Plant Molecular Biology **46:** 347–359, 2001.

A Kunitz trypsin inhibitor gene family from trembling aspen (*Populus tremuloides* Michx.): cloning, functional expression, and induction by wounding and herbivory

Miyoshi Haruta, Ian T. Major, Mary E. Christopher, Joseph J. Patton and C. Peter Constabel*
*Department of Biological Sciences, CW405 BioSciences Center, University of Alberta, Edmonton, Canada T6G 2E9 (*author for correspondence; e-mail: peter.constabel@ualberta.ca)*

Received 22 June 2000; accepted in revised form 6 March 2001

Key words: plant defense, plant-insect interactions, protease inhibitor, systemic induction

Abstract

Three Kunitz trypsin inhibitor genes were isolated from trembling aspen (*Populus tremuloides*) by PCR and cDNA screening. Based on sequence similarity, they were grouped into two classes. Southern blots showed complex banding patterns and a high level of restriction fragment polymorphism between different aspen genotypes, suggesting that these trypsin inhibitors are members of a large, rapidly evolving gene family. One of the trypsin inhibitor genes, PtTI2, was over-expressed in *Escherichia coli* and its product shown to inhibit bovine trypsin *in vitro*. Both classes of PtTI genes are induced by wounding and herbivory, permitting rapid adaptive responses to herbivore pressure. The response appears to be mediated by an octadecanoid-based signaling pathway, as methyl jasmonate treatments induced the trypsin inhibitors. Wound-induced accumulation of trypsin inhibitor protein was also observed by western blot analysis. The pattern of expression, the apparent rapid evolution of TI genes, and the *in vitro* trypsin inhibitory activity are consistent with a role in herbivore defense. This work establishes the presence of a functional protein-based inducible defense system in trembling aspen.

Abbreviations: FTC, forest tent caterpillar; MeJa, methyl jasmonate; PI, protease inhibitor; TI, trypsin inhibitor

Introduction

In order to protect themselves against insects and other herbivores, plants have evolved a variety of defense mechanisms. Many plant defenses involve toxic, deterrent, or anti-nutritive secondary plant metabolites and proteins (Harborne, 1993; Duffey and Felton, 1991); these can be constitutive, or inducible and expressed only upon herbivore damage. Induction of defenses in plants involves complex signaling pathways, and usually requires rapid changes in gene expression (for an overview, see Agrawal *et al*., 1999). The importance of induced defenses in reducing herbivore damage has been established in both laboratory and field studies. For example, transgenic tomato plants inhibited in their ability to induce defense proteins are hypersusceptible to herbivory by *Manduca sexta* (Orozco-Cardenas *et al*., 1993). Studies with natural plant populations have confirmed that induced defenses can have significant fitness advantages for plants under herbivore pressure (Baldwin, 1998; Agrawal, 1998).

Biochemical defenses have been described in many plants, but the types of defenses found will vary depending on the species, and there are no universal defense chemicals or proteins (Constabel, 1999). Very common defense proteins are the protease inhibitors (PIs). PIs inhibit the proteolytic enzymes found within herbivore guts, leading to hypersecretion of digestive enzymes and loss of essential amino acids, resulting in an inhibition of insect growth (Broadway and Duffey, 1986). Inhibitors of diverse

digestive proteases, including serine, cysteine, aspartic and metallo-proteases, have been identified in plants (Heitz *et al*., 1999). Serine PIs are the most common, and this group comprises several protein families including potato inhibitors I and II, Bowman-Birk inhibitors, and Kunitz trypsin inhibitors (Ryan, 1990; Richardson, 1991). PIs frequently accumulate in plant organs most vulnerable to herbivory, for example in seeds (Richardson, 1991). In leaves, PIs are often an inducible defense, and it was the discovery of the rapid accumulation of PIs in herbivore-damaged tomato leaves by Ryan and co-workers which first demonstrated the dynamic nature of plant anti-herbivore responses (Green and Ryan, 1972). Herbivore- and wound-induced PIs have now been described from diverse crop species in the Solanaceae, Fabaceae, Poaceae, Brassicaceae, and Salicaceae (reviewed in Ryan, 1990; Richardson, 1991; Constabel, 1999). The efficacy of PIs as defense proteins has been directly demonstrated by numerous studies; the most direct proof comes from PI over-expression in transgenic plants, which results in heightened resistance to insect pests (reviewed in Schuler *et al*., 1998; Heitz *et al*., 1999; Lee *et al*., 1999).

We have recently undertaken investigations on the biochemical anti-herbivore defenses in trembling aspen (*Populus tremuloides*). Trembling aspen is a widespread forest tree in North America and is susceptible to damage by a variety of lepidopteran and coleopteran pests. In many parts of its range, it is subject to massive defoliation by periodic outbreaks of forest tent caterpillar (FTC, *Malacasoma disstria*). Outbreaks occur on average every ten years (Peterson and Peterson, 1992). What drives the cyclical nature of FTC outbreaks is not understood, but may involve a combination of factors including changes in food quality. Delayed inducible plant defenses have been postulated to be a driving force in other northern herbivore cycles (Haukioja, 1980). Understanding trembling aspen biochemical defenses is thus of interest from both a practical and a theoretical perspective, and the widespread distribution and ecological importance of trembling aspen makes this an excellent system for studying large-scale ecological consequences of plant defense.

Major secondary metabolites of trembling aspen have been identified and studied for their anti-herbivore potential (reviewed by Lindroth and Hwang, 1996). These include condensed tannins and phenolic glycosides, which can be found at concentrations of up to 18% and 7% dry weight, respectively (Lindroth and Hwang, 1996). Both of these phytochemicals have been shown to negatively impact FTC performance (Lindroth *et al*., 1988; Constabel and Spence, unpublished data), as well as other insect herbivores (Clausen *et al*., 1989; Lindroth and Kinney, 1998). Phenolic glycosides can be considered to be constitutive defenses, although the levels of phenolic glycosides can increase modestly following wounding. Clausen *et al*. (1989) found a 20% increase in phenolic glycosides upon herbivory by the large aspen tortrix (*Choristoneura conflictana*), whereas Lindroth and Kinney (1998) reported a 35% induction after artificial defoliation. Condensed tannins, however, were not found to be induced by damage in this study.

By contrast, the protein-based herbivore defenses of trembling aspen have not been extensively studied. We had previously observed that mechanical wounding and herbivory stimulates a local and systemic increase in polyphenol oxidase, and have suggested it may in fact be involved in the mechanism of toxicity of phenolic glycosides (Haruta et al. 2001). This oxidative enzyme can have anti-nutritive effects, and is induced in tomato as part of a battery of defense proteins including a number of PIs (Constabel *et al*., 1995). The herbivore induction of polyphenol oxidase in trembling aspen suggested that other protein-based defenses such as PIs might also be induced, as has been reported in hybrid poplar (Bradshaw *et al*., 1989; Hollick and Gordon, 1993). We adopted a molecular strategy to address this question, and took advantage of available sequence data from the closely related poplar hybrid (*P. trichocarpa* × *P. deltoides*) and *Salix viminalis* (Bradshaw *et al*., 1989; Saarikoski *et al*., 1996) to isolate Kunitz trypsin inhibitor (TI) genes from trembling aspen. We then used these cDNAs as tools for the molecular characterization of trembling aspen TI expression. Our results indicate that trembling aspen contains a small TI gene family, and that at least one of these cDNAs encodes a functional trypsin inhibitor protein. We further demonstrate that real and simulated herbivory induces the accumulation of both TI mRNA and protein. The pattern of expression of TI is consistent with a role as an inducible defense in trembling aspen.

Materials and methods

Plant material and treatments

Trembling aspen (*Populus tremuloides*) were obtained locally. Plants were propagated as aseptic multishoot cultures as described (Wann and Einspahr, 1986), acclimated, and transferred to soil (Terra-lite Redi-Earth, WR Grace, Ajax, Ontario). All plant material was maintained in the University of Alberta Biotron's growth chambers under 16 h of light (250 μE m^{-2} sec^{-1}) at 18 °C and 75% relative humidity. Plants were watered daily with a solution containing 1 g/l 20-20-20 NPK fertilizer with micronutrients (Plant Products, Brampton, Ontario). Routine experiments were carried out with 10–12-week old plants (20–25 leaves) in 15 cm pots. For some experiments, larger plants in 25 cm pots were substituted.

To simulate herbivory by mechanical wounding, leaf margins were crushed three times at 2 h intervals with a hemostat. Leaf numbers were assigned basipetally, with the first unfolded leaf being designated as leaf 1. Wounding of stems was carried out by making a series of cuts with a clean razor. Methyl jasmonate (MeJa0 treatment was performed by placing plants in glass boxes for 24 h with 20 μl of a 10% solution of MeJa in ethanol on a cotton wick. Herbivore experiments were carried out in 75-cm high insect cages within the environmental chambers, by placing ten starved fourth- and fifth-instar forest tent caterpillar (FTC) larvae on leaves allowing them to feed for 36 h. For time course experiments, six leaves per plant were wounded, and both wounded and upper unwounded leaves were harvested at various times. For all experiments, tissue was frozen in liquid N_2 and stored at −80 °C until analyzed.

Cloning and DNA sequencing of TI genes and cDNAs

Molecular biology procedures were carried out by standard methods (Sambrook *et al*., 1989). Nucleotide primers were designed to amplify coding regions of TI based on sequence data from both *P. trichocarpa* × *P. deltoides* (gwin3; Genbank accession number X15516) and *S. viminalis* (swin1.1; U29090). PCR was carried out with upstream primer P-1 (5′-GATAACTAC/ATACTCATATTC-3′) and downstream primer P-4 (5′-CTCAATATCATTCC/TGACACC-3′), as well as with primer P-1 and downstream primer P-3 (5′-TGCCCAACATATGATAGTAG-3′). Amplification was performed from genomic DNA templates using 19 cycles of 94 °C (60 s)/51 °C (60 s)/72 °C (30 s) followed by 19 cycles of 94 °C (60 s)/54 °C (60 s)/72 °C (30 s). PCR products were gel-purified (QIAEX II, Qiagen) and cloned into pBluescript using a T-tailing strategy (Marchuk *et al*., 1991).

A wounded leaf cDNA library was constructed from 5 μg poly(A)$^+$ RNA, isolated from wounded aspen leaves, in λZAP II (Stratagene) according to the manufacturer's instructions. For library screening, 5×10^5 plaques were probed with a ^{32}P-labeled PtTI1 gene. DNA sequencing of cDNAs was carried out on both strands with T3 and T7 primers using the Thermosequenase dye terminator cycle sequencing kit (Amersham) and the Applied Biosystems 377 Automatic Sequencer. For genes amplified by PCR, nucleotide sequences were confirmed by sequencing three clones from at least two independent PCR reactions.

DNA and RNA hybridization analysis

Genomic DNA was isolated from trembling aspen as described (Doyle and Doyle, 1990), with the addition of one phenol and one phenol/chloroform extraction step. DNA (10 μg) was digested with 50 U of *Hin*dIII, *Xba*I or *Eco*RV (Gibco/BRL), and Southern blots carried out with full-length ^{32}P-labeled cDNAs according to standard procedures (Sambrook *et al.*, 1989). Washes were performed at low stringency (65 °C in 1× SSC, 0.1% SDS for 30 min and 10 min), or high stringency (65 °C in 0.1× SSC, 0.1% SDS for 30 min and 10 min). Hybridization signals were detected with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) or recorded by autoradiography.

Total RNA was extracted from 500 mg crushed leaves using the RNA extraction protocol of Chang *et al*. (1993), with the addition of one phenol and one phenol/chloroform extraction step after the LiCl precipitation. About 1 mg of total RNA was obtained from 500 mg aspen leaf tissue by this method. For cDNA library construction, poly(A)$^+$ RNA was isolated from total RNA with the PolyATtract mRNA Isolation System (Promega). For RNA blots, 20 μg of total RNA was electrophoresed through a 1.2% agarose-formaldehyde gel, blotted, and hybridized as described above, but without the high-stringency washes.

Bacterial over-expression, purification, and assay of recombinant TI2

The coding sequence of PtTI2 (minus the signal sequence) was PCR-amplified from the PtTI2

plasmid clone with primers P-4 and P-5 (5′-GAA/GGATCCTA/GCAGCAGTGC-3′) and cloned into a pBluescript T-tailed vector. The fragment was then subcloned into the *Bam*HI and *Hin*dIII restriction sites of the pQE31 bacterial expression vector (Qiagen). This plasmid (pQETI) contained the entire mature coding sequence of PtTI2, beginning with the signal sequence clip site, immediately downstream of the N-terminal His tag of pQE31. Junctions were sequenced to confirm that the TI coding sequence was in frame, and the plasmid moved into *E. coli* strain M15(pREP4). To produce recombinant TI, 50 ml of superbroth was inoculated with 5 ml of an overnight culture of *E. coli* harboring pQETI and grown at 37 °C to an OD_{600} of 0.5. IPTG was added to a final concentration of 1 mM, and the culture shaken at 30 °C for 5 h. The cells were then harvested by centrifugation, the pellet resuspended in 15 ml resuspension buffer (50 mM Na_2HPO_4 pH 8, 300 mM NaCl) and passed twice through a French Press (American Instrument Company, Silver Spring, MD) at 383 MPa. Inclusion bodies containing TI were harvested from the lysate by centrifugation at 10 000 × *g* for 30 min. The recombinant TI was purified on a nickel affinity resin (Ni-NTA agarose, Qiagen) and eluted using denaturing conditions (8 M urea) as described in the manufacturer's protocol. Fractions were concentrated with Centricon-10 microconcentrators (Amicon), and the TI protein purified by preparative 15% SDS-PAGE and electroelution. Gel-purified TI was again concentrated using Centricon-10 microconcentrators.

To renature recombinant TI, the purified protein was diluted in 100 mM Na_2HPO_4/10 mM Tris/6 M urea pH 7.0 and dialyzed at 4 °C against the same buffer in a decreasing series of urea concentrations. Renatured TI protein was recovered and concentrated using Centricon-10 concentrators. Protein concentrations were determined spectrophotometrically (A_{280}) using molar extinction coefficients calculated from the predicted protein sequences (Gill and von Hippel, 1989), and verified on Coomassie-stained SDS-PAGE gels. TI activity was determined by measuring the change in A_{247} due to cleavage of the trypsin substrate TAME (p-toluene-sulfonyl-L-arginine methyl ester) as described (Worthington, 1988). Serial dilutions of TI protein were assayed with a standard quantity of bovine trypsin (Sigma). Pure soybean Kunitz trypsin inhibitor (Sigma) was assayed under identical conditions. For antibody production, two white New Zealand male rabbits were each immunized with 100 μg TI protein in PBS and Freund's complete adjuvant (Sigma), with booster injections of 50 μg TI protein in Freund's incomplete adjuvant 21 and 40 days later.

Protein extraction and western blotting

Frozen leaves were ground in liquid N_2 and extracted with Na_2HPO_4 buffer (100 mM, pH 7.0) containing 0.1% Triton X-100, 5% w/v polyvinylpolypyrrolidone, and 1% 2-mercaptoethanol. Extracts were clarified by centrifugation, and soluble protein quantified using the method of Bradford (1976). For western blotting, proteins were separated by SDS-PAGE and electro-transferred onto PVDF membranes (BioRad), and the efficiency of transfer visualized by Ponceau S staining. Western blots were developed using standard procedures (Harlow and Lane, 1988) using the anti-TI antibody at a 1:15 000 dilution and a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit, BioRad) at a 1:10 000 dilution. Blots were developed by chemiluminescence (Boehringer-Mannheim Chemiluminescence Substrate Kit) or colorimetrically with 3,3′-diaminobenzidine tetrahydrochloride (Harlow and Lane, 1988). For TI assays, leaf extracts were prepared as above, but with 0.1% 2-mercaptoethanol in the extraction buffer. Extracts were partially purified by heat treatment (55 °C for 10 min) and centrifugation, followed by gel filtration through Sephadex G-50 (Centri-Sep, Princeton Separation) as per manufacturer's instructions. TI assays were conducted as described above.

Results

Isolation and characterization of TI genes in trembling aspen

In order to isolate trypsin inhibitor genes from genomic DNA of trembling aspen, we used a PCR strategy with degenerate oligonucleotide primers and trembling aspen genomic DNA as a template. Upstream primer P-1, and downstream primers P-3 and P-4 were designed to amplify coding regions of aspen TIs, based on published nucleotide sequences encoding trypsin inhibitor from *P. trichocarpa* × *deltoides* and *S. viminalis* (see Materials and methods). PCR products were obtained with both primer combinations P-1/P-4 and P-1/P-3, and these were purified and cloned. Sequence analysis indicated that both PCR products encoded trembling aspen TI (Figure 1);

```
PtTI1      1:MKITKFLGLSFLLFAFAAT-SFPEGVQAEDPEAVLDFYGDKVKAGAPYLIQDLTFIPHDN 59
PtTI2      1:MKITKFLGLSFLLFAFAAT-LFPEGVHAEDTAAVLDFYGREVQAGTPYLIQDLSYEP-GN 58
PtTI3      1:MKISNFLVLSFLLFAFTATSIFPRAVHA---AVVIDAFGDEVKAGDRYVIGAASNDFAIT 57
gwin3      1:MKITKFLGLSFLLFAFAAT-SFPDGVHAEDPAAVLDFYGREVQAGASYLI-D-----QED 53
swin1.1    1:MKITKFLALSFLLFAFTATS-FPHAVHAEDPAAVLDFYGNEVTAGASYFID-HEDSLAVS 58
             ***  ** ******** **  **  * *     * *  *  * **  * *

PtTI1     60:TTNYVVGATKSNDGVNRDVILSYGNEGLPVTFSPVTKSTDGVIREGSLITVSFDADTCKM 119
PtTI2     59:TSNFVVGAT-INPICNSDVVLSYENDGLPVTFSPVTESTDGVIREGTLITVSFDAATCKM 117
PtTI3     58:ATSPII----CN---S-DVVFSPMSDGLPVIFSKVVESNDSVINEDSYLNVDFDAPSCRM 109
gwin3     54:-F-RVVNAT-INPICNSDVILSTGIEGLPVTFSPVINSTDGVIREGTLITVSFDASTCGM 110
swin1.1   59:AATRII----CN---S-DVTLSPMSDKLPITFSPVVESTDSVIREGAYLNVNFNAILCRM 110
                    *     **  *     **  ** *  * * ** *     * * *  * *

PtTI1    120:AGVTPMWKIGFNSTGTGYIVTTGGVDQLNQFTITKYEKEGSFYQLSYCPNSDPFCECSCV 179
PtTI2    118:ADVTPMWKIGFNSTGTGYIVTTGGVDQLNQFMITKDKNESSFYQLSYCPKSDPFCECSCV 177
PtTI3    110:AGVSTMWKIELRLTARGFVVTTGGVAGLNRFTITKYEGGTNQYQLSYCPISEPICECSCV 169
gwin3    111:AGVTPMWKIGFNSTAKGYIVTTGGVDRLNLFKITKFESDSSFYQLSYCPNSEPFCECPCV 170
swin1.1  111:AGVTTMWKIELRATMRGFVVTTGGVDRLNLFKITKLEGDNSLYQLSYCPVSDPFCECSCV 170
             * *  ****    *  *  ******  ** * ***       ******* * * *** **

PtTI1    180:PVGANDDKYLAPKAADVVQVRFKPELNIYGDKMVSE                        215
PtTI2    178:PVGATNDKYLAPKAAVVVDVRFKPELNIYGDKMVSE                        213
PtTI3    170:PLGNVVNR-LAPST-VPFPVVFIPSDRASKIEYKMM                        203
gwin3    171:PVGANSDKYLAPNVSY-ADFRFKPDARIEST-----                        200
swin1.1  171:PVGNSCNR-LVPNARTPLLVVFEPDTDTAS------                        199
             * *      * *        * *
```

Figure 1. Multiple sequence alignments of three new trembling aspen trypsin inhibitors with similar sequences from Salicaceae. The three PtTI nucleotide sequences have been deposited in GenBank (accession nos. AF349441-AF349443). gwin3 (GenBank accession number P16335) and swin1.1 (AA68962) are from hybrid poplar (*P. trichocarpa* × *P. deltoides*) and *Salix viminalis*, respectively. The asterisks indicate regions of identity among all proteins.

comparison of the nucleotide sequences, however, indicated that the two primer combinations amplified very similar but distinct TI genes, which were subsequently named PtTI1 and PtTI2 (Figure 1). No introns were detected in either PtTI1 or PtTI2, consistent with the gene structure of this family of TIs.

We also screened a cDNA library constructed from wounded trembling aspen leaves using PtTI1 as a probe. Both weakly and strongly hybridizing plaques were identified. Plaques of both types were purified and the plasmids excised. Sequence analysis of inserts from both types of phage indicated that the strongly hybridizing inserts contained sequences identical to PtTI2, while the more abundant, weakly hybridizing plaques contained a distinct TI sequence. A full-length cDNA of this distinct type of TI was isolated, sequenced on both strands, and named PtTI3.

Conceptual translation and computer analysis of the sequence data predicted proteins with N-terminal signal sequences, which upon cleavage would give rise to mature polypeptides with molecular masses of 20.3, 20.0, and 18.8 kDa for PtTI1, PtTI2, and PtTI3, respectively. Sequence comparisons confirmed that PtTI1 and PtTI2 are very closely related, showing 91.7% identity at the nucleotide level, and 83.1% identity at the amino acid level (Figure 1, Table 1). By contrast, PtTI3 appears quite distinct from both of these, with 52.2% and 53.7% amino acid identity and 75.2% and 75.8% nucleotide identity with PtTI1 and PtTI2, respectively. As expected, BLASTX GenBank searches (Altschul *et al*., 1997) identified gwin3 and swin1.1 from hybrid poplar and willow, respectively, as the sequences most similar to the PtTI genes. Pairwise comparisons of the three new PtTI coding sequences with the two Salicaceae TIs indicated that PtTI1 and PtTI2 are much more similar to gwin3 (75–76% identity) than to PtTI3 (53% identity; Table 1). This suggests that PtTI1/2 and gwin3 could be orthologues and PtTI3 represents a divergent member (paralogue) within this gene family. By contrast, swin1.1 is slightly more similar to PtTI3 than are PtTI1 and PtTI1 (Table 1). Among the next closest matches to the PtTIs in the databases are tobacco NF34 isolated

Table 1. Comparison of protein sequence identities[1] (%) of three new trembling aspen trypsin inhibitors with similar sequences from Salicaceae.

	PtTI1	*PtTI2*	*PtTI3*	*gwin3*	*swin1.1*
PtTI1	100	83.1	52.2	75.0	58.5
PtTI2		100	53.7	76.0	60.8
PtTI3			100	53.0	63.3
gwin3				100	65.3

[1]Sequence identities were calculated with the Peptools Align Algorithm (Biotools, Inc) using the Biotools scoring matrix (Wishart *et al*., 2000).

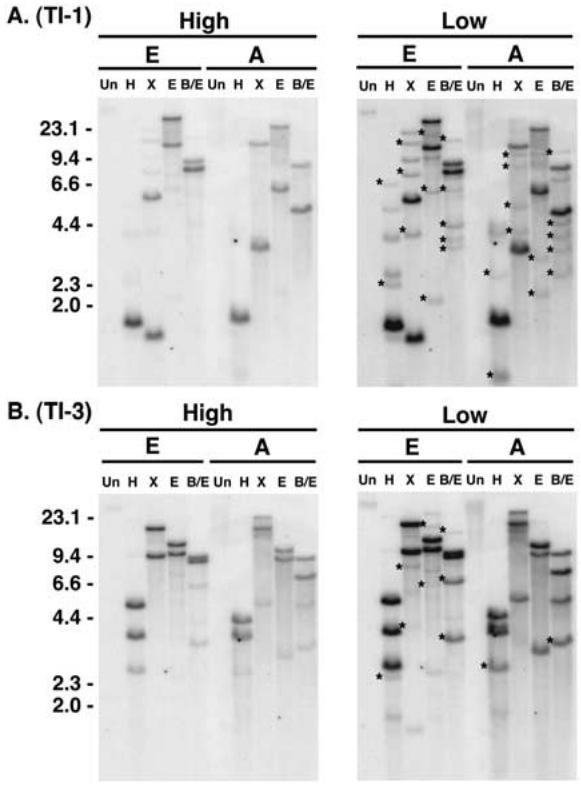


Figure 2. Southern analysis of the *PtTI* gene family in trembling aspen. Genomic DNA from two different genotypes was digested with restriction enzymes, electrophoresed and blotted according to standard protocols. The same blots were probed with *PtTI1* (top panels) and PtTI3 (bottom panels) and washed under high and low stringency conditions (left and right panels, respectively). Stars indicate those bands which did not hybridize strongly to either *PtTI1* or PtTI3, and thus represent other TI-like sequences. E and A are different genotypes of aspen. Un, unrestricted DNA; H, *Hin*dIII; X, *Xba*I; E, *Eco*RV; B/E, *Bam*HI and *Eco*RI.

during a screen for hypersensitivity-inducing proteins (Karrer *et al*., 1998), the nematode-induced LeMir gene from tomato (Brenner *et al*., 1998), and storage proteins from cocoa and sweet potato (Hattori *et al*., 1985; Spencer and Hodge, 1991). These all show identities of between 28% and 39% with the three PtTIs (not shown). However, only for swin1.1 and the sweet potato protein (sporamin) has trypsin inhibitory activity of the gene product been demonstrated (Saarikoski *et al.*, 1996; Yeh *et al*., 1997).

In order to estimate the size of the PtTI gene family in trembling aspen, Southern analyses of genomic DNA was carried out. Restricted DNA from two trembling aspen genotypes (genotypes 'A' and 'E') was used, and hybridized with *PtTI1*. This probe is 91.7% identical to *PtTI2* at the nucleotide level, and thus

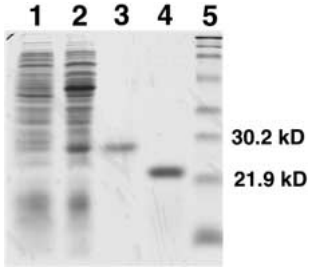


Figure 3. Coomassie-stained SDS-PAGE gel showing bacterial over-expression and purification of recombinant TI protein. PtTI2 was over-expressed in *E. coli* and purified as described in Materials and methods. Lanes 1 and 2, lysates of bacterial cultures harboring over-expression construct before (lane 1) and after (lane 2) induction with IPTG; lane 3, purified recombinant TI2; lane 4, commercial soybean Kunitz TI; lane 5, molecular weight markers.

will hybridize to all *PtTI2* genes as well. After high-stringency washes, one or two bands were detected in both genotypes, depending on the restriction enzyme (Figure 2). The pattern observed for each of the genotypes was distinct, although some bands were present in both. Two other aspen genotypes tested also showed unique TI bands on Southern blots (data not shown). Since our plant material is from wild, outbreeding individuals and highly heterozygous, these bands likely represent different alleles. The blots were then stripped and reprobed with PtTI3. With this probe, generally two bands were detected (Figure 2B). The bands are clearly different from those observed with PtTI1 (compare Figure 2, panels A and B). As with PtTI1, the two genotypes again gave distinct patterns, suggesting that distinct alleles are being detected. Under low-stringency washing conditions, the same Southern blots revealed additional bands which were not recognized by either the PtTI1 or PtTI3 probe under higher-stringency conditions (Figure 2, right panels; see bands marked with an asterisk). Therefore, there appear to be additional TI-like genes in the aspen genome.

Over-expression and functional characterization of PtTI2

To demonstrate that the TI genes we had isolated encode functional proteins, we over-expressed PtTI2 in *E. coli* as a His-tagged fusion protein. The construct contained the entire coding region of PtTI2 but without the predicted ER signal sequence, fused in frame to an N-terminal His tag. Induction of the *E. coli* strain with isopropyl-1β-D-thiogalactopyranoside (IPTG) resulted in the appearance of a new protein in bacterial

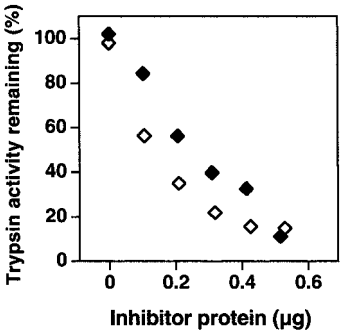
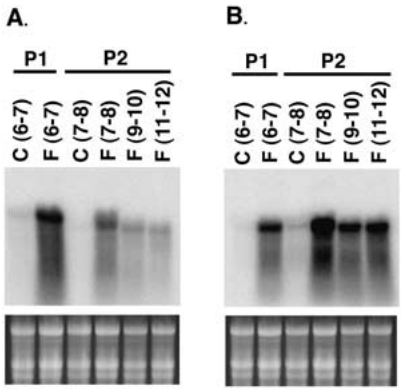


Figure 4. In vitro trypsin inhibition by recombinant TI. Bovine trypsin (660 ng per assay) was incubated with increasing amounts of renatured recombinant TI protein, and the remaining trypsin activity assayed spectrophotometrically. For comparative purposes, soybean TI was assayed in parallel. Filled symbols, recombinant TI; open symbols, soybean TI.



C.

Plant	leaf number	damage (%)
P1	6-7	10-15%
P2	7-8	15-20%
P2	9-10	8-10%
P2	11-12	~ 5%

Figure 5. Induction of TI mRNAs by herbivory. Plants were subjected to herbivory by FTC for 36 h and analyzed by northern blot. P1 and P2 represent individual plants with two main stems, so that control and herbivore-induced leaves were harvested from the same individual (leaf number indicated in parenthesis). Panel A was probed with PtTI1, panel B with PtTI3. Replica ethidium bromide-stained gels are shown as RNA loading controls. Panel C indicates the extent of material removed by FTC from each leaf during the experiment. C, control leaves; F, FTC-treated.

extracts (Figure 3). This protein migrated more slowly than expected given its predicted molecular mass of 22 kDa; however it was detected only in induced cultures harboring the pQETI over-expression construct, and was specifically bound by Ni-NTA-agarose. The recombinant TI protein was affinity-purified on Ni-NTA-agarose resin and renatured as described in Materials and methods, and its purity checked on SDS-PAGE (Figure 3). We assayed the renatured recombinant TI protein for trypsin inhibitory activity by incubating a standard amount of bovine trypsin with increasing amounts of recombinant TI. Trypsin activity, as measured using TAME as the substrate, was reduced proportionally to the amount of recombinant TI added (Figure 4). From our data we estimate that 0.75 μg of TI was required to maximally inhibit 1 μg of trypsin. This was comparable to the maximal inhibition by 0.48 μg commercial soybean Kunitz trypsin inhibitor, assayed under identical conditions (Figure 4). These experiments clearly demonstrate that PtTI2 encodes a functional trypsin inhibitor, and are consistent with our hypothesis that TIs contribute to a protein-based anti-herbivore defense system of trembling aspen.

Herbivore- and wound-induced expression of TI genes

In order to determine if the PtTI genes act as inducible anti-herbivore defenses in trembling aspen, we tested the effects of herbivory on PtTI gene expression. Two-month old aspen plants were subjected to herbivory by FTC larvae as described under Materials and methods. Since the FTC were allowed to move and feed on the entire plant, the amount and distribution of damage was difficult to control, and by their own preference the larvae fed mostly on leaves 6–12 (not shown). Therefore, only leaves with significant herbivore damage (leaves 6–7 for Plant I, leaves 7–12 for Plant II) were harvested for RNA analysis, 36 h after the onset of herbivory.

Analysis of the RNA by northern blot indicated that PtTI gene expression is strongly stimulated by FTC feeding. We hybridized replicate blots with both PtTI1 (which also hybridizes with PtTI2) and PtTI3 probes, and in both cases, the patterns of expression obtained were similar. Both probes clearly show that FTC damage results in an induction of both PtTI1/2 and PtTI3 mRNA in damaged leaves, with low transcript levels in control leaves (Figure 5A and B). In general, PtTI mRNA accumulation was proportional to the amount of tissue removed by the herbivores (Figure 5C). However, the relative signal profiles detected by the PtTI1 and PtTI3 probes were found to differ somewhat (compare panels A and B, Figure 5). This suggests that although both genes are FTC damage-induced, there may be some differential expression of the two genes. While PtTI1 and PtTI3 cross-hybridize under our hybridization con-

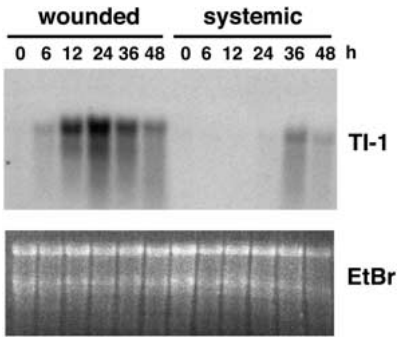


Figure 6. Time course of wound-induced TI gene expression. Leaves were wounded as described in Materials and methods, and harvested at the indicated times and analyzed by northern blot. Systemic leaves are unwounded leaves on wounded plants. The blot was probed with PtTI1. A portion of the ethidium bromide-stained gel is shown for a loading control.

ditions (see Figure 2), this was weak and unlikely to contribute significantly to the signals detected in Figure 5. We conclude that both PtTI1 and PtTI3 are herbivore-inducible, but that there are minor differences in expression levels between them. Additional blots probed with both types of probes showed similar profiles as well, suggesting that both types of transcripts accumulate in parallel (data not shown). Therefore, in subsequent RNA analyses, we used only PtTI1 as a probe.

We next performed time course experiments to determine the kinetics of TI induction in aspen. To better control the timing and extent of damage, we simulated herbivory by the use of pliers. Previous work in poplar had indicated that the defense response is activated plant-wide, i.e., systemically (Parsons *et al*., 1989; Constabel *et al*., 2000). We therefore analyzed both wounded and unwounded ('systemic') leaves from wounded plants. Figure 6 shows that PtTI1/2 mRNA accumulated within 6 h of damage in the wounded leaves, with a maximum at 24 h. By contrast, in the unwounded leaves the response was visible only after 36 h, and less intense that in the wounded leaves. Overall, this experiment demonstrated that TI expression is induced rapidly and that the induction is systemic, consistent with an adaptive role in plant defense.

Jasmonates are common inducers of defense responses in a variety of plants (Weiler, 1997). Preliminary experiments on wound-induced trembling aspen genes indicated that methyl jasmonate is an inducer of the defense response in trembling aspen, and that the induction of defense responses varies with leaf age (not shown). We therefore tested methyl jasmonate-

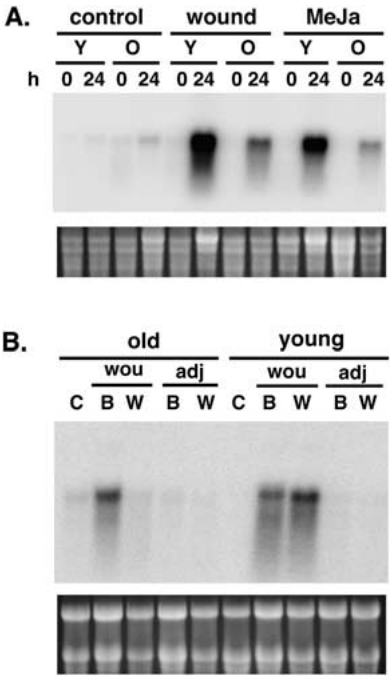


Figure 7. Northern analysis of TI gene expression. Panel A. Inducible expression of TI in leaves from 4-week old (Y) and 12-week old plants (O) before and after a 24 h wounding or MeJa treatment. Panel B. Wound induction of TI gene expression in stems of 4-week old (young) and 12-week old (old) plants. Replica ethidium bromide-stained gels are shown as RNA loading controls. Wou, wounded stems; adj, adjacent unwounded tissue on wounded stems; C, control (entire stems); B and W indicate bark and wood, respectively, from wounded stems.

induced TI expression in trembling aspen leaves from young (4-week old) and older (12-week old) plants. For comparison, a second set of trees was wounded with pliers. In both young and old trees, methyl jasmonate induced TI gene expression, but the response was consistently stronger in the leaves from young trees (Figure 7A). A similar age-dependent pattern was observed for wounded leaves, although overall response levels were stronger for wounded than methyl jasmonate-treated leaves.

In addition, we tested stem tissues for TI induction. Stems from older and younger plants were wounded using a razor, and mRNA from both the bark and the developing wood were analyzed individually by northern blot. In both tissues, TI was wound-induced, and again in younger tissues the response was stronger (Figure 7B). Both bark and developing wood appeared to have wound-induced TI mRNA. However, the induction was restricted to the wounded area, as no TI mRNA induction could be detected in adjacent unwounded stem tissues (Figure 7B).

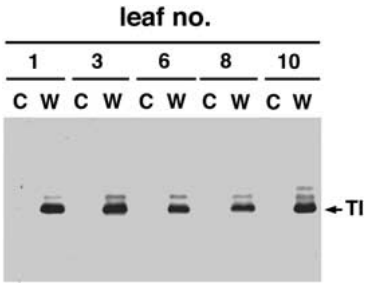


Figure 8. Analysis of TI protein in a series of leaves along the axis of an aspen sapling, before and after wounding. Soluble protein was extracted and analyzed by western blot using an anti-TI antibody. Leaf numbers are assigned beginning with the youngest unfolded leaf. C, control leaf; W, wounded leaf.

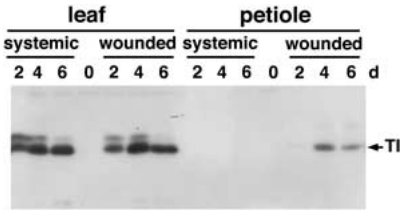


Figure 9. Time course of TI protein accumulation following wounding. Leaves were wounded, and petioles and leaves harvested at the indicated times for western blot analysis using an anti-TI antibody. Systemic tissues are unwounded tissues on wounded plants. The zero time point is the control for both systemic and wounded samples.

Western blot analysis and assays of TI expression

The purified recombinant PtTI2 protein was used to immunize rabbits and obtain an antibody specific for aspen TI. The antibody enabled us to follow the induction of TI in wounded trembling aspen at the protein level using western blot analysis. Leaves at various positions along the main axis of the plants were wounded as before, and protein extracts prepared after 96 h for western blot analysis. The antibody recognized a strongly induced protein in wounded leaf extracts which was not present in unwounded leaves (Figure 8). Although the predicted molecular mass of the PtTIs is 22 kDa, the band migrated at ca. 27 kDa in SDS-PAGE gels, as was observed for the purified recombinant TI (see Figure 3). The identities of two slightly larger but much fainter protein bands recognized by the antibody are uncertain; they may represent isoforms or size variants of the induced TI protein, since they consistently appeared together. As expected from our RNA expression studies, the inducibility of the protein appeared to be somewhat greater in the younger leaves.

In time course experiments, TI protein accumulation was observed in both wounded and systemic leaves two days after wounding and continued to be present until the end of the experiment at day 6 (Figure 9). Previous experiments had established that at the one-day time point, no TI protein had accumulated (not shown). By comparing the intensity of the signal in samples from wounded leaves to a serial dilution of recombinant TI protein, the concentration of TI protein in wounded leaves was estimated to be 250 μg per gram fresh weight (data not shown). Interestingly, in petioles from wounded leaves, TI protein was induced 4 and 6 days after wounding, but no TI protein accumulated in the petioles of systemically induced leaves (Figure 9).

To confirm that the induced accumulation of TI protein demonstrated above did in fact result in increased active trypsin inhibitor in aspen leaves, we carried out trypsin inhibition assays using protein extracts prepared from aspen leaves before and after a 72 h wound treatment. However, UV-absorbing pigments and other unknown substances interfered with the spectrophotometric assay of crude protein extracts (not shown). We therefore heat-treated the extracts, which precipitated some interfering proteins but not TI, and used gel filtration to remove small molecules. This method facilitated TI assays of aspen leaf extracts, although the purification steps required might have resulted in some loss of activity. However, extracts prepared from wounded and systemically wounded leaves consistently contained severalfold more trypsin inhibitor activity than extracts made from control leaves. An amount of 532 ± 131 μg ($n = 3$) soluble leaf protein from control leaves was calculated to maximally inhibit 1 μg of bovine trypsin, compared to an amount of 170 ± 51 μg ($n = 3$) and 232 ± 39 μg ($n = 3$) of leaf protein isolated from wounded and systemically wounded leaves, respectively. The high constitutive trypsin inhibition activity is likely due to the presence of additional unrelated protease inhibitors in aspen. Overall, this experiment confirmed that the induced accumulation of TI mRNA and TI protein we had observed also resulted in increased trypsin inhibitor activity in wounded aspen leaves.

Discussion

Trembling aspen is a widespread and economically important forest species in North America, and is

highly susceptible to insect pests. The herbivore defenses and biochemical ecology of trembling aspen have been studied at the level of secondary plant metabolites, but little is known about defense proteins and their corresponding genes. In this report we describe the cloning and characterization of three trypsin inhibitors from trembling aspen, demonstrate the *in vitro* TI activity of the PtTI2 gene product, and show induction of both TI mRNA and protein in response to tissue damage. Our data strongly suggest that TIs constitute an inducible protein-based defense system of trembling aspen against insect herbivores.

Three TI sequences, belonging to two classes, were isolated by a combination of PCR and cDNA library screening. PtTI1 and PtTI2 constitute one class and may be alleles at the same locus; the previously isolated hybrid poplar gwin3 genes are most similar to this group. PtTI3 defines a second, divergent class. Southern analysis suggests that the PtTI gene family of trembling aspen is large; in addition to the genes characterized here, there appear to be additional TI-like genes, as indicated by weakly hybridizing bands on Southern blots (Figure 2). Altogether we estimate that there are at least three to four Kunitz type TIs in trembling aspen. In hybrid poplar, Hollick and Gordon (1993) identified five distinct Kunitz TI (gwin3-like) sequences in a genomic library, and there are at least three TI genes (swin1-like) in *Salix viminalis* (Saarikoski *et al*., 1996). This multiplicity of TI genes is consistent with a defensive function, since a single TI is effective against proteases found in some but not other herbivores (Ryan, 1990; Richardson, 1991). A battery of TIs with differing specificities for gut proteases could be important for defending against different herbivores with distinct digestive enzymes. Furthermore, several insect pests have been shown to contain a diversity of trypsin-like enzymes which may protect them against the effects of PIs. Jongsma *et al*. (1995) demonstrated that feeding *Spodoptera exigua* tobacco foliage over-expressing potato PI2 caused these herbivores to switch to PI2-resistant proteases, and thus resist the effects of PI2. A similar shift to PI-resistant proteases was seen with several other lepidopteran pests including *Lymantria dispar* and *Helicoverpa zea* when fed diets containing cabbage PI (Broadway, 1995). The presence of several related TI genes in trembling aspen may therefore be a counter-adaptation to the diversity of digestive enzymes in insect.

At the population level, additional TI diversity could reside in multiple TI alleles, as suggested by the restriction fragment polymorphisms detected on Southern blots (Figure 2). Wild populations of trembling aspen have been shown to contain substantial genetic diversity (Yeh *et al*., 1995). TI genes appear to be particularly divergent, since Southern blots using the defense enzyme polyphenol oxidase as a probe detected no restriction fragment polymorphisms between the genotypes tested here (Haruta et al. 2001). Furthermore, PPOs from aspen and hybrid poplar are 92% identical; the highest identity scores for the most similar TIs from these plants (PtTI2 and gwin3) is only 76% (data not shown). It thus appears that the TI genes are evolving more rapidly than other genes. Previous studies of hybrid poplar and *S. viminalis* had described a hypervariable region near the C-terminus of gwin3 and swin1 (Hollick and Gordon, 1993; Saarikoski *et al*., 1996). Given the adaptability of insect pests and their multiple digestive enzymes described above, rapid evolution of TI genes could be very adaptive. A similar situation has been described for disease resistance genes from crop plants, where resistance genes have been shown to evolve more rapidly than other regions of the genome, facilitating rapid adaptation to new pathogen races (Richter and Ronald, 2000).

As demonstrated for hybrid poplar and *S. viminalis*, the PtTI genes of aspen are systemically wound- and herbivore-induced. Northern blots with both PtTI1 and PtTI3 as probes indicated that both classes of TI are inducible, although there appear to be some differences in expression pattern. Such differences will have to be further defined using gene-specific probes, since the full-length cDNA probes used here show some weak cross-hybridization (Figure 2). Induction of Kunitz TI genes by wounding and herbivory has been previously documented in hybrid poplar and *S. viminalis*, as well as in sweet potato (Bradshaw *et al*., 1989; Saarikoski *et al*., 1996; Yeh *et al*., 1997). We also show TI induction at the protein level with a TI-specific antibody. On western blots no TI was detected in control leaves, but accumulated within two days of wounding (Figure 9). In addition, we confirmed that protein extracts of damaged aspen leaves contain significantly higher levels of TI activity than extracts of undamaged leaves. Over-expressed recombinant PtTI2 was able to inhibit trypsin in *in vitro* assays at concentrations comparable to pure soybean Kunitz TI, which directly demonstrates that at least one of the cloned genes encodes a functional protein. Furthermore, preliminary insect bioassays indicated that the recombinant TI protein, when painted onto *Brassica napus* leaves, reduced survival and growth of

Mamestra configurata (bertha army worm), and limited the amount of herbivore damage sustained by the treated leaves (I. Major and P. Constabel, unpublished data). These experiments all suggest that the induction of the TIs is likely to be an important factor in trembling aspen defense.

Similar to other inducible herbivore defense systems, PtTIs appears to be regulated via the octadecanoid pathway (Farmer and Ryan, 1991), as MeJa induced the TI genes in aspen leaves (Figure 7). Octadecanoid-mediated defense in other species, such as tomato, is complex, and at least 19 induced genes are up-regulated by this pathway (Bergey *et al*., 1996). Most likely, trembling aspen induced defenses will be equally diverse, and in addition to TIs, other defense proteins may also be induced. Besides the already mentioned polyphenol oxidase, other up-regulated defense genes in trembling aspen have been identified (D. Peters and P. Constabel, unpublished data).

The significance of identifying the PtTI gene family in trembling aspen is in clearly establishing the presence of an inducible, protein-based defense system in this forest tree. Since toxic phytochemicals, such as phenolic glycosides and condensed tannins, are already well characterized in trembling aspen, this provides an opportunity for studying both inducible protein-based defenses and constitutive secondary metabolite-based defenses within the same experimental system. Potential trade-offs have been proposed to exist between constitutive and induced defense strategies (discussed in Agrawal, 1999), and this could be tested in trembling aspen. A high level of genotype-dependent variability in secondary metabolites has been observed in trembling aspen; for example, condensed tannin levels can vary by a factor of 8–10 (Lindroth and Hwang, 1996; P. Constabel and J. Spence, unpublished data). It will be interesting to assay inducible defenses in these genotypes. The availability of the TI antibody will facilitate studies monitoring inducible trembling aspen defenses.

Alternatively, constitutive and induced defenses may not represent a trade-off, but rather perform complementary functions. This is consistent with Havill and Raffa (1999) who reported no negative correlation in constitutive and induced resistance in a group of diverse poplar hybrids. Multiple defense mechanisms should help to slow the evolution of resistance within herbivore populations, or may represent defenses specialized against particular pests. Furthermore, a different defense strategy may be required in different plant organs or at different developmental stages. For example, young leaves are generally preferred by herbivores because of higher nutritive content and lower structural components, such as lignin (Coley, 1980). For trembling aspen, there is direct evidence that FTC are adapted to these leaves, since under natural conditions, FTC hatch out synchronously with spring leaf flush and feed on the very young leaves. If the hatch is delayed and the larvae are forced to feed on older leaves, development is significantly slowed (Parry *et al*., 1998). Since the first leaves of the season contain lower levels of condensed tannins than later ones (Lindroth and Hwang, 1996; Constabel and Spence, unpublished data), it is tempting to speculate that the young leaves rely on rapidly inducible protein-based defenses, rather than the more slowly accumulating secondary metabolites, for their defense. This idea is supported by our data indicating that the youngest leaves show strongest wound induction of PtTIs (Figure 7). Further studies on PtTI expression in trembling aspen in relation to phytochemical defenses in a variety of genotypes should lead to a clearer picture of the interaction of constitutive and induced defenses of trembling aspen.

In summary, we have characterized a family of Kunitz TI genes from trembling aspen, a species for which PIs had not previously been described. The presence of a family of rapidly diverging TI genes in trembling aspen, their induction in response to herbivory, wounding, and MeJa, and the *in vitro* trypsin inhibitory activity of the PtTI2 gene product all point to a defensive role of PtTI genes. Trembling aspen appears to have evolved multiple defense strategies which includes inducible defense proteins as well as secondary metabolites. Future work will focus on insect bioassays using both recombinant TI protein and TI-over-expressing transgenic plants.

Acknowledgements

We thank Andrew Keddie for providing the forest tent caterpillar larvae, Simon Landhauser for aspen saplings, Om Rajora for advice with aspen micropropagation, and Caroline Constabel (Phytolab, Quebec) for help with aspen tissue culture. This work was supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada.

References

Agrawal, A.A. 1998. Induced responses to herbivory and increased plant performance. Science 279: 1201–1202.

Agrawal, A.A., Tuzun, S. and Bent, E. 1999. Induced Plant Defenses Against Herbivores and Pathogens. APS Press, St. Paul, MN.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl. Acids Res. 25: 3389–3402.

Baldwin, I.T. 1998. Jasmonate-induced responses are costly but benefit plants under attack in native populations. Proc. Natl. Acad. Sci. USA 95: 8113–8118.

Bergey, D., Howe, G. and Ryan, C.A. 1996. Polypeptide signaling for plant defensive genes exhibits analogies to defense signaling in animals. Proc. Natl. Acad. Sci. USA 93: 12053–12058.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quanitities of protein utilizing the principles of protein-dye binding. Anal. Biochem. 71: 248–254.

Bradshaw, H.D. Jr., Hollick, J.B., Parsons, T.J., Clarke, H.R.G. and Gordon, M.P. 1989. Systemically wound-responsive genes in poplar trees encode proteins similar to sweet potato sporamins and legume Kunitz trypsin inhibitors. Plant Mol. Biol. 14: 51–59.

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.

Broadway, R. M. 1995. Are insects resistant to plant proteinase inhibitors? J. Insect Physiol. 41: 107–116.

Broadway, R.M. and Duffey, S.S. 1986. Plant proteinase inhibitors: mechanism of action and effect on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exigua*. J. Insect Physiol. 32: 827–833.

Chang, S., Puryear, J. and Cairney, J. 1993. A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. Rep. 11: 113–116.

Clausen, T., Reichardt, P., Bryant, J., Werner, R., Post, K. and Frisby, K. 1989. Chemical model for short-term induction in quaking aspen (*Populus tremuloides*) foliage against herbivores. J. Chem. Ecol. 15: 2335–2346.

Coley, P.D. 1980. Effects of leaf age and plant life history patterns on herbivory. Nature 284: 545–546.

Constabel, C.P. 1999. A survey of herbivore-inducible defensive proteins and phytochemicals. In: A.A. Agrawal, S. Tuzun and E. Bent (Eds.) Induced Plant Defenses Against Herbivores and Pathogens, APS Press, St.Paul, MN, pp. 137–166.

Constabel, C.P., Bergey, D. and Ryan, C.A. 1995. Systemin activates synthesis of wound-inducible tomato leaf polyphenol oxidase via the octadecanoid defense signaling pathway. Proc. Natl. Acad. Sci. USA 92: 407–411.

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.

Doyle, J.J and Doyle, J.L. 1990. Isolation of plant DNA from fresh tissue. BRL Focus 12: 13–15.

Duffey, S.S. and Felton, G.W. 1991. Enzymatic antinutritive defenses of the tomato plant against insects. In: P.A. Hedin (Ed.) Naturally Occurring Pest Bioregulators, ACS Press, Washington, D.C., pp. 167–197.

Farmer, E.E. and Ryan, C.A. 1992. Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. Plant Cell 4: 129–134.

Gill, S.C. and von Hippel, P.H. 1989. Calculation of protein extinction coefficients from amino acid sequence data. Anal. Biochem. 182: 319–326.

Green, T. and Ryan, C.A. 1972. Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. Science 175: 776–777.

Harborne, J.B. 1993. Introduction to Ecological Biochemistry. Academic Press, London.

Harlow, E. and Lane D. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Plainview, NY.

Haruta, M., Pedersen, J.A., and Constabel, C.P. (2001) Trembling aspen (*Populus tremuloides*) polyphenol oxidase: cDNA cloning, defense-related expression, and identification of a potential substrate. Physiologia Plantarum (in press).

Hattori, T., Nakagawa, T., Maeshima, M., Nakamura, K. and Ashai, T. 1985. Molecular cloning and nucleotide sequence of cDNA for sporamin, the major soluble protein of sweet potato tuberous roots. Plant Mol. Biol. 5: 313–320.

Haukioja, E. 1980. On the role of plant defences in the fluctuation of herbivore populations. Oikos 35: 202–213.

Havill, N.P. and Raffa, K.F. 1999. Effects of elicitation treatment and genotypic variation on induced resistance in *Populus*: impacts on gypsy moth (Lepidoptera: Lymantriidae) development and feeding behavior. Oecologia 120: 295–303.

Heitz, T., Geoffroy, P., Fritig, B. and Legrand, M. 1999. The PR-6 family: proteinase inhibitors in plant-microbe and plant-insect interactions. In: S.K. Datta and S. Muthukrishan (Eds.) Pathogenesis-Related Proteins in Plants, CRC Press, Boca Raton, FL, pp. 131–155.

Hollick, J. and Gordon, M. 1993. A poplar tree proteinase inhibitor-like gene promoter is responsive to wounding in transgenic tobacco. Plant Mol. Biol. 22: 561–572.

Jongsma, M., Bakker, P., Peters, J., Bosch, D. and Stiekema, W. 1995. Adaptation of *Spodoptera exigua* larvae to plant proteinase inhibitors by induction of gut proteinase activity insensitive to inhibition. Proc. Natl. Acad. Sci. USA 92: 8041–8045.

Karrer, E.E., Beachy, R.N. and Holt, C.A. 1998. Cloning of tobacco genes that elicit the hypersensitive response. Plant Mol. Biol. 36: 681–690.

Lee, S.I., Lee, S.H., Koo, J.C., Chun, -H.J., Lim, C.O., Mun, J.H., Song, Y.H. and Cho, M.J. 1999. Soybean Kunitz trypsin inhibitor (SKTI) confers resistance to the brown planthopper (*Nilaparvata lugens* Stal) in transgenic rice. Mol. Breed. 5: 1–9.

Lindroth, R.L., Hsia, M.T.S. and Scriber, J.M. 1988. Chemical ecology of the tiger swallowtail: mediation of host use by phenolic glycosides. Ecology 69: 814–822.

Lindroth, R.L. and Hwang, S.-Y. 1996. Diversity, redundancy, and multiplicity in chemical defense systems of aspen. In: J.T Romeo, J.A. Saunders and P. Barbosa (Eds.) Phytochemical Diversity and Redundancy in Ecological Interactions, Plenum Press, New York, pp. 25–56.

Lindroth, R.L. and Kinney, K.K. 1998. Consequences of enriched atmospheric CO_2 and defoliation for foliar chemistry and gypsy moth performance. J. Chem. Ecol. 24: 1677–1695.

Marchuk, D., Drumm, M., Saulino, A. and Collins, F.S. 1991. Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nucl. Acids Res. 19: 1154.

Orozco-Cardenas, M., McGurl, B. and Ryan, C.A. 1993. Expression of an antisense prosystemin gene in tomato plants reduces resistance toward *Manduca sexta* larvae. Proc. Natl. Acad. Sci. USA 90: 8273–8276.

Parry, D., Spence, J.R. and Volney, W.J.A. 1998. Budbreak phenology and natural enemies mediate survival of first-instar forest

tent caterpillar (Lepidoptera: Lasiocampidae). Envir. Entomol. 27: 1368–1374.

Parsons, T., Bradshaw, H. Jr. and Gordon, M. 1989. Systemic accumulation of specific mRNAs in response to wounding in poplar trees. Proc. Natl. Acad. Sci. USA 86: 7895–7899.

Peterson, E.B. and Peterson, N.M. 1992. Ecology, Management, and Use of Aspen and Balsam Poplar in the Prairie Provinces. Forestry Canada Northwest Region Special Report No. 1.

Richardson, M. 1991. Seed storage proteins: the enzyme inhibitors. Meth. Plant Biochem. 5: 259–305.

Richter, T.E. and Ronald, P.C. 2000. The evolution of disease resistance genes. Plant Mol. Biol. 42: 195–204.

Ryan, C.A. 1990. Protease inhibitors in plants: genes for improving defenses against insects and pathogens. Annu. Rev. Phytopath. 28: 425–449.

Saarikoski, P., Clapham, D. and von Arnold, S. 1996. A wound-inducible gene from *Salix viminalis* coding for a trypsin inhibitor. Plant Mol. Biol. 31: 465–478.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Press, Plainview, N.Y.

Schuler, T.H., Poppy, G.M., Kerry, B.R. and Denholm, I. 1998. Insect-resistant transgenic plants. Trends Biotechnol. 16: 168–175.

Spencer, M.E. and Hodge, R. 1991. Cloning and sequencing of the cDNA encoding the major albumin of *Theobroma cacao*. Identification of the protein as a member of the Kunitz protease inhibitor family. Planta 183: 528–535.

Wann, S.R. and Einspahr, D.W. 1986. Reliable plantlet formation from seedling explants of *Populus tremuloides* (Michx.). Silv. Gen. 35: 19–24.

Weiler, E.W. 1997. Octadecanoid mediated signal transduction in higher plants. Naturwissenschaften 84: 340–349.

Wishart, D.S., Stothard, P. and van Domselaar, G.H. 2000. PepTool and GeneTool: platform-independent tools for biological sequence analysis. Meth. Mol. Biol. 132: 93–113.

Worthington, C.C. 1988. Worthington Enzyme Manual. Worthington Biochemical Corporation, Freehold, NJ, p. 346.

Yeh, F.C., Chong, D.K.X. and Yang, R.C. 1995. RAPD variation within and among natural populations of trembling aspen (*Populus tremuloides* Michx.) from Alberta. J. Hered. 86: 454–460.

Yeh, K., Chen, J., Lin, M., Chen, Y. and Lin, C. 1997. Functional activity of sporamin from sweet potato (*Ipomoea batatas* Lam.): a tuber storage protein with trypsin inhibitory activity. Plant Mol. Biol. 33: 565–570.