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# Engineering Proteinase Inhibitor Genes For Plant Defense Against Predators

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## Engineering Proteinase Inhibitor Genes For Plant Defense Against Predators<sup>1</sup>

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Small proteinaceous inhibitors ( $M_r < 20,000$ ) of the digestive serine proteinases of animals and microorganisms are found as moderately abundant proteins in storage organs and leaves of many plant genera. The proteins are powerful inhibitors of the digestive enzymes of plant predators and therefore are considered to be part of the array of defensive chemicals of plants. Proteinase inhibitor genes show excellent promise, using DNA technology, to manipulate plant genomes to express these biologically active proteins in order to improve natural defense systems. Members of two unrelated families of serine proteinase inhibitors found in tomato and potato plants, called Inhibitor I (monomer  $M_r$  8000) and Inhibitor II (monomer  $M_r$  12,300), are under both environmental and developmental regulation in different tissues of the plants. Genes coding for wound-inducible Inhibitors I and II have been isolated from both tomato and potato genomes and characterized. Tobacco plants have been transformed with the chimeric genes containing wound-inducible promoters fused with the reporter gene, chloramphenicol acetyl transferase, to assess promoter function and signal transmission.

Transacting factors that regulate their expression in response to wounding are also being identified and purified. Intact genes are being employed to transform agriculturally important crop plants to determine their potential usefulness to enhance defensive capabilities of plants against herbivores and pathogens.

INDEX DESCRIPTORS: Proteinase Inhibitors Genes, Plant Defense, Herbivores, Plant transformation, Potato, Tomato, Alfalfa.

Proteinase inhibitor proteins are among the many chemicals present in plants that help protect both storage organs and vegetative tissues against attacking herbivores (Rhodes, 1979) and pathogens (Senser, et al. 1974). Serine proteinase inhibitors are found in storage organs of plants, such as seeds and tubers, where they are developmentally regulated (Goldberg, 1986) and are also found in some plants in vegetative tissues, such as leaves, where they are synthesized in response to insect attacks or other types of severe wounding (Ryan, 1978). The inhibitors are considered as defensive proteins against herbivores and pathogens because they can interfere with the digestive processes of animals, including insects, and microorganisms that utilize proteinases to hydrolyze food proteins to obtain amino acids for growth, development and maintenance. All effective proteinase inhibitors bind the proteinases with  $K_i$ s of from  $10^{-7}$  M to less than  $10^{-10}$  M (Kato and Laskowski, Jr. 1980; Travis, 1981).

Inhibitor proteins have been isolated that are specific for each of the four known mechanistic classes of proteolytic enzymes; serine, thiol (cysteiny), aspartyl and metalloproteinases but the majority of proteinase inhibitors found in plants are specific for members of the serine class of proteinases (Laskowski, Jr. and Kato, 1980). Ten families of serine proteinase inhibitors have been identified in nature (Laskowski, Jr. et al. 1987) and six of these families are found in plants. The families appear to be totally unrelated to each other, suggesting they have evolved independently from different ancestral proteins, and it is likely that more inhibitor families will be found. The first serine proteinase inhibitor was isolated from plants in the 1930s when a trypsin inhibitors, called the Kunitz inhibitor, was crystallized from extracts of soybeans (Kunitz and Northrop, 1939). Later, inhibitors were isolated from beans, seeds of many legumes and grasses, and from vegetative storage organs of various plants, such as potato tubers. It is now recognized that some seeds and vegetative storage organs can contain over 10% of their proteins as serine proteinase inhibitors (Richardson, 1977; Ryan, 1981).

Inhibitors of metalloendopeptidases, cysteinyl proteinases (previ-

ously called thiol proteinases) and aspartyl proteinases (previously called acid proteinases) are known. However, proteinaceous inhibitors of aspartyl proteinases and metalloendopeptidases have rarely been reported in plant tissues, although an inhibitor of the metalloexopeptidases, carboxypeptidase A and B, has been purified from potato tubers (Hass and Ryan, 1981). Several cysteinyl proteinase inhibitors have been purified from plants. These inhibitors apparently are related to the cystatins, a thiol proteinase inhibitor family found in animals (Barrett, 1987). Like the serine proteinase inhibitors, the thiol proteinase inhibitors may have defensive roles in plants, directed against thiol proteinases of herbivores and microorganisms. Since so few examples of this class of inhibitors have been isolated, it is not possible to generalize about their broad distribution or importance in the plant kingdom.

### Wound Inducible Serine Proteinase Inhibitors.

In the early 1970s, members of two serine proteinase inhibitor families were found to be regulated in plant leaves in response to severe wounding by insects or by other severe mechanical damage (Green and Ryan, 1972; Ryan, 1978). The inhibitors accumulated in wounded leaves as well as in leaves several cm away, indicating that a wound signal or wound hormone was released at the wound site that travelled throughout the plants to activate the accumulation of the two inhibitor proteins (Green and Ryan, 1972; Ryan, 1974). This implied that the plants were responding to insect damage by producing proteins that could potentially disrupt the digestive physiology of the attacking insects. This was the first biochemical evidence that plants can change the levels of cellular proteins that have potential defensive properties in response to insect attacks. These data suggested that wounding changed the expression of the genes in leaves coding for the proteinase inhibitors in response to systemically mediated signals produced by the attacks. These observations were the basis of research efforts in this laboratory spanning several years to seek the chemical nature of the signal(s) that are released upon wounding. More recently, wound-inducible genes have been isolated to help unravel the molecular events that regulate their expression in response to insect attacks.

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### Wound Signals for Proteinase Inhibitor Synthesis in Excised Tomato Leaves.

By using small excised tomato leaves to assay tomato plant extracts for the presence of a proteinase inhibitor inducing factor (PIIF) (Green and Ryan, 1972), the major inducing activity was found to reside in a small (DP~30; Mr~ 5000) pectin-like oligosaccharide (Bishop, et al. 1984). This oligosaccharide contained about 70% highly methylated galacturonic acid, and its overall composition was similar to pectic polysaccharides found in plant cell walls. Neutral sugars were removed from this oligosaccharide by hydrolysis with trifluoroacetic acid, which produced a polygalacturonic acid oligomer with a DP of about 20 that retained full proteinase inhibitor inducing activity (Bishop et al. 1984). This oligouronide was further fragmented by PGase or acid hydrolysis to yield small oligomers from which di- and triuronides were isolated and purified (Moloshok, T., Pearce, G. and Ryan, C. A., in preparation). The oligouronide was also fragmented with pectin lyase (pectin transeliminase) and delta-4,5 di- and triuronides were purified. The saturated and unsaturated di- and trisaccharides were as fully active as the parent oligomer when assayed by supplying them to young excised Castlemart variety of tomato plants. In our early studies using Bonny Best variety of tomatoes, we had found the dimers and trimers to be only about half as active as the parent oligomer (Bishop, et al. 1984). In surveying several tomato varieties, we found that Bonny Best is among the poorest varieties in responding to wounding or to oligosaccharides in synthesizing proteinase inhibitors. The Castlemart variety was among the best responders (Broadway et al. 1986) and is presently being used for experimentation. A comparison of the levels of Inhibitor I typically induced in leaves of Bonny Best and Castlemart tomato varieties in response to wounding and addition of oligosaccharide fragments is presented in Table I.

The induction of proteinase inhibitor accumulation in excised leaves by oligouronides implied that the cell wall fragments could be a part of the early signalling process (Ryan, 1986). Supplying radioactive oligomers with DPs of about 20 to young tomato plants resulted in their apparent fragmentation into smaller molecules, suggesting that the plants possessed enzymes that could hydrolyze oligogalacturonic acid (Bishop et al. 1984). However, no polygalacturonases have as yet been isolated from tomato leaves. We are

Table 1. Comparisons of Induction of Proteinase Inhibitor I In Leaves of Bonny Best and Castlemart Tomato Varieties

Treatment	Inhibitor I Levels Induced (ug/g leaf tissue)	
	BONNY BEST	CASTLEMART
Wounded Intact Plants*	98	131
Control	0	0
Excised Plants Supplied With Oligouronides**		
PIIF (DP = 30)	66	114
Unsaturated Digalacturonic Acid	44	118
Control***	27	33

\*Plants (5 cm in height having two expanding leaves) were wounded every hour for 4 hours by crushing the leaves with a hemostat, incubated under constant light for 24 hours and assayed for Inhibitor I levels.

\*\*Excised plants were supplied with carbohydrate inducers at 250 ug/ml for 30 minutes and then water for 24 hours under constant light.

\*\*\*Excision of the plants causes a low level of induction of Inhibitor I.

investigating possible mechanisms for the fragmentation of the cell walls in response to wounding to establish if and how cell wall fragments can be released from wounds in quantities large enough to account for their participation in signalling proteinase inhibitor synthesis throughout the plant. A recent study using radioactive cell wall pectic fragments added to wounds on tomato plants has indicated that the radioactivity is confined to the wound sites (Baydoun and Fry, 1985; Moloshok, T. and Ryan, C. A. in preparation). This argues against the fragments having a role as systemic signals. If the fragments do not move throughout the plants, then other messenger molecules must be involved. We are currently engaged in seeking such molecules and have partially purified a highly active material that does not exhibit any carbohydrate component. The full purification of this molecule may provide new clues to elucidate the systemic signalling that activates the proteinase inhibitor genes in response to wounding.

### Wound-inducible Proteinase Inhibitor Genes.

Leaves of tomato plants accumulate Inhibitor I and II proteins in response to severe wounding at a cumulative rate of about 9 ug/h (Graham et al., 1986). In excised leaves supplied with oligouronide fragments, the cumulative rates of accumulation can be over twice that amount (Gustafson and Ryan, 1976). The response is transcriptionally regulated in tomato leaves, resulting in levels of mRNA in leaf cells of severely wounded leaves that approach 1% (0.5% Inhibitor II mRNA and 0.15% Inhibitor I mRNA) of the total poly A + messenger present within 6-8 h following wounding (Graham et al., 1986). These data were obtained with the Bonny Best variety that accumulates the two inhibitors poorly. Thus, the levels of mRNA coding for the inhibitors is probably much higher in leaves of varieties such as Castlemart.

Wound-inducible Inhibitor I and Inhibitor II genes have been isolated from both tomato (Lee et al., 1986; Fox, 1986) and potato (Thornburg, et al., 1987; Keil, et al., 1986; Cleveland et al., 1987) genomes. These genes have been characterized and are being utilized to identify the cis-acting and trans-acting factors that regulate the wound-inducibility of the genes. A potato Inhibitor II gene (Thornburg et al., 1987) and a tomato inhibitor I gene (Lee et al., 1986) have been under intensive study in our laboratory. The general features of these genes are shown in Figure 1. The 5' promoter region of the potato Inhibitor II gene was fused with the open reading frame of a reporter gene, chloramphenicol acetyl transferase (CAT), and terminated with either the 3' regions of the Inhibitor II gene or a terminator from a constitutive plant gene (Figure 2). Tobacco plants transformed with the fused gene having both the 5' and 3' regions of the Inhibitor II gene exhibited wound-inducible CAT activity (Thornburg et al., 1987). The time course of induction of CAT activity in response to wounding tobacco leaves is shown in Figure 3. The construct containing the 5' region of the Inhibitor II gene with the constitutive terminator did not exhibit wound-inducible CAT activity. The reason for the lack of activity isn't known. It is possible that the constitutive terminator produces an mRNA that is much less stable than that produced by the Inhibitor II terminator. The wound-inducible tomato Inhibitor II mRNA has a half time of degradation of about 10 hr (Graham et al., 1986). If the half time of degradation of CAT, terminated with the constitutive promoter, was 1-2 hr or less, then CAT activity may not have been detectable, as found in Figure 3.

The wound-inducibility of CAT indicated that the 3' and 5' regions of the potato Inhibitor II gene contained all of the information necessary and sufficient to produce wound-inducibility. This also indicated that biochemical components in the tobacco leaf cells could regulate wound expression of the potato Inhibitor II gene. The wound-induced expression of the CAT gene was also found in leaves distal to the wounded leaves, indicating that the systemic signalling

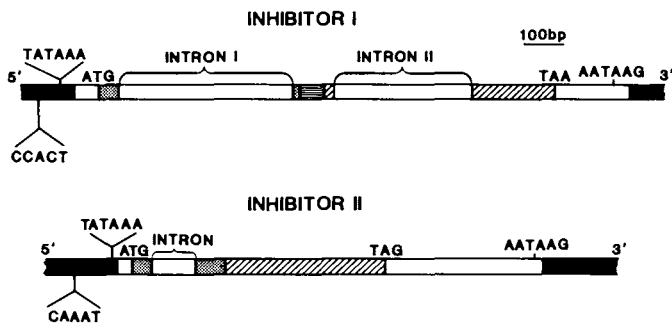


Fig. 1. Illustration of the structural and regulatory features of the Inhibitor I and II families of proteinase inhibitors from tomato and potato genomes. The dotted areas represent the transit (also called signal or leader) sequences. The slashed areas represent the open reading frames of the native, or processed inhibitor proteins found in leaf tissues. The open areas represent introns. Putative regulatory sequences, such as the TATA boxes, CAT boxes, polyadenylation signals and initiation and termination codons are shown.

mechanism was operational in tobacco plants transformed with the potato Inhibitor II promoter.

On the other hand, the induction of CAT expression in the tobacco plants was not as strong as expected, considering the high levels of wound-inducible proteinase inhibitors found in wounded potato leaves. We have now found that the levels of CAT expression can be increased 50 fold by excising leaf discs, wounding them, and floating them on a 1% sucrose solution (Figure 4, R. Johnson and C.A. Ryan, manuscript in preparation). A similar enhancement was found when the wounded discs were floated on glucose, fructose or maltose. One explanation for this behavior is that intact tobacco leaf tissue may not have the ability to divert enough carbon or energy to drive the wound-induced synthesis of CAT. However, when supplemented with sucrose or other sugars, the leaf cells are able to produce large quantities of the inhibitor. This response suggests that the tobacco tissues may not be geared to respond to the wound signals so efficiently as tomato or potato plants, where the wound response is very strong. These experiments suggest that some plants may not be equipped biochemically to express foreign wound-inducible genes and that some plant species may not be ideal candidates for transformation with strong wound-inducible promoters.

The wound-inducible potato Inhibitor II-CAT chimeric gene has provided the basis for a new assay for the proteinase inhibitor inducing factor. Transformed tobacco cells in suspension culture can be induced to increase CAT expression in response to a soluble, partially purified, non-carbohydrate preparation that induces proteinase inhibitors when added to excised tomato plants. When the inducer, for convenience called 'super-PIIF', is added to cultures of transformed tobacco cells, a several fold increase in CAT activity is induced. Oligogalacturonides do not induce CAT activity in the transformed cells. The transformed suspension cultures provide a unique system with which to study the receptors in the cells that

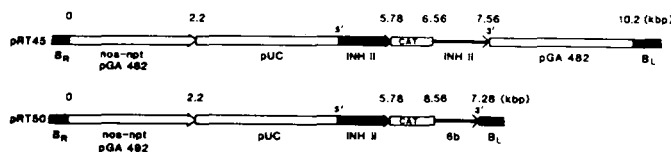


Fig. 2. Constructs in the binary transformation vector (An, 1986) containing 1000 bp of the 5' of the wound-inducible Inhibitor II gene and 1000 bp of the 3' of Inhibitor II gene (top) or 720 bp of the terminator of the constitutive 6b gene from the Ti plasmid. (Thornburg et al. 1987).

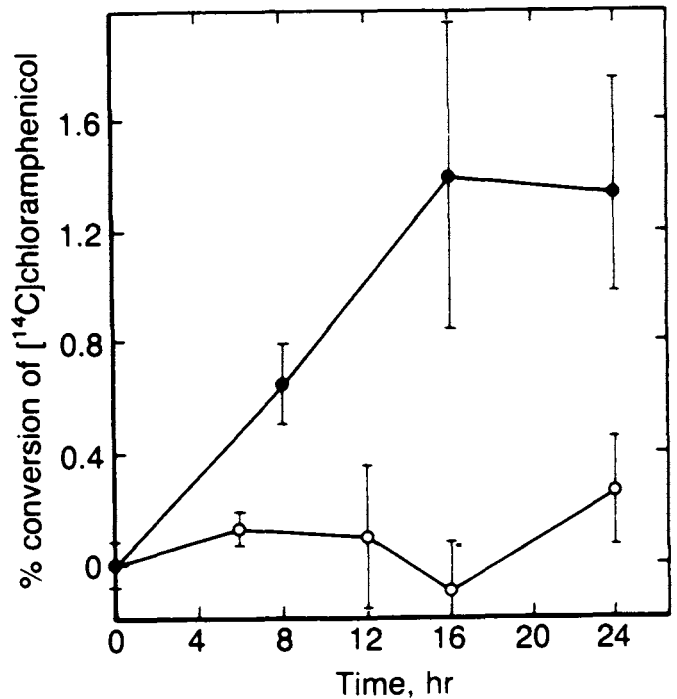


Fig. 3. Time course of the increase in CAT activity in leaves of transgenic tobacco plants transformed with constructs shown in Figure 2. (●-#45) pRT45. (○-#50) pRT50. (Thornburg et al. 1987)

regulate the wound-inducible expression of CAT separately from the induction by oligouronides.

The 5' promoter region and 3' terminator of a wound-inducible tomato Inhibitor I gene has also been fused with the open reading frame of the CAT gene to study the wound-inducibility of the gene. The fused gene was expressed when electroporated into tobacco protoplasts (Hall, G. and Ryan, C.A., in preparation).

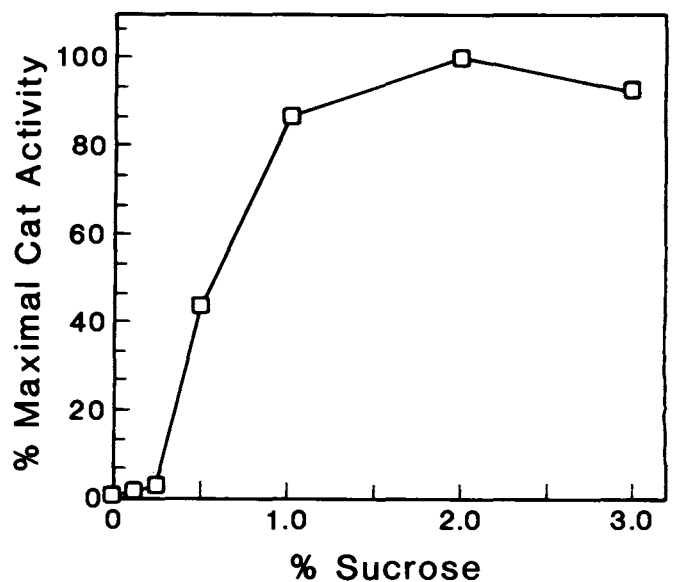


Fig. 4. CAT activity in wounded leaf discs from tobacco plants transformed with construct pRT45 when floated on water (0 time) or on solutions containing increasing concentrations of sucrose.

Deletion analyses of both the potato Inhibitor II gene and the tomato Inhibitor I gene are in progress to determine which sequences of the genes are responsible for wound induction. In conjunction with these experiments, gel retardation assays are being performed to identify those regions of the genes that can tightly bind nuclear proteins. In these assays, short (50-200 bp) DNA fragments of the promoter regions of the genes have been purified, end-labeled with  $^{32}\text{P}$ , mixed with nuclear extracts of the plants, and then subjected to electrophoresis to detect proteins that very strongly bind to specific sequences present in the gene fragments. In Figure 5 is shown results from one of our initial experiments. A 150 bp fragment from the 5' of the Inhibitor II gene near transcription initiation has been isolated and further cleaved into two fragments of 85 and 65 bp respectively (Figure 5). Each fragment was mixed with nuclear extracts from wounded and unwounded tomato plants. Nuclei from both wounded and unwounded leaves contain proteins that strongly bind to both fragments (Figure 5). The presence of the binding proteins in nuclear extracts does not appear to be related to wounding. If these proteins are involved with wound induction then other factors, induced by wounding, may interact with them to regulate wound-induction. Further gel retardation experiments are underway to explore regions of the gene further upstream for protein binding sequences. Data from these experiments should complement data obtained by deletion analysis of the genes in order to help identify those regions responsible for regulating wound-inducibility and to isolate and characterize the transacting factors that are directly involved in the process.

Table 2. Reactive Sites of Tomato, Potato and Alfalfa Proteinase Inhibitors

PROTEINASE INHIBITOR	P <sub>1</sub> -P <sub>1</sub>	SPECIFICITY
TOMATO I	-Leu-Asp-	CHYMOTRYPSIN
POTATO I	-Met-Asp-	CHYMOTRYPSIN SUBTILISIN ELASTASE
TOMATO II Domain I	-Lys-Gln-	TRYPSIN
Domain II	-Arg-Asn-	TRYPSIN
POTATO II Domain I	-Arg-Glu-	TRYPSIN
Domain II	-Leu-Asn-	CHYMOTRYPSIN
ALFALFA Domain I	-Arg-Ser-	TRYPSIN
Domain II	-Lys-Ser-	TRYPSIN

#### Strategies to Manipulate Proteinase Inhibitor Genes for Natural Plant Defense.

The variability in the specificities of the serine proteinase inhibitors provides the basis for a strategy to transform plants with different inhibitor genes to improve natural plant defense. The specificities of the inhibitors is determined by the amino acid residue at the P<sub>1</sub> site (Laskowski, Jr. and Kato, 1980). Mutations of the P<sup>1</sup> amino acid residue can change the specificity of the inhibitors, changing the type of proteinases that they inhibit. By selecting inhibitor genes that contain different amino acids at the P<sup>1</sup> site it is possible to transform plants with inhibitors targeted against specific serine proteinases of insects or microorganisms. A number of proteinase inhibitor genes and/or cDNAs are now available that have different specificities against various serine proteinases of animals, including insects, and microorganisms. In Table II is listed several of the inhibitor genes and/or cDNAs that our laboratory has isolated. The amino acids at their reactive sites that determine specificity against proteinases are shown for each inhibitor. These sites determine the specificities of the inhibitors against digestive enzymes of animals and microorganisms. Plants can be transformed with these

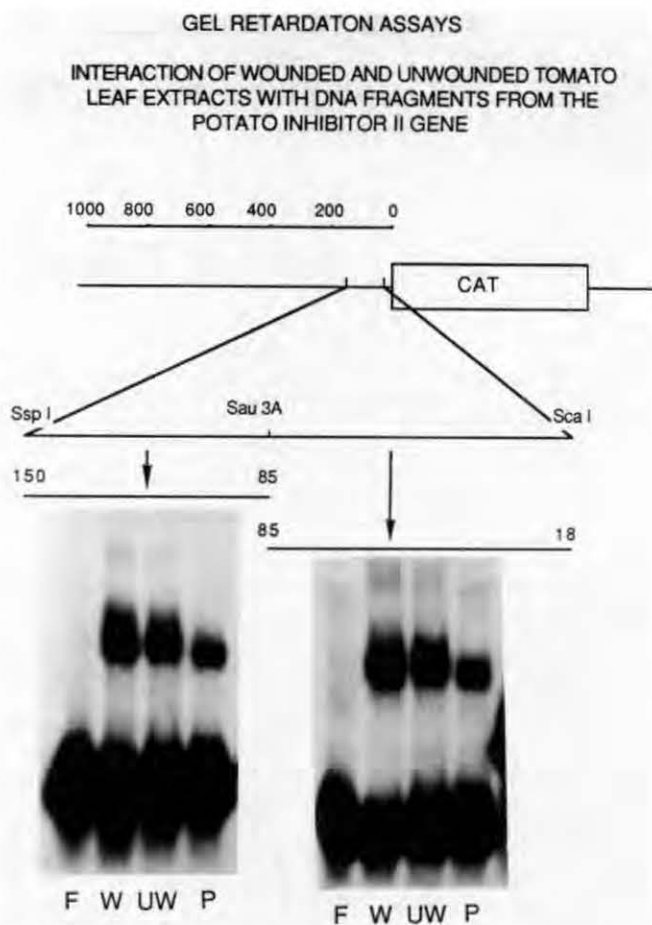


Fig. 5. Gel retardation assays in which the restriction fragments (F) shown above each experiment were mixed with nuclear extracts and subjected to electrophoresis. Nuclear extract from wounded tomato leaves (W); from unwounded leaves (UW); or with proteins partially purified from wounded tomato leaves by binding and releasing (with a NaCl gradient) from an affinity column containing an immobilized 200 bp Ssc I-Sca I restriction fragment.

genes or cDNAs in order to strongly express inhibitory activities in their leaves, roots, stems or other tissues. A variety of promoters and terminators are available for selecting maximal expression of the inhibitors. It may also be possible to select terminators that enhance the stabilities of the mRNAs in the target cells, thereby increasing the efficiency of inhibitor accumulation. With the variety of possibilities now available, it may be possible to create a spectrum of transformed plants strongly expressing the inhibitors in a tissue specific manner. With selected signal or transit sequences inserted into the open reading frames of the inhibitor genes the inhibitor proteins may be targeted to specific organelles where they can accumulate to high levels. Plants expressing the proteinase inhibitors will provide new materials for plant breeders to assay for resistances against a spectrum of plant pests. This sort of approach has already been initiated. A cDNA that codes for a Bowman-Birk type of trypsin inhibitor was obtained from the mRNA from developing cowpeas and the open reading frame was fused with a strong constitutive promoter and incorporated into the genome of tobacco plants (Hilder, et al., 1987). The fused gene was strongly expressed in tissues of the plants, resulting in inhibitor levels in leaves nearing 1% of the soluble proteins. The plants exhibited a strong resistance toward *Heliothis virescens*, the tobacco bud worm that thrives on

tobacco leaves. In similar experiments, potato and tomato Inhibitor II, driven by the 35<sup>s</sup> promoter, were expressed in leaves of tobacco and inhibited growth of *Manduca sexta* larvae that fed on them (Johnson et al., 1989). These experiments demonstrated that the expression of foreign proteinase inhibitor genes in plants can confer resistance toward insect pests and provide encouraging examples for pursuing the strategy described above.

#### Concluding remarks.

The chemistry, biochemistry, physiology and molecular biology of proteinase inhibitors and their genes now provides a fundamental foundation of knowledge to further understand the regulation of the genes that are under both environmental and developmental regulation in plants. This information can now be employed to fashion transgenic plants with foreign proteinase inhibitor genes to provide new materials that may be useful to improve the natural defensive systems of plants.

#### ACKNOWLEDGEMENTS

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