Use of Proteinase Inhibitors for Crop Protection

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Abstract -- Proteinase inhibitors are generally small proteins that specifically inhibit the action of proteinases. They are produced by plants in great quantities, yet they have no known function within plant tissues. They have been hypothesized to specifically interact with insect proteinases to protect plants against insect attack. Proteinase inhibitors accumulate in the seeds or storage organs of all plants, however, in the solanaceae, proteinase inhibitors also accumulate in the foliage of these plants. Further, they are normally expressed in the foliage at low levels, but following attack by insects, the levels of proteinase inhibitors increase dramatically.

We have isolated from a Russet Burbank Potato DNA genomic library, several genes coding for proteinase Inhibitor II. These genes have been analyzed at the molecular and functional level. Characterization of these genes has increased our understanding of the function of these proteinase inhibitors in plant tissues. The proteinase inhibitors can interact with a variety of proteinases, but the Inhibitor II's that we have isolated are specific for both trypsin and chymotrypsin.

We have prepared chimeric genes that express marker genes under the control of the wound-inducible Proteinase Inhibitor II promoter. These chimeric genes have been used to transform both tobacco plants and poplar trees. The wound-induction of these chimeric genes in the transgenic plants is similar to the induction of the genes in wild-type potatoes and tomatoes. When transgenic plants are placed in the field, the plants are fully capable of responding to insect attack by inducing new marker protein synthesis.

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INTRODUCTION

Plant proteinase inhibitors were first recognized in wheat flour 54 years ago by Read and Haas (1938). Since that time, many researchers have considered possible roles of proteinase inhibitors in plants. Early work considered plant proteinase inhibitors as possible regulatory proteins or storage proteins. However, the function of proteinase inhibitors as a regulator of endogenous proteinases was questioned because Ofelt et.al. (1955) showed that soybean proteinase inhibitors did not inhibit endogenous soybean proteinase and this conclusion was affirmed by Birk and Waldman (1965). Therefore, the storage role for the inhibitors was supported by their presence in large quantities in seeds and tubers. For example, the inhibitors are present in about 6% of the total soybean proteins (Rackis and Anderson, 1964) and up to 10% of the soluble proteins of potato tubers (Ryan et.al., 1968a).

Interest in plant proteinase inhibitors has expanded from earlier research on their regulatory or storage roles in plant and effects in the human food chain to more recent interest in their possible contributions to natural protection systems of plants. In 1964, Applebaum first proposed the role of plant proteinase inhibitors as a defense mechanism against insects based upon the study on the effect of soybean trypsin inhibitor on legume beetles (Applebaum, 1964). Moses Kunitz and co-workers established that trypsin inhibition by trypsin inhibitor was a result of a protein-protein association between the enzyme and inhibitor to form a complex that blocks trypsin activity (Kunitz and Northrop, 1936, Kunitz, 1947a and 1947b).

Potato Proteinase Inhibitors

In addition to the soybean trypsin inhibitor, other proteinase inhibitors have also received much attention. The chymotrypsin inhibitor from potato tubers (Ryan and Ball, 1962, Ball and Ryan, 1963), called Inhibitor I (Ryan, 1968a), was found to have inhibitory activity against proteinases from mammalian, bacterial, and fungal origins not from plant origin (Ryan, 1966). The potato Inhibitor I was shown to consist of four subunits and the molecular weight of Inhibitor I was found to be 39,000 ± 2,000 and that of its complex saturated with chymotrypsin, 140,000 ± 4,600, (Melville and Ryan, 1972), indicating that four molecules of chymotrypsin could make a complex with one molecule of Inhibitor I. Inhibitor I also was found in the detached potato (Ryan, 1968a) and tomato (Ryan, 1968b) leaves, which were incubated in water, but not in the attached leaves.

The possible involvement of proteinase inhibitors in plant protection received considerable support with the discovery in 1972, that attack of Colorado potato beetles on potato and tomato plants induced the accumulation of proteinase inhibitors in the leaves (Green and Ryan, 1972). This accumulation occurred even in leaves distant from the attack sites.

After this discovery, research focused on the characterization of proteinase inhibitor proteins as well as the chemical signals that induce the proteinase inhibitor accumulation. Several proteinase inhibitors are found in tomato and potato. The most thoroughly characterized of these inhibitors have been Inhibitor I (Melville and Ryan, 1972, Richardson, 1974, Richardson and Cossins, 1974) and Inhibitor II (Gustafson and Ryan, 1976, Bryant et.al.,1976).

The tomato inhibitors were shown to be highly homologous with potato inhibitors since tomato Inhibitors I and II strongly cross-reacted with antibodies prepared against each respective potato inhibitors (Gustafson and Ryan, 1976). Inhibitors I and II were also purified from wounded tomato leaves and were shown to be very similar to potato tuber Inhibitors I and II in subunit molecular weight, composition, and inhibitor activities (Plunkett et.al., 1982).

In an effort to understand the function and significance of the proteinase Inhibitor II gene family, we have isolated a series of proteinase inhibitor genes from a potato genomic library. The first of these proteinase Inhibitor II genes was previously isolated and characterized (Thornburg, et al., 1987). We have since isolated and characterized a second Proteinase Inhibitor II gene from the potato library (Park, 1991). Some of the general characteristics of the proteinase inhibitor genes are presented below.

Characteristics of Potato Inhibitor II genes

In general, the Inhibitor II open reading frame is composed of two exons separated by a single small intron. The intron-exon junctions obey the GT-AG rule (Breathnach, et al., 1978, Brown, 1986), in which intron sequences usually start with GT and end with AG.

The Inhibitor II protein is synthesized as a preprotein and the signal sequence targets the vacuolar membrane to transport the mature Inhibitor II protein into the vacuole (Nelson and Ryan, 1980). The Inhibitor II genes are processed during or shortly after synthesis between amino acid residues 25 and 26 to produce the mature inhibitor. The deduced amino acid sequence of *pin2T* indicates that it is initially translated with a

sequence of 147 amino acids, which is 7 amino acid shorter than that of the previously characterized *pin2K.* Of the 147 amino acids, the first 25 translated amino acids (30 amino acids in *pin2K),* apparently function as a signal sequence (von Heijne, 1983) to facilitate transport of the mature protein into the vacuole where it is stored until it is needed to combat insect attack (Walker-Simmons and Ryan, 1977). The signal sequence maintains cleavage specificity with the "-3/-1" rule (von Heijne, 1983), in which the signal sequence has small, neutral amino acid residues in positions -3 and -1 (counting from the cleavage site between positions -1 and +1) but are rare in -2. In the case of the Inhibitor IIT open reading frame, Ala and Val are located in positions -1 and -3, respectively. In addition to this (-3, -1) rule, the signal sequence of Inhibitor IIT contain charged residues near both termini of its sequence (in positions 5, 6, and 24) and an extended hydrophobic core. Therefore, signal sequence of Inhibitor IIT seems to be fit very well to the general properties of the known signal peptide. Hydropathy plots (Kyte and Doolittle, 1982) of the signal sequence of the Inhibitor IIT protein clearly shows that the amino terminal segment is rich in hydrophobic amino acid resides.

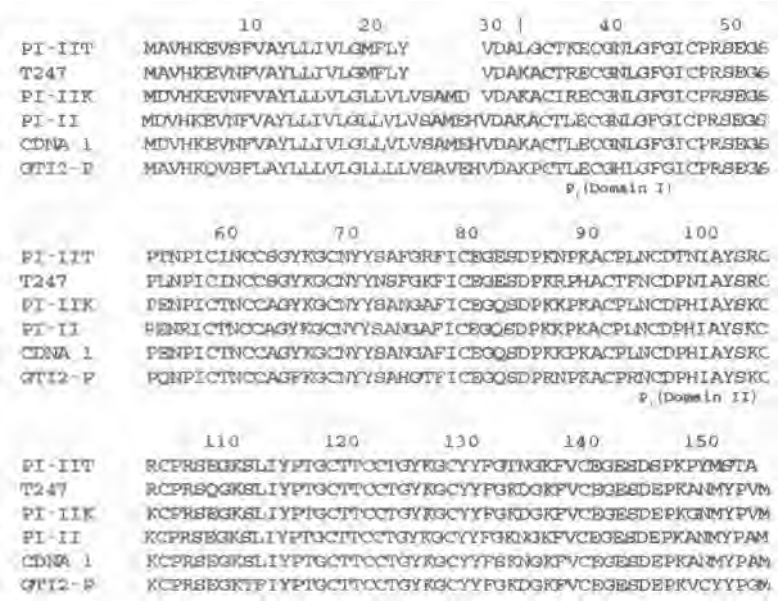

Figure 1. Comparison of the deduced amino acid sequence of the Potato Inhibitor II T with those of the other Inhibitor II genes. The single letter amino acid code is used. The identity between the inhibitors is indicated by bold face. The putative cleavage site of the transit sequence is shown with an arrow. Gaps were introduced for best fit of alignment in the transit sequence. Active site at P1 is shown by an asterisk in both domains. All of the 16 cysteine residues are underlined. The amino acid sequences of the T247, PI-11K, PI-11, cDNA1 and GTI2-P arc from Graham, et al., 1985; Thornburg, et al., 1987; Keil, et al., 1986; Sanchez-Serrano, et al., 1986; and Fox, 1986, respectively.

The entire pre-Inhibitor IIT amino acid sequence exhibits around 80% identity with deduced amino acid sequences from other Inhibitor II genes. The major difference lies in the length of the signal sequence. The mature proteinase inhibitors are very similar. Figure 1 shows the comparison of the deduced amino acid sequences of the potato Inhibitor IIT with those of the other Inhibitor II proteins.

Potato Inhibitor HT protein, like other Inhibitor II proteins, consists of two domains that share 50% amino acid identity. However, these domains differ in their active site P1-P1' residues (Schechter and Berger, 1967). The active site PI-P1' at amino acid residues 30-31 of the Inhibitor IIT protein for domain 1, is Lys-Glu, and amino acid residues 87-88 for domain 2, Leu-Asn. Figure 2 shows the comparison of internal homology within the Inhibitor IIT amino acid sequence. The boxed regions indicate the amino acid identity with each other in two domains.

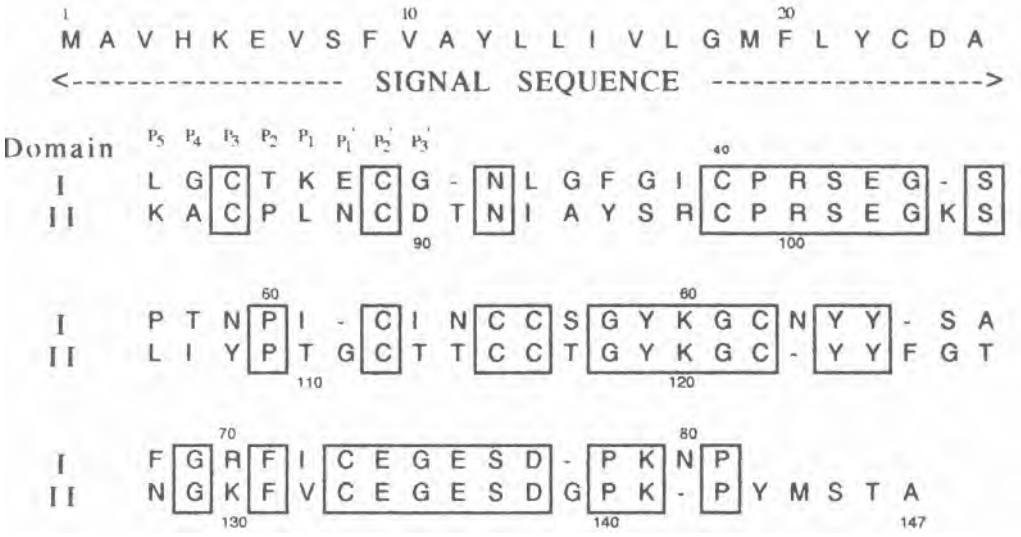

Figure 2. Alignment of the trypsin domain (Domain I) with the chymotrypsin domain (Domain II) of the Inhibitor IIT protein sequence. The single letter amino acid code is used throughout. The signal sequence is shown above the alignment of the two domains. The amino acid sequences arc aligned such that the conserved cysteine residues show maximum homology. Gaps were introduced for best fit of alignment. The N-terminal domain is labeled Domain I while the C-terminal domain is labeled Domain II. The boxed regions indicate the homologous sequence between two domains. Labeling of the inhibitory site amino acid residues is indicated (P5-P'3).

The common rules for inhibitory sites have been proposed by Kowalski and Laskowski (1972) in which inhibitors with P1 Lys and Arg tend to inhibit trypsin and trypsin-like enzymes, and those with P1 Tyr, Phe, Leu and Met inhibit chymotrypsin and chymotrypsin-like enzymes. According to the common rules for inhibitory sites, the site in domain 1 would be specific for trypsin-like proteases, while in domain 2, the reactive site is specific for chymotrypsin-like proteases. This "double headedness" is typical for members of both the Inhibitor II family (Ryan and Hass, 1980) and the Bowman-Birk family (Ikenaka and Odani, 1978) of proteinase inhibitors from plants. Both the Inhibitor II and Bowman-

Birk families contain two inhibitory domains, although these two families are unrelated.

Potato Inhibitor II family, which is one of 13 different families of serine proteinase inhibitors in nature (Laskowski, 1986), is known to inhibit both chymotrypsin-like and trypsin-like proteins from either animal or microbial origin (Ryan, 1973). The comparison of the reactive sites in potato Inhibitor II family (Table 1) indicated that three subgroups are present in this family. They are Inhibitor II which has two trypsin-specific domains, Inhibitor II which has two chymotrypsin-specific domains, and Inhibitor 11 which has one trypsin-specific domain and one chymotrypsin-specific domain. Inhibitors IIT and IIK genomic clones of potato and tomato Inhibitor II cDNA contain one trypsin-specific domain and one chymotrypsin-specific domain. Tomato Inhibitor II genomic clone contains two trypsin-specific domains. Another potato Inhibitor II cDNA and genomic clones contain two chymotrypsin-specific domains. In fact, isoforms of Inhibitor II which has two trypsin-specific domains have been isolated in Dr. Clarence A. Ryan's laboratory (Washington State University) (Fox, 1986). This result supports the presence of three subgroups in the potato Inhibitor II family.

The potato Inhibitor II gene codes for a protein which shares high homology with two small molecular weight polypeptides that were isolated from potato tubers (Hass et.al., 1982). The potato polypeptide trypsin inhibitor, PTI (MW. 5,100), exhibits 82% amino acid homology with the middle sequence of the Inhibitor IIT protein, whereas the potato polypeptide chymotrypsin inhibitor, PCI (MW. 5,400), shows 84% homology with the Inhibitor IIT protein. Figure 3 shows the alignment of the deduced amino acid sequence of *pin2T* with the amino acid sequences of PTI and PCI. PTI and PCI do not dimerize like Inhibitor II and thus exist as single

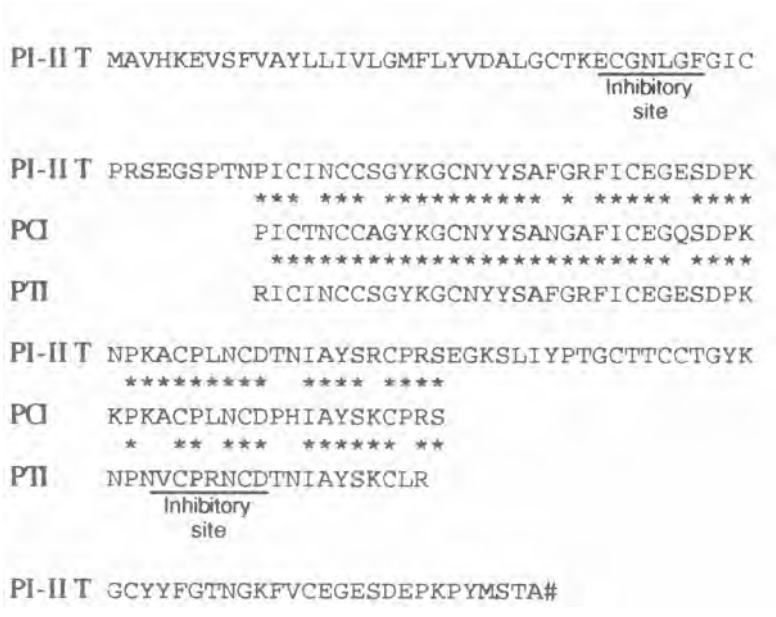

Figure 3. Alignment of the deduced amino acid sequence of *pin2T* with the amino acid sequences of polypeptide trypsin inhibitor (PTI) and polypeptide chymotrypsin inhibitor (PCI). The single letter amino acid code is used throughout. The identity between the two inhibitors is indicated by asterisks. The symbol, #, represents the stop codon of the Inhibitor IIT protein.

monomeric subunits (Pearce et.al., 1982). The PCI and PTI, represent only half the sequence of the Inhibitor II. They contain only a single inhibitory site, (amino acid 38). In both small inhibitors, the active site corresponds to the second active site (amino acid 87) of the full length Inhibitor IIT. It is thought that PCI and PTI may be derived from the Inhibitor II molecules by the action of plant proteinases.

Use of Proteinase Inhibitors to protect Crop Plants

Several studies have previously been performed indicating that the levels of proteinase inhibitors in plant tissues are correlated with insect resistance (Gatehouse and Boulter, 1983; Broadway, et al., 1986). Because of the ability of the proteinase inhibitors to effectively block the action of digestive proteases of animals, but not of plants, the proteinase inhibitors have been proposed as components of plant defenses that could be transferred from one species to another.

Much of the pioneering work that has been done in this area has been done in easily manipulated plants such as tobacco and tomato. In the first studies transgenic tobacco plants were constructed that contained the cowpea trypsin inhibitor under the control of the constitutive CaMV 35S promoter (Hilder, et al., 1987). When larvae of the tobacco budworm *(Heliothis virescens)* were placed on the plants that expressed the cowpea trypsin inhibitor at significant levels, there was reduced leaf damage on the transgenic plants relative to untransformed controls. This reduction in the leaf damage also was correlated with the level of inhibitor present in the tissues. In other recent studies, Johnson et al., (1990) demonstrated that the potato Proteinase Inhibitor II also can function to limit insect growth in transgenic tobacco plants. The transfer of these proteinase inhibitors to a wide variety of crop species is currently underway in a number of laboratories, where the inhibitors should provide plants with antinutrient properties thereby preventing herbivorous insects or fungi from deriving proper nourishment from the plant tissues. Transfer of such genes into trees has yet to be confirmed, but the success of the tobacco experiments indicate that similar results could be obtained in woody species (Thornburg, 1990).

In addition, the wound-inducible phenotype of the proteinase inhibitor gene system (Thornburg, et al., 1987) is another aspect about these genes that could be used for agricultural purposes in transgenic plants. It is known from field studies of transgenic plants, both tobacco (Thornburg, et al., 1990; Thornburg. 1991) and poplar trees (Klopfenstein, et al., 1991; McNabb, et al., 1991) that the proteinase inhibitor promoter is capable of expressing novel chimeric genes in response to insect attack, and it is further known that the Inhibitor II promoter induces these

chimeric genes in precisely those tissues that the insect preferentially consume (Thornburg, et al., 1990).

LITERATURE CITED

Applebaum, S.W. (1964) Physiological aspects of host specificity in the Bruchidae-I. General considerations of developmental compatibility. *J. Ins. Physiol.* 10:783-788.

Ball, A. K. and Ryan, C.A. (1963) Concerning a crystalline chymotryptic inhibitor from potatoes, and its binding capacity for the enzyme. *J. Biol.Chem.* 238:2976-2982.

Birk, Y. and Walman, M. (1965) Amylolytic-, trypsin-inhibiting-, and urease-activity in three varieties of soybeans and in soybean plant. Qual. Plat. Mater. Veg. 12:199-209.

Breathnach, R., Benoist, C., O'Hare, K., Gannon, F. and Chambon, P. (1978) Ovalbumin gene: Evidence for a leader sequence in mRNA and DNA sequences at the intron-exon boundaries. Proc. Natl. Acad. Sci. USA. 75:4853-4857.

Broadway, R., M., Duffey, S.S., Pearce, G. and Ryan, C.A. (1986) Plant proteinase inhibitors: A defense against herbivorous insects? Entomol. exp. appl. 41:33-38.

Brown, J. W.. (1986) A catalogue of splice junction and putative branch point sequences from plant introns. Nucleic Acids Res. 14:9549-9559.

Bryant, J., Green, T. R., Gurusaddaiah, T., and Ryan, C.A. (1976) Proteinase inhibitor II from potatoes: Isolation and characterization of its protomer components. Biochemistry 15:3418-3423.

Fox, E.A. (1986) Isolation and characterization of a proteinase inhibitor II gene from Lycopersicon esculentum. PhD Dissertation, Washington State University.

Gatehouse, A.M.R. and Boulter, D. (1983) Assessment of the anti-metabolic effects of trypsin inhibitors from cowpea (Vigna unguiculata) and other legumes on development of bruchid beetle (Callosobruchus maculatus). 34:345-350.

Graham, J., Pearce, G., Merryweather, J., Titani, K., Ericsson, L. and Ryan, C. (1985) Wound-induced proteinase inhibitors from tomato-leaves. H. The cDNA-deduced primary structure of pre-inhibitor II. J. Biol. Chem. 260:6561-6564.

Green, J. S. and Ryan, C.A. (1972) Wound-induced proteinase inhibitor in plant leaves: A possible defense mechanism against insects. *Science* 175:776-777.

Gustafson, G and Ryan, C.A. (1976) Specificity of protein turnover in tomato leaves. J. Biol. Chem. 251:7004-7010.

Hilder, V.A., Gatehouse, A.M.R., Sheerman, S.E., Barker, R.F. and Boulter, D. (1987) A novel mechanism of insect resistance engineered into tobacco. Nature. 330:160-163.

Hass, G. M., Hermodson, M. A., Ryan, C. A., and Gentry, L. (1982) Primary structures of two low molecular weight proteinase inhibitors from potatoes. *Biochemistry* 21:752-756.

Ikenaka, T. and Odani, S. (1978) Structure-function relationships of soybean double-headed proteinase inhibitors. *in* Mugnusson, S., Ottesen, M., Foltmann, B., Done, K., and Neurath, H. eds Regulatory Proteolytic Enzymes and Their Inhibitors. Pergamon Press. Oxford. pp. 207-216.

Johnson, R., Narvaez, J., An, G. and Ryan, C.A. (1990) Expression of proteinase inhibitors I and II in transgenic tobacco plants: Effects on natural defense against *Manduca sexta* larvae. Proc. Natl. Acad. Sci. USA. 86:9871-9875.

Keil, M., Sanchez-Serrano, J., Schell, J. and Willmitzer, L. (1986) Primary structure of a proteinase inhibitor II gene from potato (Solanum tuberosum). Nucl. Acids Res. 14:5641-5650.

Klopfenstein, N.B., Shi, N.-., Hall, R.B., McNabb, S.E. and Thornburg, R.W. (1991) A wound-inducible promoter from an herbaceous dicot functions correctly in the woody perennials: Potato proteinase inhibitor 11 in poplar trees. Can. J. For. Res. (in press).

Kowalski, D. and Laskowski, M., Jr. (1972) Inactivation of enzymatically modified trypsin inhibitors upon chemical modification of the a-amino group in the reactive site. Biochemistry 11:3451-3455.

Kunitz, M. (1947a) Crystalline soybean trypsin inhibitor. II. General properties. J. Gen. Physiol. 30:291-310.

Kunitz, M. (1947b) Isolation of a crystalline protein compound of trypsin and of soybean trypsin-inhibitor. J. Gen. Physiol. 30:311-320.

Kunitz, M. and Northrop, J.H. (1936) Isolation from beef pancreas of crystalline trypsinogen, trypsin, a trypsin inhibitor and an inhibitor-trypsin compound. J. Gen. Physiol. 19:991-1007.

Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157:105-132.

Laskowski, M. Jr. (1986) Proteinase inhibitors of serine proteinase: Mechanism and classification. *in* Friedman, M. ed. Nutritional and Toxicological Significance of Enzyme Inhibitors in Foods. Plenum Press, New York and London. pp. 1-17.

McNabb, H.S., Klopfenstein, N.B., Hanna, R.D., Hall, R.B., Hart, E.R., Heuchelin, S.A., Allen, K.K. and Thornburg, R.W. (1991) A field test of transgenic hybrid poplar trees: Establishment and growth through the second season. *in* Biological monitoring of genetically engineered plants and microbes., MacKenzie, D.R., eds Bethesda, MD. Agricultural Research Institute. pp. 155-159.

Melville, J. C. and Ryan, C.A. (1972) Chymotrypsin inhibitor I from potatoes. J. Biol. Chem. 247:3445-3451.

Nelson, C. E. and Ryan, C. A. (1980) *In vitro* synthesis of pre-proteins of vacuolar compartmented proteinase inhibitors that accumulate in leaves of wounded tomato plants. Proc. Natl. Acad. Sci. USA. 77:1975-1979.

Ofelt, C. N., Smith, A. K., and Mills, J.M. (1955) Proteinase of the soybean. Cereal Chem. 32:53-63.

Park, S. (1991) Characterization of a potato proteinase inhibitor II gene whose expression is not wound-responsive. PhD Dissertation, Iowa State University, Ames.

Pearce, G., Sy, L., Russell, C., Ryan, C. A., and Hass, G.M. (1982) Isolation and characterization from potato tubers of two polypeptide inhibitors of serine proteinases. Arch. Biochem. Biophys. 213:456-462.

Plunkett, G., Senear, D. F., Zuroske, G., and Ryan, C.A. (1982) Proteinase inhibitors I and II from leaves of wounded tomato plants: Purification and properties. Arch. Biochem. Biophys. 213:463-472.

Rackis, J. J. and Anderson, R.L. (1964) Isolation of four soybean trypsin inhibitors by DEAE-cellulose chromatography. Biochem. Biophys. Res. Commun. 15:230-235.

Read, J. W. and Haas, L.W. (1938) Studies on the baking quality of flour as affected by certain enzyme actions. V. Further studies concerning potassium bromate and enzyme activity. Cereal Chem. 15:59-67.

Richardson, M. (1974) Chymotryptic inhibitor I from potatoes. Biochem. J. 1 3 7:1 0 1-1 1 2.

Richardson, M. and Cossins, L. (1974) Chymotryptic inhibitor I from potatoes: The amino sequences of subunits B, C, and D. FEBS Lett. 4 5 :1 1-13.

Ryan, C.A. (1966) Chymotrypsin inhibitor I from potatoes: Reactivity with mammalian, plant, bacterial, and fungal proteinases. Biochemistry 3:1592-1596.

Ryan, C.A. (1968a) Synthesis of chymotrypsin inhibitor I protein in potato leaflets induced by detachment. Plant Physiol. 43:1859-1865.

Ryan, C.A. (1968b) An inducible protein in potato and tomato leaflets. Plant Physiol. 43:1880-1881.

Ryan, C.A. (1973) Proteolytic enzymes and their inhibitors in plants. Ann. Rev. Plant Physiol. 24:322-326.

Ryan, C. A. and Ball, A.K. (1962) An inhibitor of chymotrypsin from *Solanum tuberosum* and its behavior toward trypsin. Proc. Natl. Acad. Sci. USA. 48:1839-1844.

Ryan, C.A. and Hass, G.M. (1980) Structural, evolutionary and nutritional properties of proteinase inhibitors from potatoes. *in* Ory, R. L. ed. Antinutrients and Natural Toxicants in Foods. Foods and Nutrition Press, Westport, CT. pp. 169-185.

Schechter, I. and Berger, A. (1967) On the size of the active site in proteases. I. Papain. Biochem. Biophys. Res. Commun. 27:157-162.

Thornburg, R.W. (1990) New approaches to pest resistance in trees. AgBiotech News and Information. 2:845-849.

Thornburg, R.W. (1991) Modes of expression of a wound-inducible gene in field trials of transgenic plants. *in* Biological monitoring of genetically engineered plants and microbes. MacKenzie, D.R., eds Agricultural Research Institute, Bethesda MD. pp.147-154.

Thornburg, R.W., An, G., Cleveland, T.E., Johnson, R. and Ryan, C.A. (1987) Wound-inducible expression of a potato inhibitor II-chloramphenicol acetyltransferase gene fusion in transgenic tobacco plants. Proc. Natl. Acad. Sci. USA. 84:744-748.

Thornburg, R.W., Kernan, A. and Molin, L. (1990) CAT protein is expressed in transgenic tobacco in field tests following attack by insects. Plant Physiol. **92:500-505.**

von Heijne, G. (1984) How signal sequences maintain cleavage specificity. J. Mol. Biol. 173:243-251.

Walker-Simmons, M. and Ryan, C.A. (1977) Immunological identification of proteinase inhibitor I and II in isolated tomato leaf vacuoles. Plant Physiol. 60:61-63.

Weiss, E. A., Gilmartin, **G. M.** and Nevins, J. (1991) Poly(A) site efficiency reflects the stability of complex formation involving the downstream element. EMBO J. 10:215-219.