healthcare Bio-Sciences Inc. (hereinafter GE Healthcare), Baie d'Urfé, Que.). RNA blots were probed with cDNA clones labeled with [$\alpha$-$^{32}$P]dCTP (Rediprime II kit, GE Healthcare). Hybridizations were performed at 65 °C and washed at high stringency according to Church and Gilbert (1984). The blots were detected with a Storm PhosphorImager® (GE Healthcare) and signal intensities were quantified using ImageQuant® (GE Healthcare Bio-Sciences Inc.). EtBr staining of RNA was used to verify equal loading of lanes, and EtBr-stained 25S rRNA bands were used to normalize quantified signal intensities.

### Protein extraction, Western blot detection, and trypsin-inhibitor activity assays

Protein was extracted with $Na_2HPO_4$ buffer (100 mmol·L$^{-1}$, pH 7.0) containing 0.1% (*v*/*v*) Triton X-100, 5% (*w*/*v*) polyvinylpolypyrrolidone, and 1% (*v*/*v*) 2-mercaptoethanol. Extracts were clarified by centrifugation, and soluble protein was quantified using the method of Bradford (1976). For Western blotting, proteins were separated by SDS–PAGE and electrotransferred onto polyvinylidene difluoride membranes (Fisher Canada, Nepean, Ont.), and ponceau S (Sigma-Aldritch, St. Louis, Mo.) staining was used to verify equal loading and transfer efficiency. Western-blot detection was carried out using polyclonal antibodies raised against poplar VSP WIN4, PtdTI3, and PtdPop3-like protein (Lawrence et al. 1997; Major and Constabel 2007*b*). Immunocomplexes were detected using acid phosphatase-conjugated or horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, Calif.) and blots were developed colourimetrically with the reagents 5-bromo-4-chloro-3-indoyl phosphate (Pierce Biotechnology, Fisher Canada, Nepean, Ont.) and nitroblue tetrazolium chloride (Pierce; acid phosphatase), or 3,3′-diaminobenzidine tetrahydrochloride (Sigma; horseradish peroxidase). Root protein accumulation was quantified from six replicate leaf-wounding experiments (six control saplings paired with six wounded saplings) using ImageQuant® (GE Healthcare Bio-Sciences Inc.) to detect bands from blots scanned at 300 dpi (Hewlett–Packard Scanjet® 3670), and the ratio of signal intensities of leaf-wounded to control extracts was used to calculate average fold induction. Trypsin-inhibitor activity was determined by titrating crude protein extracts with a standard quantity of bovine trypsin (Sigma), and measuring residual trypsin activity as the change in $A_{247}$/min due to cleavage of the trypsin substrate TAME (*p*-toluene-sulfonyl-L-arginine methyl ester (Sigma)) as described (Worthington 1988). Proteinase-inhibitor activity of root extracts was determined against trypsin, since leaf extracts and recombinant *PtdTI3* have strong trypsin-inhibitor activity (Major and Constabel 2007*b*). For analysis of these assays, percent inhibition of trypsin was plotted against the square of the amount of root protein (μg) in each assay. For statistical comparison of trypsin-inhibitor activity in roots in control and wounding leaves, we compared slopes (percent inhibition/μg protein extract) from linear-regression analysis and calculated a *P* value (two-tailed) testing the null hypothesis that the slopes are identical (trypsin-inhibitor activity in control and wounded leaves is equal).

### Macroarray analysis

Macroarray analysis was performed as described previously (Major and Constabel 2006). Macroarrays were constructed from 580 cDNA inserts generated from leaves of hybrid poplar saplings that were systemically wounded (569 cDNAs; Christopher et al. 2004) or challenged by forest tent caterpillar (11 cDNAs; J.J. Patton and C.P. Constabel, unpublished data). Briefly, for array analysis, total RNA was isolated from roots of three independent biological replicates for wounding and MeJa treatments, as well as the corresponding paired controls for each. Leaf gene expression from this experiment had been previously analyzed (Major

and Constabel 2006, 2007*a*), and the data for the local response of wounded or MeJa-treated plants are used to compare with root responses. Each replicate was analyzed on an individual macroarray. Macroarrays were hybridized with $^{33}$P-labeled target cDNA at 65 °C and washed at high stringency according to Church and Gilbert (1984). Exposed PhosphoImager® screens were scanned with a Storm PhosphoImager® (GE Healthcare) and the signals were quantified using ArrayVision® 7.0 (Imaging Research, St. Catherines, Ontario, Canada). Background-corrected spot intensities were normalized to the standard deviation of the entire array (Richmond and Somerville 2000). Normalized intensities from the three biological replicates were used to calculate average expression ratios, and a Student's *t* test (paired, two-tailed) of $\log_2$-transformed data was used to determine statistical significance. *Q* values were calculated using R (www.r-project.org Ihaka and Gentleman 1996; Storey and Tibshirani 2003). Because the macroarrays were constructed with cDNAs derived from leaves and not all genes are expected to be expressed in roots, we filtered out genes with very low signal strength. We determined a threshold for reliable expression at 35% of the average transcript abundance of control roots. Thus, genes *i* with normalized signal intensity $SI_i < 0.35 \times \overline{SI}$ were removed, where $\overline{SI}$ is the mean normalized signal intensity of all genes in control roots. This threshold was selected to maximize removal of genes with functions in photosynthesis (Christopher et al. 2004) that are clearly not expressed in non-photosynthetic tissues such as roots, while minimizing the removal of genes that were significantly up-regulated. To corroborate that the genes we excluded were indeed not expressed in roots, we queried all databases containing poplar expressed sequence tags (ESTs) generated from root tissues, including PopulusDB (poppel.fysbot.umu.se; Sterky et al. 2004), PoplarDB (mycor.nancy.inra.fr/PoplarDB/index.html; Kohler et al. 2003), and The Gene Index Databases (Quackenbush et al. 2001). This in-silico analysis suggested that our threshold is conservative and that some root-expressed genes were likely excluded. However, our threshold greatly reduced the proportion of false positives (genes identified as induced by the array but without root expression support in the databases); considering that the composition of the arrays is biased towards leaf-expressed genes, reducing false positives was a priority. To create a non-redundant set of genes induced by our treatments in roots, we queried the Joint Genome Institute (JGI) poplar genome database (genome.jgi-psf.org/Poptr1/Poptr1.home.html) and obtained full-length sequences and JGI accessions for each candidate gene.

## Results

### MeJa induces acropetal and basipetal shoot–root signaling

Our previous work on poplar leaves demonstrated that simulated herbivory by means of mechanical wounding or application of insect regurgitant from forest tent caterpillar induced strong transcriptional changes in both treated and untreated (systemic) leaves on treated plants (Major and Constabel 2006). To investigate the possibility of systemic defense signaling between shoots and roots, we treated poplar shoots or roots with MeJa in experiments designed to test reciprocal induction. Shoots were treated by spraying leaves three times with MeJa at 1 h intervals, and roots were treated by supplementing water with MeJa over a 24 h period. Both plant organs were then assayed for an inducible defense response by monitoring expression of poplar trypsin inhibitor 3 (*PtdTI3*), since we have previously shown it to be an excellent defense marker (Major and Constabel 2006) and to be a functional proteinase inhibitor with anti-insect properties (Major and Constabel 2007*b*). MeJa applied to roots by irrigation resulted in a strong induction of *PtdTI3* mRNA in roots, and a moderate response in leaves (Figs. 1A and 1B); the average changes in the roots and leaves from four replicate experiments were 43- and 6.6-fold, respectively. This systemic response implies the movement of an acropetal (upwardly mobile) systemic defense signal. Likewise, application of MeJa to shoots induced *PtdTI3* mRNA not only in the leaves but also in the roots (Figs. 1C and 1D). In this case, leaf and root tissues responded to shoot treatment with equal up-regulation, since the average changes in the leaves and roots from three replicate experiments were 9.4- and 8.9-fold, respectively, compared with untreated controls. A small part of this induction may have been due to the surfactant used in the leaf spray (see mock spray; Figs. 1C and 1D); however, this does not detract from the key result that an elicitor treatment of leaves strongly effects defense gene expression in roots, demonstrating a basipetal (downward) systemic defense signal.

### Mechanical wounding induces basipetal shoot–root signaling

Since MeJa applied exogenously to plants can be hydrolyzed to JA, which may be transported to leaves and roots (Zhang and Baldwin 1997), it is not clear whether MeJa application induces a de novo systemic response. To determine if bidirectional systemic defense signaling can be induced in the absence of exogenous MeJa application, we wounded leaves of LPI 9–17 with pliers and measured *PtdTI3* mRNA-levels in unwounded tissues above and below the wounded leaves using Northern blots (Fig. 2). Transcripts of *PtdTI3* were induced in the apical bud and leaves of LPI 3–5, indicating an acropetal systemic response, as was previously shown for poplar (Davis et al. 1991*b*; Constabel et al. 2000). However, transcripts of *PtdTI3* were also clearly induced below the wounded region in leaves of LPI 18–20 and 27–29. Furthermore, *PtdTI3* transcripts were induced in roots, albeit at a low level. This experiment showed that, at least as seen for accumulation of *PtdTI3* mRNA, simulated herbivory elicits a defense response basipetal to the damaged region, and confirms our previous findings with MeJa treatment (Fig. 1). Moreover, the results suggested that mechanical wounding of leaves, a useful proxy for herbivory in poplar, is capable of inducing a defense response in poplar roots.

### Wounding of leaves induces levels of PtdTI3 mRNA, protein and trypsin-inhibitor activity in roots

To confirm that a defense response is induced in roots of wounded plants (Fig. 2), we conducted more wounding experiments and used additional techniques to analyze defense responses in roots. To measure transcript levels, we repli-

**Fig. 1.** Accumulation of *PtdTI3* mRNA in leaves of plants treated with methyl jasmonate (MeJa) applied to the roots by irrigation (A and B) or to the shoots by spraying leaves (C and D). Leaves with a leaf plastochron index (LPI) of 9–11 and mature roots were harvested 24 h after start of MeJa treatment and analyzed by Northern-blot analysis. Experiments were replicated 4 times for MeJa applied to roots and 3 times for MeJa applied to shoots. A and C show representative Northern blots. Transcript abundance from Northern blots was quantified and normalized to levels of 25S rRNA from ethidium bromide (EtBr) staining (B and D). Open bars denote untreated control plants, hatched bars mock-treated control plants (B and D only), and shaded bars MeJa-treated trees. Error bars are standard deviation.

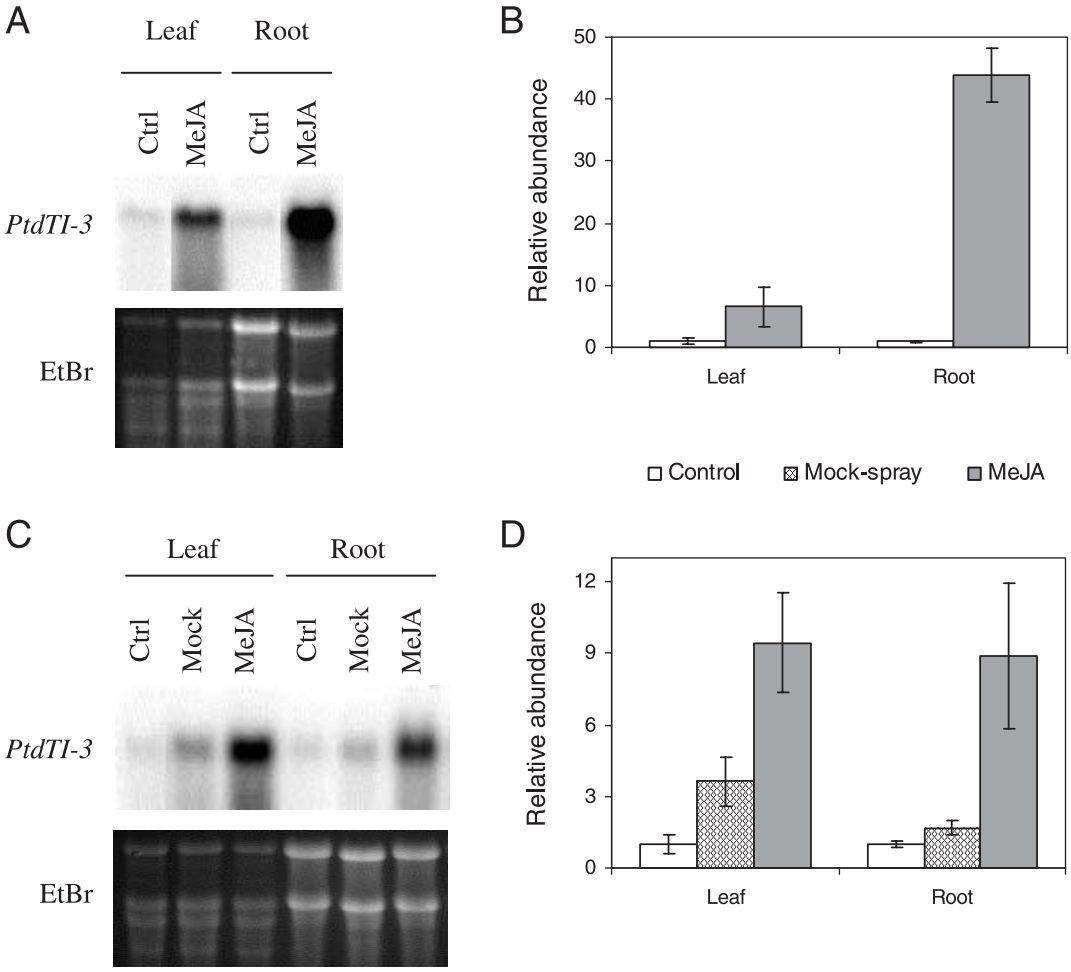



**Fig. 2.** Accumulation of *PtdTI3* mRNA in tissues of plants wounded with pliers. Leaves of LPI 9–17 were wounded, and 24 h after the start of treatment, tissues were harvested and analyzed by Northern-blot analysis. AB, shoot apex; L3, leaves of LPI 3–5; L9, leaves of LPI 9–11; L18, leaves of LPI 18–20; L27, leaves of LPI 27–29; R1, main root (root <10 cm from surface); R2, roots <5 cm from main root; R3, root 10–20 cm from main root; R4, peripheral roots (root <10 cm from root tip). EtBr staining is shown as a loading control.

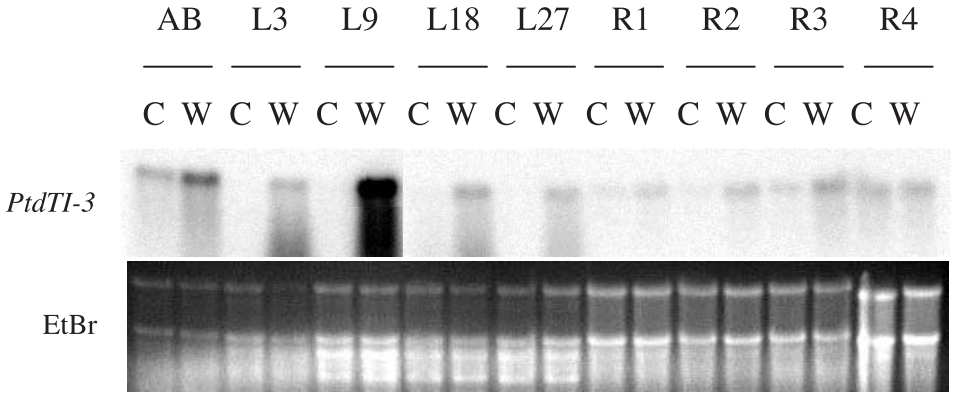
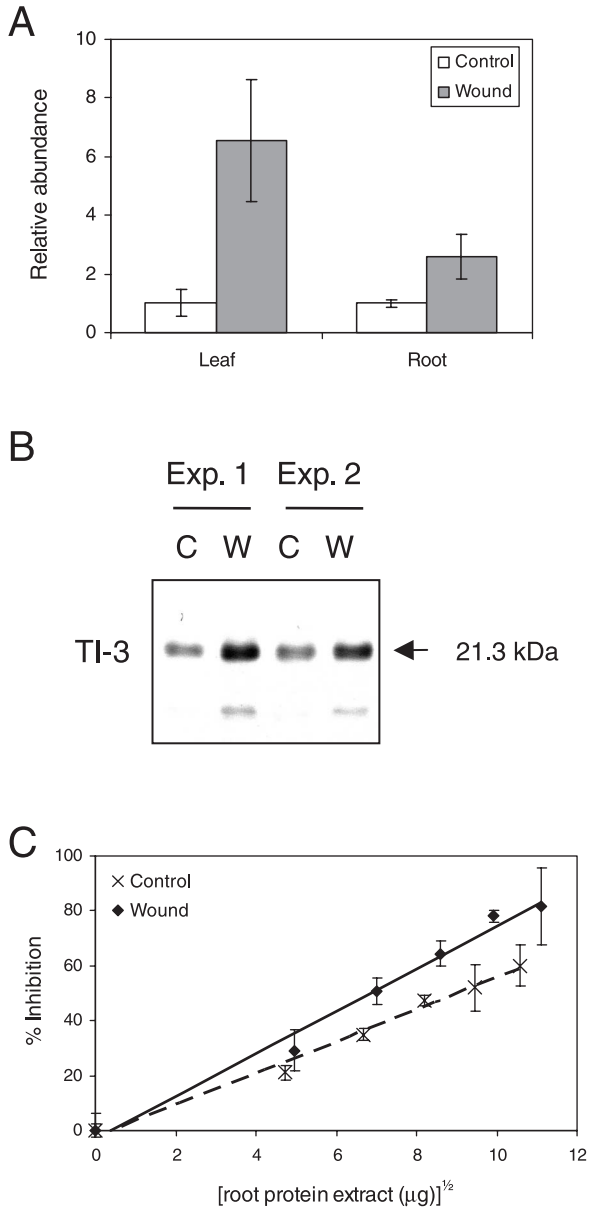


**Fig. 3.** Induction of trypsin inhibitor in roots of plier-wounded trees. Leaves of LPI 9–17 (A) or all unfolded leaves (LPI ≥ 1) (B and C) were wounded with pliers, and leaves of LPI 9–11 and mature roots were harvested 24 h (A) or 4 d (B and C) after the start of treatment. (A) Accumulation of PtdTI3 mRNA was quantified from Northern blots and normalized to levels of 25S rRNA from EtBr staining. Open bars denote untreated control trees and shaded bars wounded trees. The experiment was replicated 8 times, and error bars indicate standard deviation. (B) Western blot of wound-induced accumulation of PtdTI3 protein. Two distinct experiments are shown ("C" is control; "W" is wounded). (C) Inhibitory activity of poplar-root extracts against trypsin. Increasing amounts of crude extract from unwounded control and wounded saplings were analyzed with a constant amount of trypsin. Linear-regression analysis revealed that the difference in inhibitory activity between extracts from control and wounded saplings is significant ($P = 0.002$). Data points represent mean inhibitory activity ± standard deviation ($n$ = three technical replicates of the assay).




cated the experiment from Fig. 2 multiple times and quantified transcripts of *PtdTI3* in leaves and roots (Fig. 3A). We measured an average change in *PtdTI3* mRNA of 7.4 times in plier-damaged leaves, comparable to our previous results (Major and Constabel 2006), and a 2.7-fold change in roots. We next analyzed levels of PtdTI3 protein; for this analysis, tissues were harvested later (4 d after foliar wounding) and PtdTI3 protein levels in roots were assessed by Western-blot analysis. In six replicate experiments, PtdTI3 protein was consistently induced in roots, with an average induction of approximately double (1.76-fold; Fig. 3B). We further measured trypsin-inhibitor activity of root extracts from control and leaf-wounded saplings in a representative experiment. Using linear-regression analysis, we found that trypsin-inhibitor activity was 34% higher in roots of leaf-wounded saplings than in control saplings (Fig. 3C), and that this difference, although small, was significant ($P = 0.002$). Together, these data demonstrate that the induced defense response in roots is not restricted to changes in transcript abundance, but that the response is also translated into increased protein accumulation and inhibitory activity.

### Expression profiling reveals that some genes wound-inducible in leaves are also induced in roots

To further study the transcriptional responses of poplar roots to wounding and MeJa treatment of leaves, we conducted an analysis using cDNA macroarrays constructed previously (Major and Constabel 2006). We treated sapling leaves with MeJa or plier wounding and analyzed the response in mature roots. Gene-expression ratios were determined from a comparison of three independent control and treated replicate plants (six plants in total). Genes were considered to be differentially expressed by either wounding or MeJa treatment on the basis of a ≥2-fold change in expression (either up- or down-regulation) and significance of $P < 0.05$ as determined by a Student's $t$ test for the three replicates (Major and Constabel 2006). We previously used the same arrays and protocols to study wound- and caterpillar-regurgitant-induced responses in leaves, and in that study we rigorously tested and validated the array data using Northern-blot analysis. Since our macroarrays contained cDNAs derived from leaves, we first inspected the transcript abundance of this gene set in root samples and found that, as expected, many had extremely low signals on the arrays. We excluded these genes from the analysis by determining a threshold for reliable signal strength, which ensured that we only examined genes that are in fact expressed in roots (see Methods).

Analysis of the induced gene set indicated that, based on our defined thresholds, MeJa and wounding leaf treatments significantly up-regulated 17 and 9 genes, respectively, in roots (Table 1). We also found genes that appeared to be down-regulated, though they were far fewer. Altogether, using our criteria we identified 18 genes that were induced in roots by either MeJa or wounding treatment of leaves; of these, 8 were induced by both MeJa and wounding ($P < 0.05$). However, five additional genes induced in roots by foliar MeJa treatment were also up-regulated by wounding ($P < 0.10$), and thus there is good correspondence between root responses induced systemically by MeJa and wounding treatment of leaves. When we compared these with our pre-

**Table 1.** Effect of leaf wounding or MeJa treatment on gene expression in roots as analyzed by macroarray.

| Putative function | GenBank accession No. | JGI ID[a] | AGI accession[b] | E-value | Wound treatment | | | MeJa treatment | | | Significantly up-regulated in leaves?[f] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | FC[c] | P[d] | q[e] | FC[c] | P[d] | q[e] | |
| Endochitinase win8 | CN192595 | 694264 | At3g12500 | e-87 | 2.61 ± 0.87 | 0.036 | 0.181 | 21.35 ± 4.28 | 0.001 | 0.034 | Yes |
| Endochitinase win6.2C | CN192741 | 649163 | At3g12500 | e-100 | 4.09 ± 1.00 | 0.009 | 0.175 | 16.07 ± 0.87 | <0.001 | 0.025 | Yes |
| Kunitz-type trypsin inhibitor PtdTI5 | CN192805 | 574326 | At1g73325 | e-09 | 6.84 ± 2.52 | 0.017 | 0.180 | 7.48 ± 4.30 | 0.025 | 0.071 | Yes |
| Kunitz-type trypsin inhibitor PtdTI3 | CN192549 | 739064 | At1g73325 | e-09 | 4.36 ± 2.36 | 0.045 | 0.184 | 8.69 ± 4.31 | 0.019 | 0.069 | Yes |
| Kunitz-type trypsin inhibitor PtdTI4 | CN193330 | 555576 | At1g17860 | e-28 | 3.68 ± 1.40 | 0.041 | 0.181 | 6.47 ± 2.94 | 0.031 | 0.071 | Yes |
| Unknown protein | CN192936 | 596936 | At3g03150 | e-26 | 3.50 ± 0.53 | 0.005 | 0.175 | 5.34 ± 1.50 | 0.010 | 0.064 | Yes |
| Vegetative storage protein win4.5 | CN192930 | 571436 | At4g24340 | e-49 | 3.50 ± 0.45 | 0.003 | 0.175 | 5.13 ± 1.44 | 0.012 | 0.066 | Yes |
| Lil3 protein | CN193322 | 702880 | At4g17600 | e-72 | 1.98 ± 0.33 | 0.021 | 0.181 | 4.66 ± 0.75 | 0.004 | 0.046 | Yes |
| Acid phosphatase, class B | — | 705837 | At4g25150 | e-67 | 2.80 ± 0.98 | 0.035 | 0.181 | 3.79 ± 0.63 | 0.006 | 0.051 | Yes |
| Cytochrome P450 | CN193273 | 589289 | At5g07990 | e-100 | 3.28 ± 1.51 | 0.050 | 0.186 | 2.86 ± 0.14 | <0.001 | 0.034 | Yes |
| Poplar Pop3 / SP1 | — | 687234 | At3g17210 | e-21 | 1.86 ± 0.46 | 0.056 | 0.192 | 3.88 ± 0.26 | <0.001 | 0.034 | Yes |
| Cytochrome P450 | CN193412 | 645826 | At5g07990 | e-102 | 2.73 ± 1.23 | 0.079 | 0.220 | 2.97 ± 0.22 | 0.001 | 0.034 | Yes |
| Unknown protein, ZIM motif | CN193314 | 645806 | At1g19180 | e-31 | 2.17 ± 0.63 | 0.055 | 0.192 | 3.52 ± 0.96 | 0.016 | 0.068 | Yes |
| Apyrase | — | 573883 | At5g18280 | e-116 | 2.41 ± 0.97 | 0.060 | 0.194 | 2.82 ± 0.15 | <0.001 | 0.034 | Yes |
| Cytochrome P450 | CN193274 | 645827 | At5g07990 | e-40 | 2.28 ± 0.85 | 0.090 | 0.232 | 2.78 ± 0.74 | 0.020 | 0.069 | Yes |
| Galactinol synthase | CN192679 | 565191 | At1g60470 | e-156 | 1.06 ± 0.27 | 0.833 | 0.459 | 3.57 ± 0.69 | 0.009 | 0.064 | No |
| O-methyltransferase | CN193109 | 582793 | At4g35160 | e-15 | 2.60 ± 0.84 | 0.042 | 0.181 | 1.99 ± 0.80 | 0.153 | 0.134 | No |
| Hydroperoxide lyase | CN192806 | 688325 | At4g15440 | e-139 | 1.95 ± 0.34 | 0.025 | 0.181 | 2.61 ± 0.60 | 0.018 | 0.069 | Yes |

[a]Joint Genome Institute (JGI) protein ID No. of the JGI gene model from the *P. trichocarpa* genome (genome.jgi-sf.org/Poptr1_1/Poptr1_1.home.html) that corresponds to the expressed tag sequence (EST).
[b]Arabidopsis Genome Initiative (AGI) code with expectation value for the best match of the poplar gene (JGI gene model) to *Arabidopsis thaliana* determined by BLASTX of TAIR6 (www.arabidopsis.org/).
[c]Fold change (mean ± SD) in gene expression in roots of wounded- and MeJa-treated plants; darker shading indicates higher fold change.
[d]Significance of treated plants compared to control plants, as determined by a Student's *t*-test. To facilitate comparison of the significance of fold change in gene expression, two categories of shading proportional to *P*-values are shown: black with white text denotes $P \leq 0.05$; shading with black text denotes $P > 0.05$ but $\leq 0.1$; white with black text denotes $P > 0.1$.
[e]False discovery rate (*q*-value) was calculated using the R statistical package (www.r-project.org/) as described by Storey and Tibshirani (2003).
[f]Signficant up-regulation in leaves by wounding or MeJa treatment derived from Major and Constabel (2006, 2007*a*).

vious data of wound- and MeJa-induced gene expression in leaves (Major and Constabel 2006, 2007*a*), we found that almost all of these root-expressed genes (16) were also significantly induced in leaves. In our previous study, we reported that 163 and 108 genes were induced by MeJa and wounding, respectively (Major and Constabel 2006, 2007*a*). Thus, the set of genes induced in roots was a small subset of the genes induced in leaves; this is not surprising, given that the array is composed of leaf-expressed genes. Closer inspection of the genes induced in both roots and leaves further highlighted similarities between leaf and root responses. The most strongly root-induced genes on the array encode known leaf defense-related poplar genes, including trypsin inhibitors, endochitinases, and the vegetative storage protein (VSP) *win4.5* (Table 1, upper portion). Furthermore, several genes that we previously reported as strongly induced in leaves were also up-regulated in roots, including apyrase, acid phosphatase, proteins of unknown function (CN192936 and CN193314), and several P450 cytochromes (Major and Constabel 2006). In addition, we found one gene (an *O*-methyl transferase) that was not inducible in leaves, yet was significantly induced in roots by leaf-wounding but not by MeJa treatment; however, this gene showed only modest transcript increases (Table 1). We also found that one gene (galactinol synthase) was significantly induced in roots by foliar MeJa treatment but not by wounding, and was also non-inducible in leaves. We note that MeJa is involved in other processes in addition to defense, and thus systemic up-regulation of this gene may be unrelated to defense. An in-silico analysis of public EST databases independently confirmed that these two genes are in fact expressed in roots (see supplementary data[4], Table S1), so they indicate potential root-specific responses.

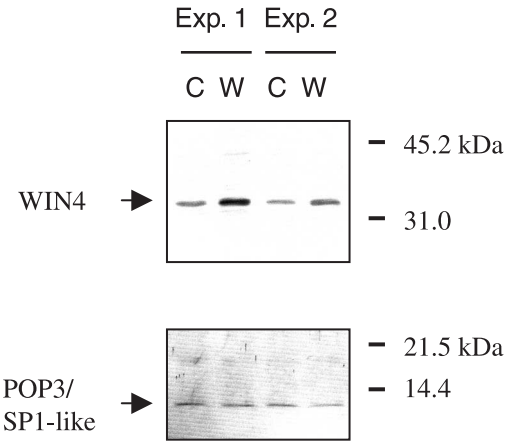
### Many genes strongly induced in leaves are not expressed in roots

We found that many genes that are strongly induced in leaves were not at all up-regulated in the roots or were expressed below our threshold for reliable expression. This list includes polyphenol oxidase, *PtdPPO1* (GenBank accession No. CN193334; JGI protein ID 568791), class 3 lipase (CN192786; 713858), VSP *pni288* (CN193425; 573862), 13-lipoxygenase (CN192531), acyl-activating enzyme (CN192663; 556023), $\beta$-amylase (CN192760; 679498), and *PtdPop3-/SP1-like* (723971), all of which we have previously shown to be among the genes most strongly induced in leaves (Major and Constabel 2006). The inclusion of polyphenol oxidase and class 3 lipase in this list is especially interesting, since these genes have the highest inductions in leaves, but were only marginally up-regulated in roots, and only after foliar MeJa treatment. Our data therefore suggest that some defense genes are specifically expressed in leaves, while others are induced in both leaves and roots.

### Defense protein levels in roots of leaf-wounded saplings correlate with gene expression

We used Western-blot analysis to validate our results and to confirm that the transcriptional changes in roots also translate into increases in the corresponding proteins. Our macroarray analysis had indicated that, like *PtdTI3*, VSP *win4.5* was up-regulated in roots, while *PtdPPO1* and *PtdPop3-/SP1-like* exhibited no change in expression (Table 1 and data not shown). We used available polyclonal antibodies raised against defense proteins to detect changes in protein levels in roots 4 d after leaf wounding. Western-blot analysis indicated that VSP WIN4.5 protein levels increased after leaf wounding in multiple experiments (Fig. 4), with average protein induction of 1.7-fold. This compared well with the 3.50-fold increase in transcript levels detected by macroarray analysis. By contrast, the PtdPop3-/SP1-like protein was present in roots, but its levels did not increase after leaf wounding (Fig. 4). This is also consistent with the small observed fold increase in transcript abundance. We also found that the PtdPPO1 protein was not present in roots (data not shown), which is again consistent with *PtdPPO1* mRNA levels below the threshold of detection on the macroarray. Therefore, these defense protein levels all corresponded well to the pattern of transcripts detected by macroarray analysis.

**Fig. 4.** Accumulation of WIN4 and Pop3-like proteins in roots of wounded trees in two separate experiments. Leaves of the entire tree were wounded with pliers and mature roots were harvested 4 d after the start of treatment and analyzed by Western blot.




## Discussion

Our results clearly demonstrate that a defense response is induced in poplar roots by simulating herbivory of leaves. Treatment of poplar sapling shoots with MeJa or mechanical wounding with pliers resulted in bidirectional systemic signaling, including the systemic induction of defense genes in roots. The response of roots to foliar wounding was observable at mRNA, protein, and activity levels. Comparative macroarray analyses indicated some similarity between the responses of leaves and roots after foliar wounding and MeJa treatment, as many genes induced in roots are poplar defense genes first described from leaves. However, some genes strongly induced in leaves were not expressed in roots. To our knowledge, this is the first application of tran-

[4] Supplementary data for this article are available on the journal Web site (http://cjb.nrc.ca) or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Building M-55, 1200 Montreal Road, Ottawa, ON K1A 0R6, Canada. DUD 5209. For more information on obtaining material refer to http://cisti-icist.nrc-cnrc.gc.ca/irm/unpub_e.shtml.

script profiling to study a systemic defense response in roots after leaf damage.

Our findings indicate that a systemic wound signal moves downwards from the shoot into the root. Since JA is known to induce defense in an acropetal fashion in tomato and other plants, it is also likely that JA or a JA derivative is transported from leaves to roots and is therefore responsible for the basipetal systemic defense response. In tobacco, radiolabeled JA applied to leaves has been shown to be translocated to roots (Zhang and Baldwin 1997). In general, the movement of the systemic signal is governed by vasculature and source–sink relations (Davis et al. 1991*b*; Jones et al. 1993; Orians et al. 2000; Schittko and Baldwin 2003). Interestingly, a recent study of *N. attenuata* showed that simulated foliar herbivory elicits rapid changes in source–sink relations, increasing the transport of photoassimilate to roots (Schwachtje et al. 2006). A similar response has also been observed for *Populus* spp., as foliar treatment with JA increases carbon allocation to roots (Babst et al. 2005). An increase in root sink strength would thus predict that a systemic signal moves basipetally into the root, although in this situation it is unclear if the response in roots is an artifact of source–sink shifts or a defense response per se. Source–sink relations may explain why we observed stronger acropetal than basipetal systemic responses because, in the young saplings we used, immature tissues above the wounded region are expected to be stronger sinks than the mature leaves and roots (Scarascia-Mugnozza et al. 1999). Irrespective of possible mechanisms, our data provide strong evidence for bidirectional systemic signaling in poplar. This was previously shown for potato, tobacco, and poplar (Peña-Cortes et al. 1988; Jones et al. 1993; Schittko and Baldwin 2003), although Davis et al. (1991*b*) did not observe basipetal induction of *win3*, another Kunitz-type trypsin inhibitor in poplar. This discrepancy is likely due to differences in the behaviour of the marker genes used, since wound-induced accumulation of *win3* transcript occurs primarily in young leaves (J.J. Patton and C.P. Constabel, unpublished data). By contrast, our marker for the inducible systemic response, *PtdTI3*, is inducible in both young and mature tissues.

The adaptive value of a defense response in roots of poplar after foliar wounding is not clear. The induced genes may constitute a general defensive response, and induction of defense genes in roots is likely to enhance resistance to belowground herbivory. Several genes we found to be up-regulated in roots are predicted to have negative effects on root pests. For example, the two genes most strongly induced in roots are endochitinases, one of which was recently shown to directly inhibit insect development (Lawrence and Novak 2006). In roots of *Citrus* species that are attacked by citrus root weevil (*Diaprepes abbreviatus* (L.)), endochitinase is an induced defense and root extracts degrade the peritrophic matrix of weevil larvae (Mayer et al. 1995). Our experiments also identified three Kunitz-type trypsin inhibitors as being strongly induced in roots. In tomato, infection and root wounding by the root-knot nematode *Meloidogyne javanica* (Treub) Chitwood increase transcript and protein levels of a Kunitz-type trypsin inhibitor in roots (Brenner et al. 1998). In sugar beet, resistance to sugarbeet cyst nematode (*Heterodera schachtii* Schmidt) is enhanced by transgenic overexpression of a Kunitz-type trypsin inhibitor (Cai et al. 2003), and thus it is likely that Kunitz-type trypsin inhibitor enhance resistance against belowground herbivores. In poplar, *PtdTI3*, in particular, is among the genes most strongly induced in leaves, and is expressed in almost all plant tissues. Recombinant PtdTI3 protein inhibits a range of proteases and is an effective inhibitor of larval midgut proteases of *M. disstria* and *Mamestra configurata* Walker (Major and Constabel 2007*b*). These root-induced poplar defense genes are therefore likely to participate in resistance to belowground herbivory and provide tantalizing clues for future experiments.

Compared with the acropetal induction of *PtdTI3* mRNA after leaf wounding, we observed only modest basipetal induction. While MeJa elicited stronger basipetal induction, we cannot exclude the possibility that the exogenous MeJa was translocated to roots (Zhang and Baldwin 1997) rather than triggering a systemic defense response. In addition, the basipetal systemic defense response may be stronger at other time points, and our analyses were only carried out at a single time point (24 h for analysis of transcript levels and 4 d for protein levels and activity). Genes with transient or delayed expression patterns have been shown in studies of inducible root defense (Alkharouf et al. 2006), and would have escaped detection by our array analysis. Furthermore, the composition of our arrays limited our analysis, since they were constructed with ESTs generated from a leaf cDNA library. This was reflected by the small number of genes up-regulated in roots compared with that in leaves. To improve the confidence of our array expression results, we performed an in-silico validation of the array results using publicly available root EST databases to independently confirm the expression of genes marked as root-expressed on the array. Among the 23 genes that were induced in roots, only 5 were not represented at least once by a root-derived EST (see supplementary data[4], Table S1). Thus the majority of array elements identified by our analysis do correspond to genes expressed in roots.

While defense responses in roots have not received as much attention as responses in shoots, herbivore-mediated interactions between below- and above-ground plant organs are known (reviewed by Blossey and Hunt-Joshi 2003; van Dam et al. 2003). However, few studies have investigated these inducible responses from a molecular perspective. We have used a molecular approach to describe basipetal systemic defense signaling in poplar. Poplar roots not only generate a systemic signal, but also respond to systemic signals generated by simulated herbivory of leaves. Moreover, we have shown that this defense response in roots is manifested at the mRNA and protein levels. Our use of transcript profiling has provided preliminary evidence that the transcriptional changes that occur as part of a root defense response has some overlap with the leaf defense response. The use of whole genome arrays or arrays with root-derived ESTs should provide a better understanding of the induced root response.

## Acknowledgements

The authors thank Dr. John Davis for providing the poplar WIN4.5 antibody; Eric Bol, Anna Isbister, Lan Tran, and Nicole Dafoe for help with recombinant proteins and anti-

body tests; and Brad Binges for maintaining trees at the Bev Glover Greenhouse. This work was supported by the Natural Sciences and Engineering Research Council of Canada in the form of postgraduate scholarships to I.T.M. and grant support to C.P.C., and the University of Victoria's Centre for Forest Biology.

## References

Alkharouf, N.W., Klink, V.P., Chouikha, I.B., Beard, H.S., MacDonald, M.H., Meyer, S., Knap, H.T., Khan, R., and Matthews, B.F. 2006. Timecourse microarray analyses reveal global changes in gene expression of susceptible *Glycine max* (soybean) roots during infection by *Heterodera glycines* (soybean cyst nematode). Planta (Berl.), **224**: 838–852. doi:10.1007/s00425-006-0270-8. PMID:16575592.

Babst, B.A., Ferrieri, R.A., Gray, D.W., Lerdau, M., Schlyer, D.J., Schueller, M., Thorpe, M.R., and Orians, C.M. 2005. Jasmonic acid induces rapid changes in carbon transport and partitioning in *Populus*. New Phytol. **167**: 63–72. doi:10.1111/j.1469-8137.2005.01388.x. PMID:15948830.

Baldwin, I.T., Schmelz, E.A., and Ohnmeiss, T.E. 1994. Wound-induced changes in root and shoot jasmonic acid pools correlate with induced nicotine synthesis in *Nicotiana sylvestris* Spegazzini and Comes. J. Chem. Ecol. **20**: 2139–2157. doi:10.1007/BF02066250.

Blossey, B., and Hunt-Joshi, T.R. 2003. Belowground herbivory by insects: influence on plants and aboveground herbivores. Annu. Rev. Entomol. **48**: 521–547. doi:10.1146/annurev.ento.48.091801.112700. PMID:12414738.

Bezemer, T.M., Wagenaar, R., van Dam, N.M., Van Der Putten, W.H., and Wackers, F.L. 2004. Above- and below-ground terpenoid aldehyde induction in cotton, *Gossypium herbaceum*, following root and leaf injury. J. Chem. Ecol. **30**: 53–67. doi:10.1023/B:JOEC.0000013182.50662.2a. PMID:15074657.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. **72**: 248–254. doi:10.1016/0003-2697(76)90527-3. PMID:942051.

Brenner, E.D., Lambert, K.N., Kaloshian, I., and Williamson, V.M. 1998. Characterization of *LeMir*, a root-knot nematode-induced gene in tomato with an encoded product secreted from the root. Plant Physiol. **118**: 237–247. doi:10.1104/pp.118.1.237. PMID:9733543.

Cai, D., Thurau, T., Tian, Y., Lange, T., Yeh, K.W., and Jung, C. 2003. Sporamin-mediated resistance to beet cyst nematodes (*Heterodera schachtii* Schm.) is dependent on trypsin inhibitory activity in sugar beet (*Beta vulgaris* L.) hairy roots. Plant Mol. Biol. **51**: 839–849. doi:10.1023/A:1023089017906. PMID:12777044.

Christopher, M.E., Miranda, M., Major, I.T., and Constabel, C.P. 2004. Gene expression profiling of systemically wound-induced defenses in hybrid poplar. Planta (Berl.), **219**: 936–947. doi:10.1007/s00425-004-1297-3. PMID:15605173.

Church, G.M., and Gilbert, W. 1984. Genomic sequencing. Proc. Natl. Acad. Sci. U.S.A. **81**: 1991–1995. doi:10.1073/pnas.81.7.1991. PMID:6326095.

Constabel, C.P., Yip, L., Patton, J.J., and Christopher, M.E. 2000. Polyphenol oxidase from hybrid poplar: cloning and expression in response to wounding and herbivory. Plant Physiol. **124**: 285–295. doi:10.1104/pp.124.1.285. PMID:10982443.

Cooper, W.R., Jia, L., and Goggin, L. 2005. Effects of jasmonate-induced defenses on root-knot nematode infection of resistant and susceptible tomato cultivars. J. Chem. Ecol. **31**: 1953–1967. doi:10.1007/s10886-005-6070-y. PMID:16132206.

Davis, J.M., Clarke, H.R., Bradshaw, H.D., Jr., and Gordon, M.P. 1991*a*. *Populus* chitinase genes: structure, organization, and similarity of translated sequences to herbaceous plant chitinases. Plant Mol. Biol. **17**: 631–639. doi:10.1007/BF00037049. PMID:1912489.

Davis, J.M., Gordon, M.P., and Smit, B.A. 1991*b*. Assimilate movement dictates remote sites of wound-induced gene expression in poplar leaves. Proc. Natl. Acad. Sci. U.S.A. **88**: 2393–2396. doi:10.1073/pnas.88.6.2393. PMID:11607168.

Haruta, M., Major, I.T., Christopher, M.E., Patton, J.J., and Constabel, C.P. 2001. A Kunitz trypsin inhibitor gene family from trembling aspen (*Populus tremuloides* Michx.): cloning, functional expression, and induction by wounding and herbivory. Plant Mol. Biol. **46**: 347–359. doi:10.1023/A:1010654711619. PMID:11488481.

Ihaka, R., and Gentleman, R. 1996. R: a language for data analysis and graphics. J. Comput. Graph. Stat. **5**: 299–314.

Jones, C.G., Hopper, R.F., Coleman, J.S., and Krischik, V.A. 1993. Control of systemically induced herbivore resistance by plant vascular architecture. Oecologia (Berl.), **93**: 452–456. doi:10.1007/BF00317892.

Kessler, A., and Baldwin, I.T. 2002. Plant responses to insect herbivory: the emerging molecular analysis. Annu. Rev. Plant Biol. **53**: 299–328. doi:10.1146/annurev.arplant.53.100301.135207. PMID:12221978.

Kohler, A., Delaruelle, C., Martin, D., Encelot, N., and Martin, F. 2003. The poplar root transcriptome: analysis of 7000 expressed sequence tags. FEBS Lett. **542**: 37–41. doi:10.1016/S0014-5793(03)00334-X. PMID:12729894.

Larson, P.R., and Isebrands, J.G. 1971. The plastochron index as applied to developmental studies of cottonwood. Can. J. For. Res. **1**: 1–11. doi:10.1139/x71-001.

Lawrence, S.D., and Novak, N.G. 2001. A rapid method for the production and characterization of recombinant insecticidal proteins in plants. Mol. Breed. **8**: 139–146. doi:10.1023/A:1013356724295.

Lawrence, S.D., and Novak, N.G. 2006. Expression of poplar chitinase in tomato leads to inhibition of development in colorado potato beetle. Biotechnol. Lett. **28**: 593–599. doi:10.1007/s10529-006-0022-7. PMID:16614898.

Lawrence, S.D., Greenwood, J.S., Korhnak, T.E., and Davis, J.M. 1997. A vegetative storage protein homolog is expressed in the growing shoot apex of hybrid poplar. Planta (Berl.), **203**: 237–244. doi:10.1007/s004250050187. PMID:9362569.

Lawrence, S.D., Dervinis, C., Novak, N., and Davis, J.M. 2006. Wound and insect herbivory responsive genes in poplar. Biotechnol. Lett. **28**: 1493–1501. doi:10.1007/s10529-006-9119-2. PMID:16955355.

Loivamaki, M., Holopainen, J.K., and Nerg, A.-M. 2004. Chemical changes induced by methyl jasmonate in oilseed rape grown in the laboratory and in the field. J. Agric. Food Chem. **52**: 7607–7613. doi:10.1021/jf049027i. PMID:15675811.

Major, I.T., and Constabel, C.P. 2006. Molecular analysis of poplar defense against herbivory: comparison of wound- and insect elicitor-induced gene expression. New Phytol. **172**: 617–635. doi:10.1111/j.1469-8137.2006.01877.x. PMID:17096789.

Major, I.T., and Constabel, C.P. 2007*a*. Insect regurgitant and wounding elicit similar defense responses in poplar leaves: Not something to spit at? Plant Signal. Behav. **2**: 1–3.

Major, I.T. and Constabel, C.P. 2007*b*. Functional analysis of the Kunitz trypsin inhibitor family in poplar reveals biochemical diversity and multiplicity in defense against herbivores. Plant Physiol. In press.

Mayer, R.T., Shapiro, J.P., Berdis, E., Hearn, C.J., McCollum,

T.G., McDonald, R.E., and Doostdar, H. 1995. Citrus rootstock responses to herbivory by larvae of the sugarcane rootstock borer weevil (*Diaprepes abbreviatus*). Physiol. Plant. **94**: 164–173. doi:10.1111/j.1399-3054.1995.tb00798.x.

Omer, A.D., Thaler, J.S., Granett, J., and Karban, R. 2000. Jasmonic acid induced resistance in grapevines to a root and leaf feeder. J. Econ. Entomol. **93**: 840–845. PMID:10902339.

Orians, C.M., Pomerleau, J., and Ricco, R. 2000. Vascular architecture generates fine scale variation in systemic induction of proteinase inhibitors in tomato. J. Chem. Ecol. **26**: 471–485. doi:10.1023/A:1005469724427.

Parsons, T.J., Bradshaw, H.D., Jr., and Gordon, M.P. 1989. Systemic accumulation of specific mRNAs in response to wounding in poplar trees. Proc. Natl. Acad. Sci. U.S.A. **86**: 7895–7899. doi:10.1073/pnas.86.20.7895. PMID:2813366.

Peña-Cortes, H., Sanchez-Serrano, J., Rocha-Sosa, M., and Willmitzer, L. 1988. Systemic induction of proteinase-inhibitor-II gene expression in potato plants by wounding. Planta (Berl.), **174**: 84–89. doi:10.1007/BF00394877.

Quackenbush, J., Cho, J., Lee, D., Liang, F., Holt, I., Karamycheva, S., Parvizi, B., Pertea, G., Sultana, R., and White, J. 2001. The TIGR Gene Indices: analysis of gene transcript sequences in highly sampled eukaryotic species. Nucleic Acids Res. **29**: 159–164. doi:10.1093/nar/29.1.159. PMID:11125077.

Ralph, S., Oddy, C., Cooper, D., Yueh, H., Jancsik, S., Kolosova, N., Philippe, R., Aeschliman, D., White, R., Huber, D., Ritland, C., Benoit, F., Rigby, T., Nantel, A., Butterfield, Y., Kirkpatrick, R., Chun, E., Liu, J., Palmquist, D., Wynhoven, B., Stott, J., Yang, G., Barber, S., Holt, R., Siddiqui, A., Jones, S., Marra, M., Ellis, B., Douglas, C., Ritland, K., and Bohlmann, J. 2006. Genomics of hybrid poplar (*Populus trichocarpa* × *deltoides*) interacting with forest tent caterpillars (*Malacosoma disstria*): normalized and full-length cDNA libraries, expressed sequence tags, and a cDNA microarray for the study of insect-induced defences in poplar. Mol. Ecol. **15**: 1275–1297. doi:10.1111/j.1365-294X.2006.02824.x. PMID:16626454.

Rasmann, S., Kollner, T.G., Degenhardt, J., Hiltpold, I., Toepfer, S., Kuhlmann, U., Gershenzon, J., and Turlings, T.C.J. 2005. Recruitment of entomopathogenic nematodes by insect-damaged maize roots. Nature (Lond.), **434**: 732–737. doi:10.1038/nature03451. PMID:15815622.

Richmond, T., and Somerville, S. 2000. Chasing the dream: plant EST microarrays. Curr. Opin. Plant Biol. **3**: 108–116. doi:10.1016/S1369-5266(99)00049-7. PMID:10712953.

Ryan, C.A. 1990. Protease inhibitors in plants: Genes for improving defenses against insects and pathogens. Annu. Rev. Phytopathol. **28**: 425–449. doi:10.1146/annurev.py.28.090190.002233.

Scarascia-Mugnozza, G.E., Hinkley, T.M., Stettler, R.F., Heilman, P.E., and Isebrands, J.G. 1999. Production physiology and morphology of *Populus* species and their hybrids grown under short rotation. III. Seasonal carbon allocation and patterns from branches. Can. J. For. Res. **29**: 1419–1432. doi:10.1139/cjfr-29-9-1419.

Schilmiller, A.L., and Howe, G.A. 2005. Systemic signaling in the wound response. Curr. Opin. Plant Biol. **8**: 369–377. doi:10.1016/j.pbi.2005.05.008. PMID:15939667.

Schittko, U., and Baldwin, I.T. 2003. Constraints to herbivore-induced systemic responses: bidirectional signaling along orthostichies in *Nicotiana attenuata.* J. Chem. Ecol. **29**: 763–770. doi:10.1023/A:1022833022672. PMID:12757332.

Schoonhoven, L.M., van Loon, J.J.A., and Dicke, M. 2005. Insect–plant biology. Oxford University Press, New York.

Schwachtje, J., Minchin, P.E.H., Jahnke, S., van Dongen, J.T., Schittko, U., and Baldwin, I.T. 2006. SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots. Proc. Natl. Acad. Sci. U.S.A. **103**: 12935–12940. doi:10.1073/pnas.0602316103. PMID:16912118.

Sterky, F., Bhalerao, R.R., Unneberg, P., Segerman, B., Nilsson, P., Brunner, A.M., Charbonnel-Campaa, L., Lindvall, J.J., Tandre, K., Strauss, S.H., Sundberg, B., Gustafsson, P., Uhlen, M., Bhalerao, R.P., Nilsson, O., Sandberg, G., Karlsson, J., Lundeberg, J., and Jansson, S. 2004. A *Populus* EST resource for plant functional genomics. Proc. Natl. Acad. Sci. U.S.A. **101**: 13951–13956. doi:10.1073/pnas.0401641101. PMID:15353603.

Storey, J.D., and Tibshirani, R. 2003. Statistical significance for genomewide studies. Proc. Natl. Acad. Sci. U.S.A. **100**: 9440–9445. doi:10.1073/pnas.1530509100. PMID:12883005.

Tindall, K.V., and Stout, M.J. 2001. Plant-mediated interactions between the rice water weevil and fall armyworm in rice. Entomol. Exp. Appl. **101**: 9–17. doi:10.1023/A:1019293403417.

Urwin, P.E., Levesley, A., McPherson, M.J., and Atkinson, H.J. 2000. Transgenic resistance to the nematode *Rotylenchulus reniformis* conferred by *Arabidopsis thaliana* plants expressing proteinase inhibitors. Mol. Breed. **6**: 257–264. doi:10.1023/A:1009669325944.

van Dam, N.M., and Raaijmakers, C.E. 2006. Local and systemic induced responses to cabbage root fly larvae (*Delia radicum*) in *Brassica nigra* and *B. oleracea.* Chemoecology, **16**: 17–24.

van Dam, N.M., Horn, M., Mares, M., and Baldwin, I.T. 2001. Ontogeny constrains systemic protease inhibitor response in *Nicotiana attenuata.* J. Chem. Ecol. **27**: 547–568. doi:10.1023/A:1010341022761. PMID:11441445.

van Dam, N.M., Harvey, J.A., Wackers, F.L., Bezemer, T.M., van der Putten, W.H., and Vet, L.E.M. 2003. Interactions between aboveground and belowground induced responses against phytophages. Basic Appl. Ecol. **4**: 63–77.

van Dam, N.M., Witjes, L., and Svatos, A. 2004. Interactions between aboveground and belowground induction of glucosinolates in two wild *Brassica* species. New Phytol. **161**: 801–810. doi:10.1111/j.1469-8137.2004.00984.x.

Wang, J., and Constabel, C.P. 2004. Polyphenol oxidase overexpression in transgenic *Populus* enhances resistance to herbivory by forest tent caterpillar (*Malacosoma disstria*). Planta (Berl.), **220**: 87–96. doi:10.1007/s00425-004-1327-1. PMID:15309534.

Winz, R.A., and Baldwin, I.T. 2001. Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. IV. Insect-induced ethylene reduces jasmonate-induced nicotine accumulation by regulating putrescine *N*-methyltransferase transcripts. Plant Physiol. **125**: 2189–2202. doi:10.1104/pp.125.4.2189. PMID:11299398.

Worthington, C.C. 1988. Worthington enzyme manual. Worthington Biochemical Corporation, Freehold, N.J.

Zhang, Z.P., and Baldwin, I.T. 1997. Transport of [2-C-14]jasmonic acid from leaves to roots mimics wound-induced changes in endogenous jasmonic acid pools in *Nicotiana sylvestris.* Planta (Berl.), **203**: 436–441.