Running Title: Functional analysis of the poplar Kunitz trypsin inhibitor family

Corresponding author:
C. Peter Constabel
Centre for Forest Biology and Department of Biology
University of Victoria
Stn CSC, PO Box 3020
Victoria, BC
V8W 3N5, Canada
Telephone: +1-250-4725140
Fax: +1-250-7216611
Email: cpc@uvic.ca

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Functional Analysis of the Kunitz Trypsin Inhibitor Family in Poplar Reveals Biochemical Diversity and Multiplicity in Defense against Herbivores

Ian T. Major and C. Peter Constabel

Centre for Forest Biology and Department of Biology, University of Victoria, Stn CSC, PO Box 3020, Victoria, BC, V8W 3N5, Canada
Footnotes:

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2Current address: Laurentian Forestry Centre, Canadian Forest Service, Sainte-Foy, Québec, Canada

*Corresponding author:
C. Peter Constabel
Telephone: +1-250-4725140
Fax: +1-250-7216611
Email: cpc@uvic.ca
ABSTRACT
We investigated the functional and biochemical variability of Kunitz trypsin inhibitor (KTI) genes of hybrid poplar (*Populus trichocarpa x P. deltoides*). Phylogenetic analysis, expressed sequence tag databases, and western blot analysis confirmed that these genes belong to a large and diverse gene family with complex expression patterns. Five wound- and herbivore-induced genes representing the diversity of the KTI gene family were selected for functional analysis, and shown to produce active KTI proteins in *E. coli*. These recombinant KTI proteins were all biochemically distinct and showed clear differences in efficacy against trypsin, chymotrypsin, and elastase type proteases, suggesting functional specialization of different members of this gene family. The *in vitro* stability of the KTIs in the presence of reducing agents and elevated temperature also varied widely, emphasizing the biochemical differences of these proteins. Significantly, the properties of the recombinant KTI proteins was not predictable from primary amino acid sequence data. Proteases in midgut extracts of *Malacosoma disstria*, a lepidopteran pest of *Populus*, were strongly inhibited by at least two of the KTI gene products. This study suggest that the large diversity in the poplar KTI family is important for biochemical and functional specialization, which may be important in the maintenance of pest resistance in long-lived plants such as poplar.
INTRODUCTION

Plants actively respond to challenge by insect herbivores with a rapid induction of a suite of biochemical defenses. These defenses can include both secondary metabolites and proteins, which may act as toxins, antifeedants or antinutrients. One of the most common inducible herbivore defenses in plants is the rapid synthesis of proteinase inhibitors, small proteins that inhibit insect digestive proteases and lead to reduced insect growth rates or increased mortality (Ryan, 1990). Other induced antinutritive proteins of plants include oxidative enzymes that destroy or modify dietary amino acids and fatty acids, such as polyphenol oxidase (PPO), peroxidase and lipoxygenase (Duffey and Felton, 1991), as well as the essential amino acid-degrading enzymes arginase and threonine deaminase (Chen et al., 2005; Chen et al., 2007). Defensive proteins that affect insect growth via other mechanisms include the cysteine proteases that permeabilize the insect peritrophic matrix (Pechan et al., 2002; Konno et al., 2004; Mohan et al., 2006). Some plants also enhance insect resistance by increasing the synthesis of alkaloids, terpenoids, glucosinolates and phenolics in response to damage (Walling, 2000; Kessler and Baldwin, 2002). The types of secondary metabolites and proteins with potential anti-herbivore activity are numerous and often taxon specific; consequently, plants can affect insects via many different mechanisms (reviewed in Constabel, 1999).

Proteinase inhibitors (PIs) are widespread in the plant kingdom and have been described from many plant species and tissues. The PIs comprise at least ten distinct protein families, classified by their amino acid sequence and the mechanistic class of proteinases they inhibit (Laskowski and Kato, 1980; De Leo et al., 2002; Rawlings et al., 2004). Most plant PIs, such as the well-characterized Kunitz and Bowman-Birk PI families, inhibit serine proteinases, in particular trypsin. The Kunitz-type protease inhibitors, referred to hereafter as Kunitz trypsin inhibitors (KTI), are proteins of approx. 20 kDa with one or two disulfide bonds and a single reactive site. By comparison, the Bowman-Birk inhibitors are smaller (approx. 8-10 kDa) with high cysteine content and two reactive sites (Richardson, 1991). The interaction of KTIs with their cognate proteases is well-characterized; the reactive site of the KTI binds tightly to the active site of the protease in a substrate-like manner, and the KTI thus acts as a competitive inhibitor. Although the reaction is reversible, some KTIs have such strong affinities for their target protease that the reaction can be considered irreversible (Beynon and Bond, 1989). The structure of plant KTIs is a β-trefoil fold of 10 to 12 antiparallel β-strands connected by long
loops (Song and Suh, 1998). The reactive site is located on a protruding reactive loop, which forms H-bonds with the active groove of the protease. Most KTI proteins have four conserved cysteine residues that form two disulfide bridges, although examples with only one or no disulfide bridges are known (do Socorro et al., 2002; Araujo et al., 2005; Macedo et al., 2007). Evidence from several studies suggests that the first disulfide bridge, which surrounds the reactive loop, is necessary for inhibitory activity of most KTIs (DiBella and Liener, 1969; do Socorro et al., 2002).

As a group, plant KTIs have extremely diverse protease targets and thus have negative effects on a broad range of phytophagous pests and pathogens. Individual KTIs typically have more specific activities. While many plant KTIs inhibit trypsin or chymotrypsin, some can inhibit other serine proteinases such as elastase and subtilisin (Terada et al., 1994; Valueva et al., 2000; Revina et al., 2004; Sumikawa et al., 2006). KTIs that inhibit cysteine or aspartic proteinases are also reported (Ritonja et al., 1990; Rowan et al., 1990; Heibges et al., 2003b). Moreover, some proteins belonging to the Kunitz family do not act as protease inhibitors (McCoy and Kortt, 1997), or may have lectin-like carbohydrate binding or invertase inhibitor activity (Glaczinski et al., 2002; Macedo et al., 2004). Some plant KTIs are antimicrobial, presumably via inhibition of microbial proteinases (Kim et al., 2005; Park et al., 2005). Thus, determining a precise function of KTI-like proteins cannot be based only on primary sequence similarities, but requires in vitro assays for confirmation. While the biochemical activity of many individual plant KTIs is known, only in potato has an extensive analysis of a large KTI family within the same species been reported (Heibges et al., 2003a; Heibges et al., 2003b). In that study, the KTI family showed surprising sequence variation including many nonsynonymous substitutions and indels, which appears to translate into functional diversity.

Poplar has been developed as a woody plant model in genomics and molecular biology, and is especially useful as an experimental system for ecological genomics. As trees, poplars are long-lived organisms faced with many generations of pest insects and pathogens. The P. trichocarpa genome is now fully sequenced (Tuskan et al., 2006), and large expressed sequence tag (EST) collections are available and can be used for digital analysis of gene expression and gene discovery (Sterky et al., 2004). Microarrays are also being used for global expression analysis. These resources all provide unprecedented access to genome-level information in a plant of significant ecological importance and world-wide distribution, and make this an
excellent system for studies of plant defense proteins. The first KTI (\textit{win3}) from hybrid poplar was described by Gordon and co-workers (Bradshaw et al., 1990; Hollick and Gordon, 1993, 1995). Two wound-inducible \textit{win3} paralogs (\textit{PtTI1}, \textit{PtTI2}) were later isolated from \textit{P. tremuloides} (Haruta et al., 2001). This study also described wound-inducible accumulation of the corresponding proteins and their \textit{in vitro} inhibitor activity, consistent with a role for these KTIs in herbivore defense. Furthermore, heterologous expression of \textit{win3} in tobacco leaves demonstrated a modest but significant reduction in growth of tobacco budworm (Lawrence and Novak, 2001). Recently, new wound-inducible KTIs from hybrid poplar were identified via EST gene discovery programs (Christopher et al., 2004; Ralph et al., 2006), and transcript profiling studies have shown that several KTI genes are among the most strongly induced genes after wounding and herbivory (Major and Constabel, 2006; Ralph et al., 2006). This work points to a significant role of the KTI gene family in induced herbivore defense of poplar.

Here we characterize the protease inhibitory activities of five representative wound-inducible members of the KTI gene family as recombinant proteins from \textit{E. coli}. We determine that all the poplar KTI genes tested encode functional inhibitors but with different inhibitor profiles, protease preferences, and stabilities. The combined expression patterns and inhibitory activities of the KTIs correlated with protease inhibitor activity of wounded poplar leaf extracts. We also demonstrate inhibitory activity of several KTIs against larval midgut proteases from \textit{Malacosoma disstria} and \textit{Mamestra configurata}, pests of \textit{Populus} and crucifers, respectively. Therefore, our study provides direct evidence of a role of the poplar KTI gene family in the inducible defense of \textit{Populus} species against insect herbivores.
RESULTS

The Poplar Kunitz trypsin inhibitor family contains many diverse members

As part of our ongoing analysis of the poplar defense response, we sought to characterize herbivore- and wound-inducible KTIs in detail. Wounding leaves induces transcript accumulation of several poplar KTIs, including win3 in hybrid poplar and the win3 orthologs PrTI1 and PrTI2 in *P. tremuloides* (Bradshaw et al., 1990; Haruta et al., 2001). Previously, we identified three novel KTIs among the most abundant and most highly induced transcripts during the hybrid poplar wound response (Christopher et al., 2004; Major and Constabel, 2006). In addition, the recent availability of the *P. trichocarpa* genome sequence facilitated a comprehensive analysis of the KTI gene family. We determined that poplar has at least 22 KTIs (Fig. 1), but with sequence ambiguities the number of KTIs could be as high as 30. In this analysis, we included only gene models that were full-length, contained the Kunitz motif 

\[(L,I,V,M)-X-D-X_2-G-X_2-[L,I,V,M]-X_2-Y-X-[L,I,V,M]\]

and at least one disulfide bond. KTI gene models with nucleotide sequences \(\geq 99\%\) identical were considered to be allelic; such sequences typically had synonymous to non-synonymous substitution ratios of 1. For comparison, sequences \(\leq 97\%\) identical and with synonymous to non-synonymous ratios of approx. 1.5 were not considered allelic. Phylogenetic analysis revealed that the KTI family is highly diverse; at the amino acid level, the similarity of the KTIs is as low as 25% (Supplemental Table S1). Inspection of the phylogenetic tree revealed that the KTI family consists of several clades. KTIs within the same group are approx. 60% to 70% similar at the amino acid level, while KTIs from different groups share only approx. 30% of amino acid residues. Moreover, at least one of these homology groups (A) can be subdivided further into subgroups A1, A2 and A3. *gwin3*, the first poplar TI to be studied (Bradshaw et al., 1990; Hollick and Gordon, 1993, 1995), and *win3*-like genes form subgroup A1. This subgroup also includes *PrTI1* and *PrTI2*, very recently duplicated genes from *P. tremuloides* (Haruta et al., 2001; Talyzina and Ingvarsson, 2006). The *gwin3* and *win3.12* KTI genes were chosen for phylogenetic analysis because hypervariability of the *win3* (*TI2*) locus prevented identification of a full-length gene model corresponding to *gwin3* in the poplar genome (v1.0 and v1.1). The *win3* locus has been shown to consist of several clustered KTI genes interspersed with repeats (Bradshaw et al., 1990; Hollick and Gordon, 1993), which is reflected by a region densely populated with repetitive
DNA and truncated KTI gene models in the poplar genome sequence. Additional analysis will be required to resolve the gene organization at this locus.

The three previously identified wound-inducible KTIs (PtdTI3, PtdTI4, PtdTI5) each belong to separate homology subgroups (Christopher et al., 2004; Major and Constabel, 2006). TI3 forms subgroup A3 with 739063, while TI4 is a member of group B with another three genes. TI5 does not fit with any of the homology groups and is found on a separate branch. The two recently reported KTIs TI6 and TI7 belong to subgroup A2 along with two other genes (Talyzina and Ingvarsson, 2006). Group C includes eight genes, none of which have been previously studied.

Inspection of sequence alignments of representative KTIs from each clade / subgroup further illustrates their sequence diversity (Fig. 2). This made aligning the sequences somewhat unreliable; to improve the quality of the alignment, secondary and tertiary structure predictions were made using JPred, SWISS-MODEL, CPHmodels and ESyPred3D, which were used to manually edit and refine the alignment. We included the extensively studied soybean KTI (GmKTI) and sporamin from Ipomoea batatas for comparison. While all KTIs contain the Kunitz motif, they may otherwise share little overall amino acid similarity, including the position of gaps. However, it is interesting that the most conserved regions correspond to predicted β-sheets and, for the poplar KTIs, the signal sequence. This is consistent with a recent analysis of molecular evolution of the poplar KTI group A, which showed that the loop regions connecting β-strands in particular are under positive selection (Talyzina and Ingvarsson, 2006).

Furthermore, while some conserved residues are found within the reactive loop of the poplar KTIs, this loop is highly variable, including the P1 residue of the reactive site (boxed area and starred residues, Fig. 2). The reactive loop of sporamin has atypical residues compared with GmKTI and other plant KTIs, but is similar to the poplar KTIs. For the P1 site of the seven aligned poplar KTIs, six different residues are predicted but none are Lys or Arg. These are most common among plant KTIs and considered specific for trypsin inhibition (Beynon and Bond, 1989). Interestingly, the entire predicted reactive loops are devoid of Lys or Arg, except for 75560. By contrast, many of the poplar KTIs have a Ser as the P1’ residue. These conserved and variable regions are presumably indications of the selective pressures exerted on the KTI family (see Discussion).
The poplar KTIs typically contain four cysteine residues that form two conserved intramolecular disulfide bonds. An exception is TI5, which has only two Cys residues that form the first disulfide bond. All the poplar KTIs except TI5 and 554072, also have two additional free Cys residues located in a loop (Cys165 and Cys167, win3 numbering). Overall, the poplar KTIs share very little similarity with GmKTI (less than 25% similarity), sporamin and other well-characterized plant KTIs. This sequence diversity, especially of the reactive loop, predicted that the poplar KTIs should have distinct inhibitory activities (see below).

**Poplar KTIs show both developmental and wound-inducible expression patterns**

To determine in which tissues the various KTI genes are expressed, we queried the PopulusDB database for digital expression profiles derived from the abundance of ESTs in diverse libraries (Sterky et al., 2004). The expression patterns varied widely across the gene family (Fig. 3). We note that these digital northerns do not distinguish the expression patterns of highly similar sequences, due to ambiguous EST clusters that correspond to several gene models. For example, KTIs TI3 and 739063, which have 87% nucleotide sequence identity (Supplemental Table S1), match EST sequences from the same cluster in PopulusDB, and so their expression patterns are reported as identical. The highest levels of expression (EST abundance) were found for TI3 / 739063. These were expressed in essentially all tissues, while expression of other KTIs was more restricted. KTIs of the A1 and A2 subgroups (e.g. GWIN3, TI6, TI7) were abundant in flowers, young leaves and the apical shoot. None of the remaining KTIs appeared to be expressed at significant levels in healthy leaf tissue, from which most cDNA libraries in PopDB are derived. Interestingly, many KTIs were expressed in floral tissues, most notably female catkins. Since the EST sets comprising this digital northern did not include any herbivore- or stress-induced tissues, we also included a summary of results from recent transcript profiling studies (Christopher et al., 2004; Major and Constabel, 2006; Ralph et al., 2006) (right panel, Fig. 3). This analysis indicated that inducible herbivore- and wound-induced expression also varies across the family; while most of the KTIs of group A are induced by wounding, except TI4 and TI5 the remaining KTIs appeared not to have this pattern. However, for some genes no wound-induced expression data are available.

In order to confirm that the observed wound-induced changes in transcript levels translate into increased protein accumulation, we performed western blot analysis using the two
antibodies available for KTIs. The TI2 antibody is specific for KTIs in the win3/TI2 subgroup and detected several isoforms in leaves (Fig. 4). For simplicity, we collectively refer to these bands as TI2. The presence of multiple TI2 isoforms is consistent with the complexity and multiplicity of this subgroup (Bradshaw et al., 1990; Hollick and Gordon, 1993; Haruta et al., 2001). By contrast, on western blots the TI3 antibody recognized only a single band corresponding to TI3 protein (Fig. 4). A survey of hybrid poplar tissues determined that TI2-like proteins were found only in wounded leaves, while TI3 was present constitutively in most organs including leaves, bark, wood and roots (Fig. 4A). TI2 and TI3 were both constitutively present in female catkins of field-grown trees, with minor levels of both proteins in male catkins (Fig. 4B). TI2 protein accumulation in leaves was strongly induced by wounding, and TI3 protein showed only a modest wound-induction. Furthermore, TI2 protein induction was clearly stronger in younger leaves, i.e. at leaf plastochron index (LPI) 3-5, while TI3 protein accumulation was relatively consistent independent of tissue age. These KTI proteins thus show very distinct patterns of protein accumulation, but both patterns were consistent with digital northern data (Fig. 3). After wounding, foliar levels of TI2 and TI3 proteins accumulated rapidly after wounding and with similar kinetics; no increase in protein was visible in the first 24 hrs, but both proteins accumulated to substantial levels 2 d after wounding (Fig. 4C). A similar pattern of TI2 protein accumulation was previously observed in *P. tremuloides* (Haruta et al., 2001).

Since both transcript and protein levels of poplar KTIs increased in leaves in response to leaf damage, proteinase inhibitor activity should also increase. We therefore assayed inhibition of serine proteinases by crude leaf extracts using a kinetic assay (Worthington, 1988); such assays are more accurate than end-point assays, since activity measurements are effectively the means of continuous time points. Different amounts of leaf extract were tested with trypsin, chymotrypsin and elastase, three common digestive enzymes found in lepidopteran pests. We first compared trypsin inhibitor activity of leaf extracts from immature leaves at LPI 3-5, and mature leaves of LPI 9-11, and found that the activity was greatest for younger leaves of both untreated and wounded saplings (data not shown). We therefore focused on young leaves (LPI 3-5) for subsequent tests of inhibitory activity against other proteases, and calculated the 50% inhibitory concentrations (IC50) for control and wounded leaf extracts against the three proteases. For undamaged saplings, leaf chymotrypsin inhibition was higher than trypsin inhibition (IC50 of 0.072 gL−1 and 0.23 gL−1 respectively; see insets to Fig. 5), but wounded leaves contained similar
levels of trypsin and chymotrypsin inhibitor activity (IC$_{50}$ of 0.039 gL$^{-1}$ and 0.029 gL$^{-1}$, respectively). Elastase inhibitor activity represented only a small fraction of the total inhibitory activities. All three proteinase inhibitory activities increased significantly following wounding (2.5 - 5.9 fold change in IC$_{50}$, $P << 0.001$). As observed in untreated leaf extracts, elastase inhibitory activity was a small proportion compared to trypsin and chymotrypsin after wounding. Therefore, as already suggested by the western analyses, these data confirm that KTI gene induction is followed by an increase in protease inhibitory activity in poplar leaves.

Recombinant poplar KTI proteins exhibit distinct inhibitory activities and biochemical properties

We predicted that the substantial sequence diversity of the poplar KTI genes should translate into diverse biochemical and biological properties of the corresponding proteins. To test this hypothesis, we characterized the functions of TI2, TI3, TI4, TI5 and TI6 proteins in detail, since these represent the diversity of the KTI family. High levels of recombinant protein expressed in *E. coli* were observed for all KTI bacterial expression constructs, with the bulk of the recombinant proteins found in inclusion bodies, as illustrated for TI3 (Fig. 6A). The proteins were purified under denaturing conditions and dialyzed in order to renature the polypeptides and recover active protein. We initially tested the renaturation conditions used successfully for TI2 (Haruta et al., 2001), but found that under these conditions most of the other KTIs formed aggregates and precipitated. We therefore optimized the renaturation conditions for each KTI by altering the ionic strength, pH and concentration of reducing agent of the buffer. These tests showed that optimal re-folding conditions varied considerably among the different KTIs (Table I). Thus, four of the KTIs were found to be soluble only in a buffer of high ionic strength, whereas TI3 was soluble in water or a buffered low-strength ionic solution. Moreover, while most KTIs were soluble near pH 8, their solubility differed greatly away from pH 8.

We verified the purity and concentrations of the successfully renatured KTIs by SDS-PAGE, which clearly showed a single major band for each KTI at approximate predicted $M_r$ (Fig. 6B). In addition, larger bands were apparent for TI3, TI4 and TI6; these may be protein dimers, as the apparent $M_r$ of these bands are twice that of the predominant bands. We previously reported putative dimers for TI2 (Haruta et al., 2001), and other plant KTIs are known to form dimers as well (Terada et al., 1994). Such dimers may be linked by an intermolecular disulfide bond.
formed between the free Cys residues of the KTIs (Fig. 2), since the predicted 3D models suggest that the residues are exposed on the surface of the proteins (data not shown). While migration of TI2, TI4 and TI6 in gels corresponded well with their predicted Mr (Table I), TI3 and TI5 migrated slower than expected. This is likely the result of residual secondary protein structure, since both recombinant proteins migrated according to their predicted Mr on denaturing urea-SDS-PAGE gels, which denature proteins more completely (data not shown).

Using in vitro assays with commercially available proteases, we confirmed that the recombinant and renatured poplar KTI proteins have protease inhibitory activity. Commercial GmKTI from soya was included for comparison since it has been extensively described in the literature. Because previous studies of plant KTIs have found a wide range of possible target proteases, we assayed inhibitor activity against several proteases: the serine proteinases trypsin, chymotrypsin, elastase and subtilisin, and the cysteine proteinase papain. We again used kinetic assays at multiple TI concentrations to test inhibition of trypsin, chymotrypsin and elastase. End point assays were used to test additional proteinases outside the chymotrypsin subfamily, the serine protease subtilisin and cysteine protease papain. These experiments confirmed that all KTIs tested are active protease inhibitors, but with remarkably different target preferences (Fig. 7). Thus, recombinant TI2 was a strong inhibitor of trypsin (Fig. 7, top panel), with activity levels similar to that of GmKTI, while TI3, TI5 and TI6 had intermediate levels of inhibitor activity. In contrast, TI4 did not inhibit trypsin at detectable levels. Against chymotrypsin, TI6 was the most potent of the recombinant poplar KTIs, though less potent than GmKTI, followed by TI3, TI5, and TI4 (Fig. 7, center panel). Although TI2 was very effective against trypsin, it failed to inhibit chymotrypsin significantly. The only poplar KTI with inhibitory activity against elastase was TI6 (Fig. 7, bottom panel). None of the poplar KTIs tested were active against subtilisin or papain.

To help compare the inhibitory profiles of the KTIs, we calculated the IC$_{50}$ for each KTI (Table II). Such IC$_{50}$ values permit the comparison of inhibitory activities relative to the various proteases tested and emphasize the very different activity profiles of the five poplar KTIs. We note that these are maximum IC$_{50}$ values, since we could not determine directly if all protein in each preparation was fully re-folded. However, we obtained similar inhibitory activities from independent recombinant protein preparations. The comparison of IC$_{50}$ indicated that several KTIs have strong preferences for specific targets (Table II). TI2 appears to be a specific inhibitor
of trypsin, while TI4 was specific to chymotrypsin. TI3 and TI5 both inhibited trypsin and chymotrypsin, and TI6 inhibited chymotrypsin and elastase. Thus all the recombinant KTIs tested were active as protease inhibitors, and as a group inhibited the major digestive enzymes trypsin, chymotrypsin, and elastase.

Recent work has suggested that protein stability is a key function of plant defense proteins, given the hostile environment of herbivore digestive systems (Chen et al., 2007). We found that KTI activity remained high even after dialysis at pH extremes (Table I). We therefore investigated KTI stability by assessing the denaturing effects of a reducing agent or increasing temperature. Purified recombinant KTIs were first incubated with increasing concentrations of DTT for 30 min, after which remaining inhibitory activities were measured. These activities changed little up to 2 h and thus only data for the 30 min incubations are shown (Fig. 8A). In general, each KTI protein was stable to a critical DTT concentration, beyond which it quickly lost activity. This critical concentration and the rate of activity loss differed for each KTI, giving rise to distinct stability profiles (Fig. 8A). TI4 and TI6 were both resistant to 0.1 mM DTT, but were differentially sensitive at higher concentrations. TI3 retained activity up to 1 mM DTT but quickly lost activity above this level. TI5 was most resistant to reducing conditions, since it retained 65% activity at 100 mM DTT, even after a 2-h incubation. Interestingly, TI2 activity increased to an optimal 31.6 µM DTT, but quickly lost activity at higher concentrations and lost all activity beyond 3 mM DTT.

Similarly, we found that the poplar KTIs had different stabilities to elevated temperatures. KTI proteins were incubated for 30 min at different temperatures, and remaining inhibitory activities were compared with samples kept on ice (Fig. 8B). TI2 was most thermolabile, losing activity above 30°C. Similarly, TI3 was susceptible to temperatures above 40°C. We also repeated these experiments for TI2 and TI3 with gradual cooling after incubation at temperature, and found similar losses of activity (data not shown). TI6 was more thermostable than both TI2 and TI3, remaining active after incubation at 60°C. Neither TI2, TI3 nor TI6 were boiling stable, since they retained less than 20% activity after boiling. By contrast, TI5 was stable at all temperatures, retaining 80 to 100% activity at all temperatures assayed. TI4 was also stable at high temperatures, but exhibited enhanced activities of 125 to 130% when incubated at 60°C or greater.
In view of the apparent temperature stability of the KTIs, we also measured long-term stabilities by assaying the recombinant KTI preparations at 4°C at 25-day intervals for extended time periods (Supplemental Fig. 1). We found no loss in activity within 150 days, though the measured activities of the KTIs fluctuated between 80% and 130%. We extended this analysis to more than one year for TI2, again measuring activity periodically, and found no loss of activity (supplemental Fig. 1). Overall, these tests demonstrated the remarkable stability of the poplar KTI proteins.

**Recombinant poplar KTIs inhibit lepidopteran midgut proteases**

The inhibitory activity of recombinant KTIs against commercially available proteases of mammalian origin prompted us to test these KTIs against digestive proteases from insects. We obtained midguts from bertha armyworm (BAW, *Mamestra configurata*), a lepidopteran pest of crucifers. The midgut proteases of BAW have been well-characterized, and like other lepidopteran larvae, consist primarily of serine proteinases, particularly trypsin-, chymotrypsin- and elastase-like enzymes (Hegedus et al., 2003). For these assays, different amounts of poplar KTIs were incubated with a standard quantity of BAW midgut extract, and the remaining protease activity was measured. Assays were performed at two different pHs, which correspond to two pH optima of BAW midgut protease activity, using an end-point assay with azocasein as a general protease substrate (Hegedus et al., 2003). In contrast to our results with commercial mammalian proteinases, TI3 was the only poplar KTI tested with significant activity against BAW midgut proteases (Fig. 9, top panel, and data not shown). GmKTI was also an effective inhibitor, consistent with the results of Hegedus et al. (2003) (Fig. 9, bottom panel). Both TI3 and GmKTI were more potent inhibitors of BAW gut protease activity at pH 11 than 8. At pH 11, TI3 inhibited approx. 40% of BAW total midgut proteolytic activity, while GmKTI inhibited at least 50%. Extrapolation of the linear portions of these plots shows that only 0.47 µg of TI3 was needed to inhibit 40% of BAW protease activity, whereas 6.8 µg of GmKTI was required to inhibit the same activity. Thus, TI3 has a much greater affinity for at least some BAW midgut proteases. A similar pattern was observed for pH 8 assays, where TI3 was more potent but had a lower maximum inhibition of gut protease activity compared to GmKTI.

We also tested poplar KTI inhibition of midgut extracts from forest tent caterpillar (FTC, *Malacosoma disstria*), a lepidopteran pest of poplars. We found that protease activity of FTC
midguts is due primarily to serine proteases with a pH optimum of 11.5 (data not shown). As with BAW protease assays, we measured residual protease activity after incubation of poplar KTIs or GmKTI with a standard quantity of FTC midgut extracts. All five KTIs inhibited FTC midgut proteolysis, although only TI2 and TI3 were strong inhibitors (Fig. 10). Comparison of inhibition curves revealed that TI3 inhibited FTC gut proteases with comparable apparent potency as GmKTI, and with greater potency than TI2 (Fig. 10). Furthermore, TI3 inhibited almost 70% of protease activity, while TI2 inhibited approx. 40%. These maximum inhibition values, which represent the proportion of the mixture of midgut proteases that are susceptible to inhibition, confirmed the potency of these KTIs against FTC and establish their ecological relevance.
DISCUSSION

Kunitz TIs have been studied in many different plants and contexts, often with a focus on their potential for biotechnology-based pest control for agriculture; however, the diversity of KTIs within a plant species has not been extensively examined. The prevalence of KTIs in the poplar genome represents a unique opportunity to explore the variation and function of these proteins. Although the precise number of KTI genes in the *P. trichocarpa* genome has not yet been definitively determined, there could be as many as 30 genes. The region surrounding the *win3/TI2* group in particular contains much repetitive DNA and appears to have evolved rapidly, making this clade of the gene family difficult to resolve. For a functional analysis in the context of plant herbivore defense, we selected five herbivory-inducible genes that represent the diversity of the entire poplar KTI family. Our analysis indicates that each KTI gene product is a functional protease inhibitor with a distinct range of target protease preferences, and shows differential stability to reducing agents and temperature.

The expression of KTI genes correlates with inducible protease inhibitor activity in poplar leaf extracts

Based on our interest in herbivory, we chose to characterize representative poplar KTIs from homology groups that were wound- or herbivore-inducible. Results from published array and northern analyses suggested that group C KTIs generally do not respond to wounding (Fig. 3). Therefore this group was not included in our analysis, though it is an important target for future studies. The inducible nature of TI2 and TI3 was demonstrated at the protein level by western blot analysis, and these proteins showed rapid accumulation after wounding of leaves (Fig. 4). This suggests that for the other KTIs, induced transcripts are also likely to result in increased levels of the gene products. Furthermore, we showed a significant induction of total PI activity in wounded poplar leaves. Interestingly, wounded poplar leaf extracts inhibited chymotrypsin and trypsin almost equally, whereas control leaf extracts were more effective against chymotrypsin. This pattern likely reflects the strong inducibility of TI2 (Fig. 4) and its strong preference for trypsin (Table II). Only low levels of elastase inhibitory activity were found in leaf extracts, as predicted by our finding that only one KTI (TI6) had any significant anti-elastase activity (Fig. 7). Therefore, total PI activity in leaf extracts generally correlated with the activities of the
expressed KTI proteins. The induction of protease inhibitory activities in crude extracts of wounded leaves is important because it demonstrates that upregulation of KTI genes has a demonstrable impact on herbivore food quality.

Analysis of transcript abundance from digital northerns showed highly diverse expression patterns across the KTI family. For example, based on EST abundance, win3/TI2 expression was highly specific while TI3 showed very broad pattern of expression, which was confirmed by western analysis (compare Fig. 3 and Fig. 4). The win3/TI2 protein accumulated to highest levels in young leaves, consistent with the corresponding transcripts being detected mostly in young leaves, the shoot apex, and floral organs (see below). Most of the other poplar KTIs also exhibited relatively tissue-specific patterns of expression, again with preferred expression in flowers (TI4) and shoot meristem (TI5) (Fig. 3). By contrast, TI3 protein and transcripts accumulated in almost all tissues (Fig. 3 and Fig. 4). The apparently ubiquitous expression of TI3 is curious, since proteinase inhibitors are thought to incur large fitness costs (Zavala et al., 2004). Presumably TI3 has acquired new regulatory sequences during evolution, leading to its broad expression pattern, similar to the promoter of a KTI pseudogene (WCI-P1) from winged bean (Psophocarpus tetragonolobus) which confers strong constitutive expression in leaves, petioles, stems, roots and seeds (Habu et al., 1997).

In general, the analysis of EST databases predicted that the highest levels of KTI transcripts are present in poplar flowers, particularly female floral buds and catkins. For win3/TI2 and TI3, western analysis confirmed the accumulation of their corresponding proteins (Fig 5). Hollick and Gordon (1995) had also previously shown that the win3.12 promoter drives developmentally regulated reporter gene expression in tobacco reproductive organs including flowers, pollen and seeds. We did not observe win3/TI2 or TI3 protein accumulation in pollen or seeds (data not shown); this may be because win3 expression is temporally restricted during pollen and seed maturation (Hollick and Gordon, 1995). PIs are also known to be expressed in the flowers of other plant species, suggesting they play functional roles in plant reproductive organs (Botella et al., 1996; van Dam et al., 2001). Such roles could include the regulation of proteolysis of storage proteins, especially in seeds, as well as defense against herbivore pests for these crucial organs. We note that other poplar defense-related proteins including PPO, WIN4, and SP1/POP3-like also accumulate in female catkins (data not shown), suggesting that at least in poplar, KTI gene expression may be part of a larger accumulation of defense proteins.
Biochemical analysis indicates functional diversification of poplar KTI genes

The poplar KTIs tested inhibited trypsin, chymotrypsin and elastase with different profiles (Fig 7). TI2 and TI4 had very specific inhibitory activities, being effective against only trypsin or chymotrypsin, respectively. By contrast, TI3 and TI5 inhibited both trypsin and chymotrypsin with similar efficiencies. TI6 was the only elastase inhibitor, a rare activity among plant KTIs (Valueva et al., 2000; Araujo et al., 2005; Sumikawa et al., 2006). These functional differences presumably reflect the sequence diversity of the KTI family. A previous study of a KTI gene family, a biochemical analysis of potato KTIs, also revealed functional diversity both between and within the homology groups (Heibges et al., 2003a; Heibges et al., 2003b). Thus potato group A and B KTIs strongly inhibit trypsin, while potato group C KTIs do so only weakly. In contrast to the poplar KTIs, the potato KTIs also inhibit other enzymes including subtilisin, the aspartic proteinases cathepsin D and aspergillopepsin, and cysteine proteinases (Ritonja et al., 1990; Rowan et al., 1990; Valueva et al., 2000; Heibges et al., 2003b; Revina et al., 2004).

In poplar, the functional properties of the individual properties KTIs could not be easily predicted based on primary sequence data. For example, although TI2 and TI6 protein sequences were most similar among the KTIs tested (Supplemental Table S1), their inhibitory activities were the most divergent. By contrast, TI3 and TI5 inhibited proteinases with similar potencies, but share little sequence similarity. Likewise, a comparison of reactive site and reactive loop residues revealed that these do not readily predict protease substrate preferences. This may relate to the atypical residues of the poplar KTI reactive loops (Fig. 2). Our prediction of the KTI reactive loops differs by a window of three amino acid residues from a previous study of Populus group A KTIs (Talyzina and Ingvarsson, 2006). We used secondary and tertiary structure predictions to predict reactive loop position, which provides a better estimate than sequence alignments with divergent plant KTIs. A similar approach has been used to predict the second reactive site of a winged bean chymotrypsin inhibitor (Dattagupta et al., 1999). However, precise definition of the reactive loops and sites of the poplar KTIs will require site-directed mutagenesis or structural determination.

By contrast, the reactive site residues of other types of plant KTIs may be useful indicators of inhibitor specificity. For example, Arg-Lys and Ile-Ser are often reactive site residues in inhibitors of trypsin, while Leu-Ser is often the reactive site for KTIs effective against...
chymotrypsin. Other plant KTIs may have more atypical reactive sites. Glu-Ser are the reactive site residues of DrTI from *Delonix regia* seeds and sporamin from *Ipomoea batatas* tubers (Pando et al., 2001; Yao et al., 2001), and these are also the predicted residues for the poplar TI2 and TI3 reactive sites (Fig. 2). The importance of Glu and Ser was shown by site-directed mutagenesis in sporamin. Here, changing reactive site residues from Glu to Arg or Ser to Ile completely abolishes trypsin inhibitor activity, despite the presence of Arg or Ile in reactive loops of other trypsin-inhibiting KTIs (Yao et al., 2001). Mutation of another negatively charged residue in the sporamin reactive loop also destroys inhibitory activity, indicating that the negatively charged residues in the sporamin reactive loop may contribute to the inhibitory activity (Yao et al., 2001). Interestingly, 19 of 22 poplar KTIs have a conserved negatively charged residue in their reactive loops. Thus, the molecular interactions between poplar KTIs and substrate proteinases may be similar to those in DrTI and sporamin.

We also observed that the biochemical stabilities of the poplar KTIs differed considerably within the group. Similar to other protease inhibitors, several poplar KTIs retain activity in the presence of the reducing agent dithiothreitol (DTT) and at high temperature (Garcia et al., 2004). However, these stability profiles varied greatly among the five poplar KTI proteins tested here. In our assays, TI5 was the most stable KTI, retaining activity at high levels of DTT and high temperature, while TI2 was very sensitive, losing one-half of its activity at 1 mM DTT and 50°C (Fig. 8). The presence of only a single disulfide bond in TI5 suggests it has evolved features that contribute to protein stability that are not affected by DTT. Although protein stability has not been measured for most plant KTIs that have one or no disulfide bonds, a recent analysis of such a KTI from *Inga laurina* seeds did in fact show high stability in the presence of DTT (Macedo et al., 2007). Studies of other plant KTIs also reveal diverse stability profiles. Some KTIs are exceptionally resistant to reducing agents, boiling and pH extremes, while others are sensitive (Garcia et al., 2004; Macedo et al., 2004). These data support the idea that anti-herbivore proteins may be inherently more stable and resistant to proteolysis. Previous work from this laboratory found that the defensive enzyme polyphenol oxidase is stable in the gut of a lepidopteran herbivore and remained active in frass (Wang and Constabel, 2004). In a preliminary analysis with poplar-fed forest tent caterpillar (*Malacosoma disstria*), western blots showed clear bands corresponding to TI2 and TI3 in frass (data not shown). This suggests that poplar KTIs are stable and resist digestion within the guts of lepidopteran and perhaps other
herbivores. Likewise, the tomato anti-herbivore enzymes arginase and threonine deaminase were shown to be stable in midguts of *Manduca sexta*, where they destroy these nutritionally important amino acids, ultimately resulting in decreased larval growth (Chen et al., 2005; Chen et al., 2007).

**The role of KTIs in poplar herbivore defense**

*In vitro* tests with recombinant proteins showed that only TI3 is an active inhibitor of midgut proteases from BAW, a lepidopteran pest of canola (Fig. 9). Comparison of the initial slopes of the TI3 and GmKTI inhibition curves indicated that TI3 has a stronger affinity for some BAW proteases than soybean GmKTI; however, neither protein completely abolishes proteolytic activity (Fig. 10). When we tested our recombinant KTIs against midgut proteases from FTC, which specialize on *P. tremuloides* in western Canada, all five KTIs inhibited proteolysis although TI2 and TI3 were clearly most potent (Fig. 10). The effectiveness of herbivore-induced poplar KTIs on the digestive proteases of a poplar pest strongly supports an important role for KTIs in poplar defense. Furthermore, the differences in effectiveness that we measured against different pests, combined with the differences in target preferences against commercial proteases exhibited for the five KTIs, further support the idea of functional specialization within the poplar KTI gene family.

 Compared with *Arabidopsis*, an annual species, the poplar genome has a larger suite of genes associated with insect resistance (Tuskan et al, 2006). Over its lifespan, a perennial such as poplar is exposed to more potential pest insects than an annual plant, and a broad array of protease inhibitors with activities against diverse proteases would seem advantageous. Lepidopteran pests contain complex mixtures of gut proteases, often differentially expressed, which can facilitate a rapid adaptation of gut protease activity to ingested PIs in the herbivore diet (Jongsma et al, 1995; Mazumdar-Leighton and Broadway, 2001; Hegedus et al, 2003). Therefore the poplar KTIs should have many potential targets, both within any one pest species, and among different poplar pests. While we are aware that *in vitro* inhibition of gut proteases does not always correlate with a reduction of insect performance *in vivo* (Jongsma et al, 1995; Bown et al, 2004), we have used artificial diet bioassays with recombinant TI3 protein at biologically relevant concentrations to demonstrate that this inhibitor does in fact significantly reduce larval growth (I.T. Major, E. Despland, and C.P. Constabel, unpublished results).
Previously, heterologous expression in tobacco also demonstrated that \textit{win3} can reduce growth of tobacco budworm (Lawrence and Novak, 2001). Thus, some poplar KTI genes have already been shown to provide protection against insects, and it is likely that many of the others do the same. We therefore suggest that the diversity of genes in the KTI family is likely to provide ecological benefits to \textit{Populus}.

The interaction of poplar KTIs with a diversity of pest protease targets should be reflected in selective pressure on the KTI genes. In evolutionary time, this would leave traces of positive selection and rapid molecular evolution in KTI genes in different poplar species or populations. We previously noted the high incidence of restriction fragment polymorphisms and high rates of non-synonymous substitutions in aspen KTIs (Haruta \textit{et al.}, 2001; Miranda \textit{et al.}, 2004). More comprehensive analyses of molecular evolution have shown convincingly that the poplar KTIs gene family is evolving rapidly, with some KTIs showing clear signs of positive selection (Ingvarsson, 2005; Talyzina and Ingvarsson, 2006). The origins of such strong selection pressures on the KTI family are difficult to demonstrate directly, but are predicted to relate to pressures from a diversity of herbivore pests and multiple digestive proteases. Therefore, gene duplication followed by strong positive selection from herbivores could lead to the biochemical diversification we observed in our experiments. Since poplars are long-lived trees, large and diverse families of defense genes may be crucial for survival against multiple pest species with short generation times capable of quick evolutionary changes.
MATERIALS AND METHODS

Plant material and wounding treatments

Poplar hybrid H11-11 (*Populus trichocarpa* × *P. deltoides*) were propagated and maintained in the Bev Glover Greenhouse at the University of Victoria as described previously (Major and Constabel, 2006). Plants were typically 12 weeks old and 1 m tall with approximately 30 leaves. Lateral shoots were removed periodically but no less than two weeks prior to experiments, so that each plant consisted of a single main stem. Leaves were numbered using the Leaf Plastochron Index (Larson and Isebrands, 1971), with the index leaf (LPI 0) defined as the first developing leaf with a lamina length of > 20 mm. For wound treatments, the margins of all leaves (LPI > 0) were crushed with pliers three times at 1 h-intervals. Male and female catkins were sampled from a stand of black cottonwood trees (*P. trichocarpa*) growing on the campus of the University of Victoria. Immediately after harvesting, tissues were frozen in liquid nitrogen or on dry ice, and stored at -80°C until analyzed.

Protein extraction and western blot analysis

Protein was extracted from leaves of greenhouse-grown poplar by tissue homogenization in sodium phosphate buffer (100 mM, pH 7.0) containing 0.1% (v/v) Triton X-100, 5% (w/v) polyvinylpolypyrrolidone, and 0.1% (v/v) 2-mercaptoethanol. For catkins from field-grown trees, proteins were extracted with Tris buffer (50 mM, pH 7.5) containing 5% (w/v) sodium dodecyl sulfate (SDS), 5% (w/v) polyvinylpolypyrrolidone, and 2.5% (v/v) 2-mercaptoethanol. Extracts were clarified by centrifugation, and soluble protein quantified using the Bradford method (1976) for sodium phosphate-buffer extracts or the RC DC protein assay (Bio-Rad, Hercules, CA, USA) for Tris/SDS-buffer extracts. Proteins were separated by SDS-PAGE and electro-transferred onto PVDF membranes (Pierce, Fisher Canada, Nepean, ON, Canada). Ponceau S (Sigma, St. Louis, MO, USA) staining was used to verify equal loading and transfer efficiency. Western blot detection was carried out using polyclonal antibodies raised against Ti2 (Haruta et al., 2001) and Ti3 (see below). Immunocomplexes were detected using acid phosphatase- or horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) and blots were developed colorimetrically with 5-bromo-4-chloro-3-indoyl phosphate (BCIP, Pierce) and
Identification of KTIs from poplar genome and *in silico* analysis

To obtain the complete poplar KTI family, the *Populus trichocarpa* v1.0 genome sequence (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) was queried for gene models annotated as Kunitz-type PIs. KTI gene models are based on the genome v1.0 annotation as the more recently curated v1.1 genome sequence (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) was missing some KTI gene models that had EST support from GenBank and several models were truncated (data not shown); however, the genome v1.1 annotations are shown for comparison (Supplemental Table S2). The gene models were combined with previously identified *Populus* KTI sequences (Bradshaw et al., 1990; Hollick and Gordon, 1993; Haruta et al., 2001; Christopher et al., 2004) and used to query the genome again for any Kunitz-like proteins not annotated as such. Gene models were removed that were not KTIs (i.e. lacking the Kunitz motif and having at least one disulfide bond). Many gene models encoded truncated KTIs that were flanked by regions of highly repetitive DNA and could not be resolved as separate TI genes (see Results, *win3* locus). Truncated gene models were used to query the GenBank plant EST database to try to identify full length sequences; in the absence of full length ESTs in GenBank, these were not analyzed further. KTI gene models with nucleotide sequences ≥ 99% identical were considered allelic, and only one gene model per locus was used for phylogenetic analysis.

Multiple amino acid alignments were made using Clustal W with default parameters, followed by manual adjustments in BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). To improve alignments, secondary structure predictions were made using Jpred (http://www.compbio.dundee.ac.uk/~www-jpred/) and tertiary structure predictions were made using SWISS-MODEL (http://swissmodel.expasy.org/), CPHmodels (http://www.cbs.dtu.dk/services/CPHmodels/) and ESyPred3D (http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/). Predicted secondary structure and tertiary structures were compared and used to help align variable sites and indels. A neighbor-joining phylogenetic tree was constructed in MEGA 3.1 (http://www.megasoftware.net/) using *p*-distance with pairwise deletion of gaps. MEGA recommends using *p*-distance (proportion of amino acid differences to the total number of sites...
compared) for pairwise deletion because it normalizes the number of differences based on the number of valid sites compared. Bootstrapping was used to test the robustness of the tree in MEGA 3.1. To determine preliminary KTI expression patterns via a digital northern, we queried the PopulusDB EST database (http://poppel.fysbot.umu.se/) which contains >100,000 EST sequences from 19 different tissue libraries (Sterky et al., 2004). An expression profile was constructed by determining the number of ESTs, according to their libraries, of each cluster.

**Heterologous expression and purification of recombinant KTI proteins**

TI2 was expressed and purified as described previously (Haruta et al., 2001). Signal peptides of each poplar KTI were predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). The coding sequence of poplar KTIs, minus the signal sequence, were PCR-amplified from plasmid cDNA clones. TI3 (EST ID H1059; GenBank accession CN192549) was amplified with the primers TI-3s (5’-CGGGATCCGAAGCAGTGATCGATGCC-3’; BamHI site underlined) and TI-3a (5’-CCCAAGCTTCATCATTTTATACTCG-3’; HindIII double underlined). TI4 (H828; CN193330) was amplified with the primers TI-4s (5’-CGGGATCCGAGGGAATCCAGTGCTTG-3’) and TI-4a (5’-CCCAAGCTTACTGAGCCGGTGCTTG-3’). PCR products were digested with BamHI and HindIII and cloned into the respective restriction sites of the pET21a (Novagen, EMD Biosciences, San Diego, CA, USA) bacterial expression vector. The resulting pET-TI3, pET-TI4, pET-TI5 and pET-TI6 plasmids were sequenced and moved into E. coli strain BL21(DE3)(Novagen) for expression.

The recombinant proteins were isolated from bacterial inclusion bodies as described previously (Haruta et al., 2001), and purified on a nickel affinity resin (Ni-NTA Agarose, Qiagen) and eluted along a pH gradient under denaturing conditions (8 M urea) as per the manufacturer’s protocol. To renature the recombinant KTIs, we initially used our previous protocol (Haruta et al., 2001) but found that the proteins differentially aggregated and precipitated in buffers of depending on ionic strength, concentration of reducing agent, and pH
(Table I). We therefore optimized the re-folding conditions for each KTI, and found a re-folding buffer suitable for all the proteins, such that subsequent in vitro assays for PI activity would not be influenced by different buffers. The recombinant KTIs were dialyzed against 50 mM Tris-HCl buffer (pH 8.0) at 4°C for 24 h with at least six changes of buffer. For comparison with in vitro assays of PI activity, soybean KTI (GmKTI; Sigma) dissolved in ddH2O was also dialyzed against 50 mM Tris-HCl buffer (pH 8.0) so that buffers were comparable. Protein concentrations were determined spectrophotometrically (A$_{280}$) using molar extinction coefficients calculated using by Vector NTI Advance 9.0 Software (Invitrogen, Burlington, ON, Canada) or reported by supplier (GmKTI; Sigma), and were verified on Coomassie-stained SDS-PAGE gels.

TI3 antiserum was generated in rats using recombinant TI3 produced as above, but in addition we precipitated TI3 protein by dialysis against PBS (phosphate buffered saline) at 4°C. Rats were immunized with 100 µg TI3 protein in Freund’s complete adjuvant (Sigma, St. Louis, MO, USA), with booster injections of 50 µg protein in Freund's incomplete adjuvant using standard procedures.

**Proteinase inhibitor assays**

All PI activity assays were done in triplicate and the results shown as the means of three replicate assays. The PI activities of recombinant poplar KTIs against trypsin (EC 3.4.21.4; Sigma), chymotrypsin (EC 3.4.21.1; Sigma) and elastase (EC 3.4.21.36; Sigma) were determined by pre-incubating increasing concentrations of each KTI with a standard quantity of proteinase (6.6 x 10$^{-4}$ g L$^{-1}$ final assay concentration) in the appropriate assay buffer. PI activity was determined as described, by measuring residual proteinase activity as the rate of hydrolysis of specific chromogenic substrates (Worthington, 1988). Trypsin activity was assayed with TAME (p-toluene-sulfonyl-L-arginine methyl ester; Sigma) by monitoring the change in ∆A$_{247}$ min$^{-1}$, chymotrypsin activity was assayed with BTEE (benzoyl-L-tyrosine ethyl ester; Sigma) by monitoring the change in ∆A$_{256}$ min$^{-1}$, and elastase activity was assayed with SucAla3pNA (N-succinyl-Ala-Ala-Ala-p-nitroanilide; Sigma) by monitoring the change in ∆A$_{410}$ min$^{-1}$. Trypsin and chymotrypsin activity assays were measured in 1 mL reactions using a UV-1601PC spectrophotometer (Shimadzu, Columbia, MD, USA). Elastase activity assays were measured in 0.2 mL reactions in 96-well, non-stick microtitre plates (Corning, Fisher) using a Sunrise microtitre plate reader (Tecan US, Durham, NY, USA). GmKTI (Sigma) and bovine serum
albumin (BSA; Sigma) were assayed under identical conditions as positive and negative controls, respectively. The BSA negative control ensured that small reductions in protease activity were due to actual inhibition and not substrate competition. KTI concentrations required to inhibit 50% of the proteinase activity were calculated from the linear portion of the plot of residual proteinase activity against KTI protein (µM).

PI activities against subtilisin A (EC 3.4.21.62; Sigma) and papain (EC 3.4.22.2; Sigma) were determined by pre-incubating increasing concentrations of each KTI with a standard quantity of proteinase in the appropriate assay buffer for 10 min (for subtilisin, 50 mM Tris-HCl pH 7.5 at 37°C; for papain, 50 mM sodium acetate pH 6.0, 2 mM DTT at 23°C). After azocasein (Sigma) was added to a final concentration of 1%, the reaction was followed for 1 h and stopped by the addition of trichloroacetic acid. Reactions were clarified by centrifugation, NaOH was added to the supernatant, and the A₄₅₀ was measured and used to calculate residual proteinase activity.

PI activity of leaf extracts was determined against trypsin, chymotrypsin and elastase by titrating leaf protein extracts with each proteinase, and residual proteinase activity measured as described above. Percent proteinase inhibition was plotted against square root-transformed protein concentration (mg mL⁻¹) of leaf extracts because the relationship between proteinase activity and protein extract was not linear. To directly compare levels of inhibitory activity in leaves, we calculated the total protein concentration of leaf extract which inhibits 50% of proteinase activity (IC₅₀). For statistical comparison of inhibitor activity in leaves of control and wounding treatments, we compared slopes (% inhibition/(protein extract)½) from linear regression analysis and calculated a P-value (two-tailed) testing the null hypothesis that the slopes are identical (PI activity is equal from control and wound treatments).

For stability tests, recombinant KTIs were incubated under different conditions, and after the denaturing treatment PI activity compared with the activity of the untreated KTIs. Inhibition of trypsin was measured for TI2 and TI3, while chymotrypsin was used for TI4, TI5 and TI6. For thermostability assays, KTIs were incubated from 10 to 100°C at 10°C intervals for 30 min, then cooled on ice for approx. 30 min before measuring residual PI activity. Some thermostability experiments were also performed with gradual cooling after heating, as well as with different incubation times (ranging from 2.5 to 150 min), but results were the same for both assay variations. To test stability in the presence of reducing agent, KTIs were incubated with
dithiothreitol (DTT) at concentrations increasing exponentially from 1 µM to 100 mM (final concentration) for 30 and 120 min. The reactions were terminated by adding iodoacetamide at twice the concentration of DTT before measuring residual PI activity. For long-term stability, KTIs were stored at 4°C and residual PI activity was measured at 25-day intervals for 100 days. For TI2 residual activity was further measured at 50-day intervals for an additional 200 days.

PI activity against insect proteases was determined from midgut extracts. Bertha armyworm (Mamestra configurata) extracts were obtained from Dr. Dwayne Hegedus (Agriculture and Agri-Food Canada, Saskatoon, SK, Canada). Dr. Emma Despland (Concordia University, Montréal, QC, Canada) provided Forest tent caterpillar (Malacosoma disstria) midguts dissected from fourth and fifth instar larvae that were reared on artificial diets (Despland and Noseworthy, 2006). FTC midguts were extracted into insect Ringer’s solution and clarified by centrifugation, as described previously (Hegedus et al, 2003). A standard quantity of midgut extract was pre-incubated with increasing concentrations of KTIs. For BAW midgut extracts, protease activity was measured in two assay buffers of different pH (0.1 M Tris-HCl pH 8.0; 0.1 M glycine-NaOH pH 11.0). These correspond to the two pH optima of midgut protease activity (Hegedus et al., 2003). For FTC midgut extracts, protease activity was measured at pH 11.5 (0.1 M glycine-NaOH pH 11.0), which corresponds to optimum protease activity (data not shown). Azocasein were carried out as above, except that the reaction was allowed to proceed for 6 h.

Supplemental Material

Supplemental Figure S1. Long-term stability of poplar KTIs stored at 4°C.

Supplemental Table S1. Amino acid sequence similarities and identities among members of the poplar Kunitz trypsin inhibitor family.

Supplemental Table S2. Kunitz-type trypsin inhibitor gene models from Populus trichocarpa genome versions 1.0 and 1.1.
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FIGURE LEGENDS

Figure 1. Phylogeny of Kunitz TI members from the genus *Populus* constructed by neighbor-joining of protein distance. Kunitz TI sequences were retrieved from the *P. trichocarpa* genome; only full-length TI sequences with a Kunitz motif sharing less than 96% amino acid identity with other sequences and having EST support were included in this analysis. Numbers at branches represent bootstrap support from 2000 replicates (only values > 80% are shown for clarity). Sequences are annotated with the protein ID from the *P. trichocarpa* v1.0 genome sequence (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html). Sequences annotated as TI3, TI4, TI5, TI6 and TI7 are the putative *P. trichocarpa* orthologs of Kunitz TIs that have been previously identified (Christopher et al., 2004; Talyzina and Ingvarsson, 2006). Accessions of annotated TI sequences (GenBank gene index or JGI protein ID from *P. trichocarpa* genome): GWIN3 (gi|20946), WIN3.12 (gi|169460), TI3 (739064), TI4 (555576), TI5 (574326), TI6 (725622), TI7 (697808). Scale bar is *p*-distance (proportion of amino acid sites at which two sequences are different).

Figure 2. Alignment of representative Kunitz trypsin inhibitor (KTI) protein sequences from poplar. Soybean KTI (GmKTI) and sporamin of *Ipomoea batatas* (IbSPOA) are shown for comparison. Shading shows conserved (black) and similar (grey) amino acid residues, and dashes represent sequence gaps. A solid line above the alignment marks the signal peptides and inverted triangles denote the Kunitz motif (L,I,V,M)-X-D-(X2)-G-(X2)-(L,I,V,M)-(X5)-Y-X-(L,I,V,M). Below the alignment, two disulfide bridges formed by four conserved Cys residues are shown by brackets. Plus signs (+) denote free Cys residues present in poplar KTIs. The known structural features of GmKTI are indicated with arrows above the alignment (<—>) delineating β-sheets, a boxed region indicating the reactive loop, and stars (*) denoting the P1 and P1’ reactive-site residues. Sequences were retrieved from NCBI with the following accession numbers: GmKTI (1AVU), IbSPOA (Q40084), WIN3 (CAA33539), TI2 (AAK32690), TI3 (AAQ84216), TI4 (AAQ84217), TI5 (AAQ84218) and TI6 (DT502517). Sequences for TIs 554072 and 75560 were retrieved from the *P. trichocarpa* v1.0 genome (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html).
Figure 3. Digital northern of expression data for KTI transcripts. The PopulusDB database (http://poppel.fysbot.umu.se/) was queried for each KTI and the number of ESTs from the corresponding cluster are shown (grey = no similar sequence in PopulusDB). For wound induction, data from Christopher et al. (2004), Ralph et al. (2006) and Major and Constabel (2006) are reported (“+” for degree of induction, “-” symbols for repression, and “NC” for no change of expression). Sequences are annotated as in Fig. 1 and the KTI phylogeny at left shows the sequence relationships among the different KTI members. Tissue abbreviations are: A + B, cambial zone; UA, dormant cambium; G, tension wood; C, young leaves; I, senescing leaves; L, cold stressed leaves; Q, dormant buds; P, petioles; F, flower buds; M, female catkins; V, male catkins; K, apical shoot; T, shoot meristem; N, bark; R, roots; S, imbibed seeds; Y, virus/fungus-infected leaves.

Figure 4. Western blot analysis of TI2 and TI3 protein accumulation following wounding of poplar saplings. A, Leaves were wounded with pliers and 4 days later tissues were harvested from leaves at leaf plastochron index (LPI) 3-5 (L3), stem between internodes at LPI3-5 (S3), leaves at LPI 9-11 (L9), bark at internodes LPI 9-11 (B9), wood at internodes LPI 9-11 (W9), and mature roots (R). Six paired control and wounded trees were separately analyzed, and representative blots are shown. B, Female and male catkins from field-grown *P. trichocarpa* trees were collected in May 2007. C, Leaves were wounded and leaves 9-11 harvested at the indicated days. Two independent experiments were conducted, and representative blots are shown.

Figure 5. Inhibitory activity of poplar crude leaf extracts against three commercial proteases. C Increasing amounts of crude extracts from young leaves (LPI 3-5) before (control, squares) and 4-days after wounding (wounded, diamond) were incubated with standard amounts of trypsin, chymotrypsin and elastase, and the residual protease activity was measured. Six paired control and wounded trees (biological replicates) were used, and each sample analyzed in triplicate (technical replicates). Steeper slopes indicate more potent inhibition. Values are mean protease activity ± SE (n = 3). Insets show the IC$_{50}$ calculated from linear regression of all six independent biological replicates.
**Figure 6.** Purification of recombinant TIs from *E. coli*. A, SDS-PAGE of recombinant TI3 protein preparations from different stages of purification. Lane 1, *E. coli* cells before induction with IPTG; 2, *E. coli* cells 24-h after induction with IPTG; 3, *E. coli* cell lysate without inclusion bodies; 4, lysed inclusion bodies containing TI3; 5, TI3 after purification on Ni-NTA resin column; 6, protein standard marker. B, SDS-PAGE of recombinant TI proteins. Lanes 1-5 are loaded with TI2-TI6, respectively. Lane 6 is loaded with soybean KTI.

**Figure 7.** Inhibitory activities of purified recombinant poplar TIs against three commercial proteases. Titration of TI2 (blue diamond), TI3 (red square), TI4 (purple triangle), TI5 (orange closed circle) and TI6 (green ‘x’) against trypsin, chymotrypsin, and elastase. Soybean KTI (GmKTI; yellow dash) is shown for comparison and bovine serum albumin (BSA; indigo open circle) is a negative control. Data points represent the mean protease activity ± SE (*n* = 3). The linear regression used to calculate the IC$_{50}$.

**Figure 8.** Effect of denaturing conditions on activity of poplar TIs. A, KTIs were incubated in the presence of increasing concentrations of DTT for 30 min followed by incubation in iodoacetamide. B, KTIs were incubated at increasing temperatures for 30 min followed by incubation on ice. Data points represent mean TI activity ± SE (*n* = 3).

**Figure 9.** Inhibition of midgut proteases from bertha armyworm (BAW, *Mamestra configurata*) by poplar TI3 and soybean KTI (GmKTI). Increasing amounts of TI3 and GmKTI were preincubated with a constant amount of BAW midgut extract and the residual total proteolytic activity was assayed at pH 8 (cross) and pH 11 (diamond) with azocasein. Note the different x-axis scales for TI3 and GmKTI data. Values are mean protease activity ± SE (*n* = 3).

**Figure 10.** Inhibition of midgut proteases from forest tent caterpillar (FTC, *Malacosoma disstria*) by two poplar TIs and soybean KTI (GmKTI). Increasing amounts of TI2 (blue diamond), TI3 (red square), and GmKTI (yellow dash) were preincubated with a constant amount of FTC midgut extract and the residual total proteolytic activity was assayed at pH 11.5 with azocasein. Values are mean protease activity ± SE (*n* = 3).
### Table I. Properties of recombinant poplar trypsin inhibitors$^a$.

| TI  | Size (aa) | Predicted pI | Predicted Molecular weight (kD) | Actual (SDS-PAGE) | Protein solubility$^b$ | 50 mM Tris (pH range) |
|-----|-----------|--------------|---------------------------------|-------------------|------------------------|------------------------|
| TI2 | 213       | 4.42         | 23.1                            | 24                | insoluble              | 2, & 5 - 12            |
| TI3 | 202       | 6.15         | 22.0                            | 26                | soluble                | 2 - 4, & 9 - 12        |
| TI4 | 212       | 4.96         | 23.1                            | 24                | insoluble              | 2 - 3, & 8 - 12        |
| TI5 | 214       | 3.98         | 22.9                            | 30                | insoluble              | 7 - 12                 |
| TI6 | 202       | 5.32         | 21.8                            | 23                | partially-soluble      | 2 - 3, & 7 - 12        |

$^a$Predicted $M_r$ and pI were calculated by Vector NTI Advance 9 software; SDS-PAGE-determined $M_r$ were extrapolated from the logarithmic-transformed migration distance of the proteins compared with protein standards (low range, Bio-Rad).

$^b$Recombinant protein solubility was assessed by dialysis of proteins in water and 50 mM Tris buffers of pH 2 - 12. For solubility in Tris buffer, the pH range for which the proteins were soluble is shown.
Table II. Summary of inhibitory activities of KTIs against different proteases.

|     | Trypsin | Chymotrypsin | Elastase | Subtilisin | Papain |
|-----|---------|--------------|----------|------------|--------|
| TI2 | 0.0280  | 2.95         | NA       | NA         | NA     |
| TI3 | 0.152   | 0.152        | 74.1     | NA         | NA     |
| TI4 | 128     | 0.294        | NA       | NA         | NA     |
| TI5 | 0.205   | 0.188        | 9.16     | NA         | NA     |
| TI6 | 0.643   | 0.111        | 0.731    | NA         | NA     |
| GmKTI| 0.0116  | 0.0567       | 3.65     | NA         | NA     |

*a*Inhibitory concentrations were calculated from the linear portion of the plot of residual proteinase activity against TI protein concentration (Fig. 7). NA: no activity.
Figure 1.
Figure 2.
**Figure 3.**
Figure 4.
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Figure 9.
Figure 10.