Transformation, Expression and Ecological Impacts of Transgenic Plants Expressing Novel Inhibitory Proteins

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Abstract: Over time and exposure, plant protein protease inhibitors and related proteins have evolved as a plant's defense to various sources of harm such as herbivory from insects and other invertebrates. This defense mechanism utilizes inhibitory proteins such as protease inhibitors and lectins in order to ward off digestive and otherwise detrimental proteases and enzymes. An increase in the plant's resistance to these attacks has been a long term goal sought by the agricultural industry for both the benefit of the economy and the health of the general public. Modern advances in technology have utilized "genetic modification" in order to make it possible to manipulate these defense proteins in plants. Increased plant defense can be achieved through the introduction of genes from other species of plants and other types of organisms into a different species of plants. This increase in plant defense may be the solution we've been looking for in order to safely increase crop yields which would effectively reduce the use of land for agriculture and world food shortages.

This is a review of these plant defenses and various attempts of genetic manipulation to increase their expression. This review will weigh the environmental pros and cons that come with the use of this technology as well as natural phenomena that would prove this technology to be as dangerous as it is helpful.

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Introduction:

With the world's population exceeding 6 billion in the year 2000 and projected to reach 8.5 billion by the year 2025, the demand for food will continue to grow with it (Babu et al., 2003). In order to meet this global need for food, more sustainable agricultural practices need to be developed. Our current agricultural processes have attempted to stop the persistent crop losses to phytophagous insects, which is at 14% of the total global agricultural output, through the deployment of *Bacillus thuringiensis* (Bt) insect resistant plants (Hilder and Boulter, 1999). According to the ISAAA, the use of genetically modified crops has increased from being grown on 4.2 million acres in 1996 to 222 million acres in 2005 (Cerda and Wright 2004). Unfortunately, strong evidence through research has shown that target insects have developed resistance to these toxins produced by crops (Sharma and Ortiz, 2000). The other alternative that farmers currently employ to keep crop losses down is through the heavy usage of synthetic chemical pesticides which have detrimental effects on the environment and human health (Christou et al., 2006).

Since the first transgenic tobacco plants that expressed foreign proteins were produced in 1984 (Horsch et al., 1985) scientists have researched the insect-plant interaction and have developed novel ways to increase plant resistance. The development of plants that can express protease inhibitors, lectins, and other inhibitory molecules has been heavily pursued as a new alternative of creating pest-resistant plants. There are many types of proteases and other digestive enzymes used by pests in order to digest their plant meals. Through the transformation of a wide array of inhibitory molecules into

crops to hinder these enzymes, crop losses due to insect herbivory can be reduced (Leo et al., 2002).

Many different inhibitory molecules have been transformed into plants cells by utilizing methods of transforming transgenic plants with a high success rate over the years (Tzfira and Citovsky, 2006). Scientists have been testing these inhibitors on a species-by-species basis in order to determine how well each inhibitor affects each insect's protease profile (Leo et al., 2002) in order to make the plant's defense more effective (Christou et al., 2006), as well as more environmentally friendly (Hilbeck, 2001).

Ever since the development of genetically modified plants, restrictions and public concern have been implemented and expressed in order to ensure that their use is safe for humans and the environment (Cowgill and Atkinson, 2004). The expression of protease inhibitors and lectins in transgenic plants is rightfully under the same scrutiny as any other issue that involves the use of genetic manipulation. This paper will review the production, regulation, ecological effects and health risks of transgenic plants as well as the target insect proteases and the inhibitors that inactivate them.

Methods for producing transgenic plants:

Agrobacterium tumefacien mediated gene transfer:

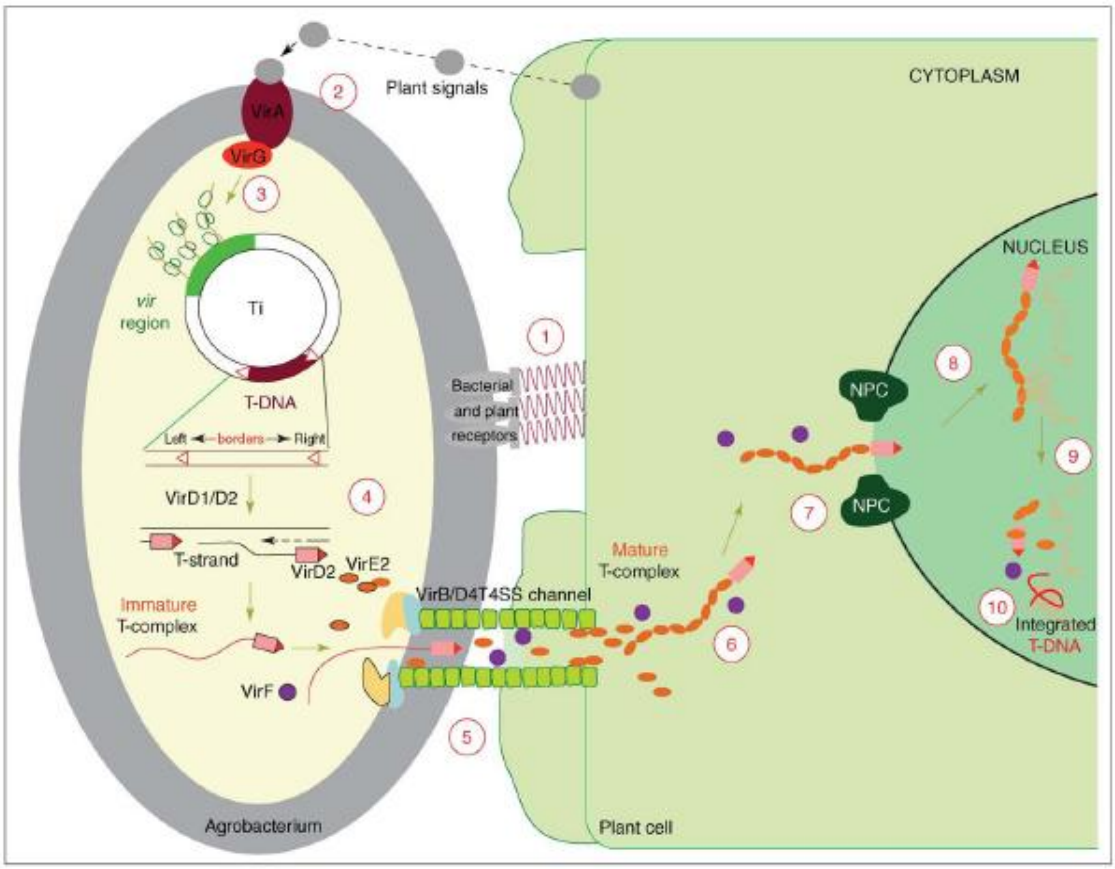
Agrobacterium tumefacien (At) is a soil dwelling bacterium that infects a large range of plants, mainly dicotyledons, through the integration of its own genetic material into a host cell. This integration is made possible through the use of its tumor-inducing

(Ti) plasmid, which codes for most bacterial virulence (Vir) proteins and a T-DNA region (Babu et al., 2003). *At* transformation, which has become the leading method of cellular transformation (Franklin et al., 2007), utilizes the Ti plasmid in order to transfer genes of interest into a target cell and incorporate them into the genome. Selected T-DNA encoded genes are deleted in order to disarm the plasmid vector and a recombinant T-DNA plasmid containing the gene of interest, such as a gene encoding for a protease inhibitor, replaces it (Tzfira and Citovsky, 2006).

The steps in which transformation are shown in (Tzfira and Citovsky, 2006) (Fig.1) produce the final product, which is a viable, transformed cell that can be regenerated into a mature plant that is expressing the transgene of interest.

Although this method of transformation has been used to effectively produce hundreds of transgenic plant species (Babu et al., 2003), there are problems associated with its use. *At* transformation's obstacles include the inability to transfect plants like legumes and cereals although transformation has been possible in some cases (Christou, 1996; Gelvin, 2000). *At* transformation has been rendered ineffective by such plants as St. John's Wort because of the antimicrobial agents, Hypericin and Hyperforin that are expressed by the plants' cells (Franklin et al., 2007). Most scientists believe that the limit to the number of hosts that *At* can infect has been reached, but can be expanded through the manipulation of the plant host's genome that will allow effective *At* transformation (Gelvin, 2000).

Figure 1



A model for the *Agrobacterium*-mediated genetic transformation. The transformation process comprises 10 major steps and begins with recognition and attachment of the *Agrobacterium* to the host cells (1) and the sensing of specific plant signals by the *Agrobacterium* VirA/VirG two-component signal-transduction system (2). Following activation of the *vir* gene region (3), a mobile copy of the T-DNA is generated by the VirD1/D2 protein complex (4) and delivered as a VirD2–DNA complex (immature T-complex), together with several other Vir proteins, into the host-cell cytoplasm (5). Following the association of VirE2 with the T-strand, the mature T-complex forms, travels through the host-cell cytoplasm (6) and is actively imported into the host-cell nucleus (7). Once inside the nucleus, the T-DNA is recruited to the point of integration (8), stripped of its escorting proteins (9) and integrated into the host genome (10). A detailed model of the host cellular mechanisms and the role of plant-specific factors in the transformation process are given in Figure 2. (This illustration was reproduced, with modifications, from [28•] with permission.).

(Source: Tzfira and Citovsky, 2006.)

Microprojectile Bombardment:

Microprojectile bombardment or biolistics is the means of transforming a cell by firing a microprojectile coated with DNA at high speeds at a target cell (Franklin et al., 2007). Particle bombardment has been used widely for the transformation of legumes and cereals because of their agronomic importance and other recalcitrant species that have problems associated with *At* transformation (Walden and Wingender, 1995).

This technique involves the coating of microparticles, made of gold or tungsten, with DNA via a suspension containing the DNA segments of interest, calcium chloride, and spermidine. These coated microparticles are suspended in ethanol and subsequently coated onto a macroparticle (Southgate et al., 1995). The group of macroparticles are accelerated either by pressurized helium, an electrical discharge or an explosion in the direction of the cell by a 'gene gun' (Walden and Wingender, 1995) and are stopped suddenly by a stopping screen. The microparticles then disassociate from the macroparticle and continue past the stopping screen to penetrate the target cell's membranes, carrying the foreign DNA into the interior of the target cell (Babu et al., 2003). In order for successful transformation the DNA must penetrate the nuclear membrane so the nuclear genome can be accessed or else cytoplasmic degradation may occur (Southgate et al., 1995).

The physical nature of bombardment has a few problems associated with it. One important problem since its development is the physical trauma inflicted on the target cell. The cell's viability after one or multiple bombardments is dependent on a number of factors that must be accounted for on a species by species basis (Franklin et al., 2007). Another setback is the low rate of transformation due to the nature of recombination in the nucleus. The chance of a particle penetrating the nucleus is around 10% and the success of recombination is even less (Southgate et al., 1995). Regardless of its problems, particle bombardment is a valuable and widely used transformation tool.

Electroporation:

Electroporation utilizes the phenomena of 'electropermeabilization' in order to deliver DNA into the tissue of intact cells (Weaver and Chizmadzhev, 1996). This technique involves the application of a high-voltage electric pulse to a solution containing a mixture of protoplasts and foreign DNA (Babu et al., 2003). The electric field causes the formation of hydrophobic pores in the lipid bilayer of the membrane by lateral thermal fluctuations of the lipid molecules. The charged free floating DNA suspended in the mixture enters the pores due to electrical drift which is the driving force of DNA induction (Weaver and Chizmadzhev, 1996). The pores are then closed up utilizing membrane repair mechanisms. The inserted DNA must now avoid being degraded by exo- and endonuclease activity and be shuttled into the nucleus for integration into the genome (Sorokin et al., 2000).

Mechanical stability or instability of membranes is important for the recovery of treated cells and is usually dependent on the applied current strength and time of exposure. Too strong a current or too long of exposure can cause irreversible electroporation which would lead to rupturing of the cell (Weaver and Chizmadzhev, 1996). The fragility of treated cells is one problem associated with this technique because of the weakening of the membrane. It isn't until treated cells have divided and regenerated their membranes that the protoplasts can be handled (Sorokin et al., 2000).

Polyethylene Glycol Mediated Gene Transfer:

Polyethylene glycol (PEG) is a polymer that is used in combination with a transformation buffer in order to insert foreign DNA into target plant protoplasts (O'Neill et al., 1993). The PEG medium is capable of producing high osmotic pressures across the

cellular membrane which induces poration of the membrane and allows the entry of cloned DNA material (Francois et al., 2002). After the cells are removed from the PEG medium the pores are closed and the cells remain intact and viable. The DNA, as in any case, must then be escorted into the nucleus for integration into the genome (O'Neill et al., 1993).

One problem that was observed in PEG transformed plants was an increase in the number of unexpected nuclear genomic mutations that are associated with the technique (Heifetz and Tuttle, 2001). Another setback that is common to most techniques is the need for establishing a regeneration system from a single transformed cell (Christou, 1996). This direct gene transfer method is an effective technique in order to successfully transform viable protoplasts.

Microinjection:

Microinjection is a simple theory but complex technique that uses microscopic needles or fibers to facilitate penetration of DNA into specific tissue types (Christou, 1996). The cloned DNA containing the gene of interest is mixed with silicone oil and galinstan and inserted into a microscopic needle on the scale of femotoliters. The mixture is then gently warmed and expelled from the microsyringe via the resulting pressure that is produced. This technique enables researchers to inject genetic material into either the nucleus or the chloroplast which depends on which genome is selected for incorporation (Francois et al., 2002).

Microinjection is a difficult technique in that it is easy to damage cells beyond recovery. Vibrations and the volume of material being injected with too much force are

among the variables that must be accounted for in order to have successful transformation (Francois et al., 2002). This technique is not utilized as frequently as the other techniques but does have applications for transferring DNA into plant tissues, and more specifically the chloroplasts of cells, which are not transformable by most techniques (Babu et al., 2003).

Construction of Transformed DNA:

In order to successfully transform DNA into a genome and express it under desired conditions, genetic constructs need to be made to facilitate the integration and expression of the foreign gene (Christou, 1996). A selectable or screenable marker gene, a promoter, a coding sequence containing your gene of interest, and a terminating sequence should be included in the transgene construct (Walden and Wingender, 1995).

Selectable or screenable markers are usually dominant genes that encode either antibiotic or herbicide resistance that enable physical differentiation between transformed and untransformed plants (Ebinuma et al., 1999). A screenable marker is a gene that encodes a protein that results in the physical expression of a visible product in plants that are successfully transformed. Selectable markers are genes that elicit a detoxifying response such as herbicidal or chemical resistance and would enable selection through the growth of plants in an antibiotic or herbicidal medium (Christou, 1996). Selectable markers are preferred as they would be the only plants able to be established and grow under the conditions that would require some kind of toxic resistance (Christou, 1996), but they have more risks when their environmental risks are considered (Hilbeck, 2001).

The promoter region of the construct is responsible for the expression level of the transgene being inserted (Walden and Wingender, 1995). Promoters can confer either constitutive expression like under the Cauliflower Mosaic Virus (CaMV) 35S promoter (Hilder and Boulter, 1999) or tissue-specific expression like the Maize Pollen specific promoter (Jouanin et al., 1998). A constitutive promoter would express the gene product in most, if not all, of the tissue types and would be expressed at some level all the times (Hilder and Boulter, 1999). A tissue-specific promoter drives the expression of transgenes in particular parts of the plant (Christou, 1996). A tissue-specific promoter can be induced by would response signals so the level of gene expression or gene regulation is dependent on the level and location of herbivorous activity (Hilder and Boulter, 1999).

Types of Proteases and Their Roles in Insect Digestion:

Phytophagous digestion is regulated by a series of digestive proteases that metabolize essential molecules like proteins and carbohydrates. The magnitudes of importance these proteases have in insects make them a great target for transgenic plant resistance. These digestive proteases are classified according to their catalytic mechanisms (Fan and Wu, 2005) with the foremost proteases involved in digestion being Serine Proteases, Cysteine Proteases , Aspartic Proteases, Metallo-Proteases (Lawrence and Koundal, 2002) , and □-Amylase which is a dietary metallo-enzyme that cleaves carbohydrates (Pereira et al., 1999). The inhibition of these classes of digestive enzymes has been the primary means for resistance development in transgenic plants.

Serine Proteases:

Serine type proteases constitute the predominant catalytic type of protein cleaving enzyme and has been found in almost all living organisms (Simonet et al., 2003). Serine proteases have been identified in the digestive tracts of insects, particularly butterflies, moths (Lepidoptera), and flies (Diptera) whose gut pHs are alkaline (pH 9-11) where serine protease activity is the highest (Lawrence and Koundal, 2002). These proteases are initially synthesized as inactive zymogens and remain that way until they are cleaved by effector proteins in response to certain stimuli. Serine proteases are characterized as peptidases whose active site consists of the catalytic residues Serine, Histidine and Aspartate (Simonet et al., 2003). Serine proteases are primary inhibitory targets because they are not used by plants for large scale protein digestion so the production of inhibitory molecules would not affect endogenous protease activity (Lawrence and Koundal, 2002).

There are three classes of serine type proteases that work in conjunction in the initial digestion of proteins in animals, which include trypsin, chymotrypsin and elastase. The majority of the known serine protease types are trypsin type proteases (Lawrence and Koundal, 2002).

Trypsin, like other serine proteases, has a conserved serine active site and it also has conserved cysteine residues that form disulfide bridges that are required for proper protein folding and activity (Zeng et al., 2002). Trypsin is known to cleave peptides at the C-terminal at the basic residues of Lysine, K, and Arginine, R (Lawrence and Koundal, 2002). This is through the attracting and stabilizing effects of the negatively charged Aspartate residue in the active site (Zeng et al., 2002). A property of trypsin is

that it is autocatalytic, meaning it cleaves itself in order to become active. Trypsin is initially produced as trypsinogen and through autocatalysis becomes active trypsin (Hartley, 1970).

Chymotrypsin, like trypsin is a digestive protease that utilizes essentially the same catalytic mechanism with a His, Ser and Asp active site (Bagley and Altman, 1996). Chymotrypsin is initially synthesized as the precursor chymotrypsinogen which is activated through cleavage by trypsin (Hartley, 1970). This serine protease targets peptide molecules and cleaves them at the C-terminal of the hydrophobic residues Phenylalanine, F, Tyrosine, Y, and Leucine, L (Lawrence and Koundal, 2002). The serine residue of chymotrypsin's catalytic triad uses a hydroxide group as a nucleophile in order to cleave the peptide bond of the peptide (Fan and Wu, 2005).

Elastase, the third major class of serine proteases, is also responsible for breaking down proteins in digestion. This smaller protease is responsible for the cleavage of peptides at the C-terminal at neutral residues of Alanine, Glycine and Valine (Lawrence and Koundal, 2002). Elastase is initially synthesized as proelastase which is subsequently activated through cleavage by trypsin (Hercz, 1969).

It is the combination of these three serine protease classes that comprises one of the main types of digestive proteases that can be targeted by inhibitors being expressed in transgenic plants.

Cysteine Proteases:

Cysteine Proteases have been isolated from the midgut of numerous insects including Coleoptera (Jouanin et al., 1998), the beetle larvae of the cowpea weevil, the

bruchid, Zabrotes subfaceatus (Lawrence and Koundal 2002) and it also plays a primary role in nematode digestion who have a major economic importance worldwide (Hilder and Boulter, 1999). The digestive role of cysteine proteases in insects is the degradation of intracellular proteins, essentially the same role as the serine proteases (Karrer et al., 1993).

The difference between the two protease types is the nature by which the protease catalyses the hydrolysis of peptide bonds in dietary proteins (Otto and Schirmeister, 1997). Cysteine proteases have their highest activity in a mid pH range of 5-7 (Lawrence and Koundal, 2002) and also differ by utilizing a thiol group supplied by a cysteine residue in the active site (Otto and Schirmeister, 1997). Cysteine Proteases can be broken down into several families including papains, calpins, asaparagines and cathepsins (Lawrence and Koundal, 2002) based on the mechanisms by which the protease breaks down proteins. These protein families are being targeted by transgenic plants.

Aspartic Proteases:

Like other insect proteases, aspartic proteases are utilized by insects, viruses, fungi and plants to digest proteins for nutrients or play roles in other pathways that involve proteolysis (Christeller et al., 1998). Unlike other insect proteases, aspartic proteases are used by a fewer number of insects which include Coleoptera and Hemiptera. Because of the limited use of aspartic proteases in insects there are a limited number of studies being conducted on creating inhibitors for them (Lawrence and Koundal, 2002).

Most of the aspartic proteases belong to the pepsin family of proteases which

includes the digestive enzymes pepsin, chymosin and lysosomal cathepsins D (Fan and Wu, 2005). The acidic nature of the aspartic acid residue in the active site (Fan and Wu, 2005) makes this class of proteases have favorable activity at low pHs of around 3-5 (Lawrence and Koundal, 2002). These proteases also differ by their catalytic mechanism in that there is no covalent tetrahedral intermediate formed during proteolysis of the peptide bond (Fan and Wu, 2005).

Metallo-Proteases:

Metallo-proteases are protein cleaving enzymes that have a distinguished metallic ion, usually zinc, in its active site which is used to effectively break peptide bonds (Fan and Wu, 2005). This class of proteases are found in the guts of insects such as the larvae of the corn earworm (Bown and Gatehouse, 2004), and in the midgut of the two spot ladybug (Walker et al., 1998). There are two main families of metallo-proteases in insects, metallocarboxypeptidases and metalloendopeptidases (Lawrence and Koundal, 2002). The metallo-proteases use a zinc ion in the active site that has specificity towards various C-terminal residues that leads to protein degradation going from the C to N terminal (Bown and Gatehouse, 2004).

α-Amylase Metallo-Enzymes:

α-amylase is a calcium metallo-enzyme that breaks down long chain carbohydrates in insects and other animals (Carbonero et al., 1993). Some pests that have been identified to contain α-amylase are bruchid beetles (Jouanin et al., 1998), and the Yellow Meal Worm. α-amylases are the most important digestive enzymes of many

insects that feed exclusively on seed products, which consist largely of carbohydrates, during larval or adult life (Pereira et al., 1999).

These enzymes constitute a family of endo-amylases that use a calcium ion to catalyze the hydrolysis of α-D-(1,4) glucan linkages which are present in starches and glycogen (Franco et al., 2002). Although α-amylase is not a proteolytic enzyme, it is a major digestive enzyme in pests. Therefore, many inhibitors that target it are being developed to be expressed in insect resistant transgenic plants.

Transgenically Expressed Inhibitors:

The translational products of the inserted transgenes are what enable plants to inhibit the digestion proteases produced by the insects that feed on them. A large array of Protease Inhibitors, Lectins and α-amylase inhibitors have been discovered and the genes that code for these inhibitors are being introduced into plants in order to elicit plant defense against pest herbivory.

Protease Inhibitors:

Protease inhibitors are natural protease antagonists that are present generally in all life forms (Fan and Wu, 2005). Aside from their role in the regulation of endogeneous proteolysis (Koiwa et al., 1997), protease inhibitors have been proven to play a large part in the plant's defense through induction in plant tissues by herbivory or wounding (Fan and Wu, 2005). Protease inhibitors are ubiquitous in plants as they have been found in the reproductive organs, storage organs and vegetative tissues of most plant families (Walker et al., 1998).

There have been many protease inhibitors isolated and they have been arranged in to the following general families: Bowman-Birk Serine protease inhibitors, Cereal Trypsin protease inhibitors, Cereal α-amylase inhibitors, Cystein protease inhibitors, Metallocarboxypeptidase inhibitors, Mustard Trypsin inhibitors, Potato type I and II protease inhibitors, Serpins, Kunitz-Soybean Trypsin inhibitors and Squash protease inhibitors (Leo et al., 2002). The general structural trend of protease inhibitors is that they vary fro 4 to 85 kDa and contain a high number of cysteine residues that readily form sulfide bonds. This makes them more resistant to high temperatures, extreme pHs and proteolysis (Fan and Wu, 2005).

Serine proteases inhibitors are the most studied class of protease inhibitors because of the large variety and importance of serine proteases in insects (Fan and Wu, 2005). Most protease inhibitors that target serine proteases are smaller, light weight molecules called Serpin and Kunitz type inhibitors that utilize a 'lock and key' mechanism that disables catalytic activity in the protease (Boigegrain et al., 2000). Most of the Serine protease inhibitors possess two active sites that are able to inhibit both trypsin and chymotrypsin (Jouanin et al., 1998).

Cystatins or phytocystatins inhibit Cysteine proteases and are the second most readily studied class of protease inhibitors (Fan and Wu, 2005). Cysteine protease inhibitors have enormous stability to heat, extreme pHs, and have high specificity for Cysteine proteases (Otto and Schirmeister, 1997). The Cysteine protease inhibitors can be found specifically in seeds and storage tissues of plants, but like any other inhibitor they can be induced by wounding and insect attack (Jouanin et al., 1998). Cysteine

protease inhibitors have been found to protect plants such as the Pearl Millet, which is the fourth major rood crop of India, from losses due to fungal diseases (Joshi et al., 1998).

Aspartic protease inhibitors are scarce in nature, but have been reported to be found in potato, wheat, yeast and nematode (Christeller et al., 1998). Pepstatin, a strong and specific inhibitor of aspartic proteases has been demonstrated to inhibit activity of the midgut enzymes of the Colorado potato beetle (Lawrence and Koundal, 2002). Potato tubers possess an aspartic protease inhibitor of cathepsin D that has also shown inhibitory properties towards other serine proteases such as trypsin and chymotrypsin (Lawrence and Koundal, 2002).

Metallo-protease inhibitors are generally represented by the metallo-carboxypeptidase inhibitor family in tomato and potato plants (Fan and Wu, 2005). These inhibitors are polypeptides around 4 kDa that strongly and competitively inhibit a broad spectrum of carboxypeptidases from both animals and microorganisms (Lawrence and Koundal, 2002). Tomato and potato plants accumulate an array of carboxypeptidase inhibitors along with serine protease inhibitors that, in combination, will have the capacity to inhibit 5 types of major digestive enzymes including trypsin, chymotrypsin, elastase, and carboxypeptidases A and B of higher animals and insects (Lawrence and Koundal, 2002).

The protease inhibitors of the 4 major protease families work best in combination with one another in ‘cocktails’ in order to reduce proteolytic activity of as many target digestive proteases as possible (Fan and Wu, 2005). The role of these 4 protease inhibitor types is to stop the digestion of proteins by the phagocytes that feed on them, which would lead to the malnourishment in those pests and eventually death (Pilon, et al.,

2006). There is increasing evidence that malnourishment related insect death is not caused directly by the inhibition of protein digestion proteases in the midgut, but rather is caused by the hyperproduction of additional digestive proteases which would lead the overuse of the limited available amino acids to the pest (Pilon et al., 1996).

α-Amylase Inhibitors:

The second type of enzyme inhibitor used in the modification of crop plants expresses proteins that inhibit insects from digesting carbohydrates (Schuler et al., 1998). α-amylase inhibitors have been divided into 6 different types which include, lectin-like, knottin-like, cereal-type, Kunitz-like, Gamma-purothionin-like and thaumatin-like (Franco et al., 2002). All these types of inhibitors have shown pest gut α-amylase inhibition which lead to increased mortality of the pests (Hilder and Boutler, 1999) or the underdevelopment of larvae (Schuler et al., 1998). α-amylase inhibitors like AAI from a Mexican crop plant (Pereira et al., 1999), WAAI from wheat, and BAAI from a common bean (Hilder and Boulter, 1999) have all been transformed into crop plants such as tobacco and have resulted in the increased mortality of the lepidopteran larvae that fed on them (Hilder and Boulter, 1999). There have been inhibitors such as the Indian finger millet bifunctional inhibitor that have displayed inhibition of not only α-amylase but also trypsin (Lawrence and Koundal, 2002). The ability of inhibitors to inhibit multiple types of catalytic enzymes can enable researchers to develop the most effective strategy of digestive enzyme inhibition for plant defense.

Lectins:

Lectins are carbohydrate binding proteins that are abundant in seeds and storage tissues of some plant species (Babu et al., 2003). Lectins were discovered by a medical student in 1888 when working on castor beans and were described then as a 'toxic proteinaceous factor in the extracts of beans that agglutinate red blood cells' (Vasconcelos and Oliveira, 2004). After this discovery and with the modern techniques of producing insect resistant plants, researchers have utilized these proteins in order to control crop pests (Christeller et al., 2005).

Lectins have been found to be toxic to insects by having deleterious binding interactions with intestinal glycoproteins (Babu et al., 2003) that form carbohydrate agglutinates and thus render carbohydrates unavailable for digestion (Hilder and Boulter, 1999). There are 4 major classes of plant Lectins which include merolectins, hololectins, chimerlectins and superlectins which differ from one another by the number of carbohydrate-binding domains they contain (Vasconcelos and Oliveira, 2004). These proteins have high resistance to proteolysis and are stable over a large range of pHs. They also have the ability to bind to epithelial cells that line the small intestines (Vasconcelos and Oliveira, 2004).

Examples of lectins that are commonly expressed transgenically are the Snowdrop lectin (GNA), and the PSA from the pea, both of which cause resistance to Leopidoptera (Jouanin et al., 1998) and the peach potato aphid (Hilder and Boulter 1999). Lectins can be co-expressed with other proteases and α-amylase inhibitors in order to defend against most if not all insect pests (Jouanin et al., 1998).

Regulating Expression of Insect Resistance Transgenes:

Plants that are under stress by herbivores or wounding are capable of activating a cascade that results in the expression or upregulation of defense related compounds and molecules (Koiwa et al., 1997). The development of transgenic plants utilizes the plant's natural defense mechanisms in order to manipulate the expression of a gene or genes of interest that have been inserted into the plant's genome (Ryan, 2000).

Jasmonic Acid Signaling Pathway:

The production of protease inhibitors and other wound response proteins are highly regulated by a signal transduction pathway that is initiated by predation (Koiwa et al., 1997). Experimentally it has been determined that the control of wound response is dependent on the formation of jasmonic acid as a result of the catalyzed break down of linolenic acid via the octadecanoid signal pathway (Moura and Ryan, 2001). The octadecanoid pathway (fig. 2) utilized systemin, which is an 18 amino acid polypeptide that is released from the wound site. Systemin, which is processed from the larger prohormone protein prosystemin, activates a lipid-based signal transduction pathway in which linolenic acid is released from plant membranes and is converted into jasmonic acid by the enzymes LOX and AOS (Ryan, 2000).

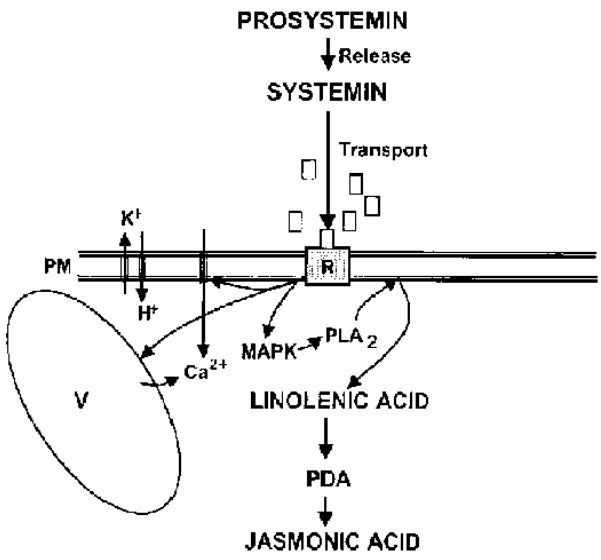


Fig. 4. A current model for the systemic signaling pathway for defensive genes in tomato plants that are activated by herbivore attacks (wounding). The interaction of systemin with its membrane receptor initiates intracellular events that activate a PLA2. The phospholipase releases LA from membranes, the production of JA, and the activation of defensive genes.

(Source: CA Ryan, 2000)

Jasmonic acid regulates the expression of wound induced protease inhibitors and other defensive proteins through the presence of a jasmonite-responsive element or a G-box present in the promoter region of wound response genes (Koiwa et al., 1997). This G-box sequence (CACGTGG) attracts a DNA-binding protein or transcription factor that would result in the gene being turned on (Koiwa et al., 1997).

There are 3 other systemic signals responsible for the translocation of the wound response which includes abscisic acid (ABA), hydraulic signs and electrical signals (Lawrence and Koundal, 2002). It is through the 'cross-talk' of these signaling pathways, which are initiated by different inducers, that an effective plant defense against pests is expressed (Koiwa et al., 1997).

Gene Pyramiding:

Gene pyramiding or 'gene stacking' (Sharma and Ortiz, 2000) is deployment of multiple genes coding for two or more toxins that possess different modes of action (Jouanin et al., 1998). The effectiveness and durability of resistance in transgenic crops is likely to be greater if they are engineered with this multi-gene approach of resistance (Hilder and Boulter, 1999). There are 6 methods of stacking genes which include: Crossing, Sequential Transformation, Co-transformation, Internal ribosome entry site, Transplastomic technology, and Polyprotein approach (Francois et al., 2002). Using these methods of gene pyramiding a plant can deploy multiple resistant gene combinations such as a cowpea trypsin inhibitor (CpTi) with a pea lectin (PSA) (Hilder and Boulter, 1999).

The need for the multiple expressions of genes is because of one type of gene either being too specific or only mildly effective against target pests. Plants eliciting a multiple gene response show higher resistance and toxicity against pests (Sharma and Ortiz, 2000).

A complete database at: http://www.ba.itb.cnr.it/srs7bin/cgi-bin/wgetz?-page+LibInfo+-id+217H11UmZdu+-lib+PLANT_PIs has been compiled by the work of researchers around the world in a collaborative effort to produce the widest possible array of effective inhibitory molecules to be applied to specific insects that the profile of proteases that they express (Leo et al., 2002). This database includes the family, subfamily, inhibited proteases, their reactive site and the common mutations of all known protease inhibitors that have been submitted to the database (Leo et al., 2002). It is through this kind of effort that safe but effective deployment will be made possible.

Insect Adaptation, Ecological Hazards and the Management of Transgenic Plants:

Plants and herbivores have been co-evolving for thousands of years and as a result plants and herbivores alike have defense mechanisms against one another (Jongsma and Bolter, 1997). The introduction of transgenically modified plants that are expressing a wide array of defensive proteins in high concentrations will alter the ecological balance between the first trophic level (producers) and the second trophic level (herbivores) (Sharma and Ortiz, 2000). Transgenic plants also pose the risk of having their genes escape into the wild through a series of processes that will lead to proliferation of plants expressing transgenes (Kuvshinov et al., 2001).

The Adaptation of Insects to Insect Resistant Plants:

The introduction of transgenically modified plants into a system that has an established equilibrium of energy transfer from one trophic level to another will have a strong impact on the organisms involved (Ferry et al., 2004). One issue concerning the deployment of transgenic crop is the evolution of new insect biotypes as a result of the selection pressure on organisms that can or can not obtain food (Sharma and Ortiz, 2000). The pressure to evolve proteases that are insensitive to host plant protease inhibitors is considerable being that is it an immediate life or death situation (Jongsma and Bolter, 1997).

An evolutionary variable as strong as the obtaining of nutrients is going to affect the ecosystem in multiple ways. The capacity of some insects to up-regulate the expression of insensitive proteases that were feeding on dietary protease inhibitors has

been experimented and observed in numerous cases (Fan and Wu, 2005; Bolter and Jongsma, 1995; Girard, 1998; Ferry, 2004; Gruden, 2004).

The exposure of insects to moderate amounts of inhibitory molecules or ineffective inhibitory molecules can cause the full restoration of protease activity by the production of protease inhibitor insensitive proteases (Jongsma and Bolter, 1997). This is because protease inhibitors are effective against a limited number of proteases and therefore they will have only a mild impact on the complex mixtures of digestive enzymes in the gut of insects (Leo and Gallerani, 2002). As a result of exposure to protease inhibitors, or other inhibitory molecules, the insect can shift their protease profile towards different types of protease, for example shifting from serine type to aspartic type proteases. They can also express the same type of proteases but with specific structural changes that render them un-inhibitable by the expressed inhibitory molecule (Gruden et al., 2004).

Another tactic insects have developed to become resistant to insect resistant plants is the development and up-regulation of detoxifying proteases (Ferry et al., 2004) (Girard et al., 1998). Insects can use cytochrome 450 monoxygenase and glutathione S-transferase to detoxify potential secondary metabolites which would result in the clearing of inhibitory molecules from the insect's system. It has been shown the corn earworms recognize plant defense signaling molecules, like jasmonic acid, in order to activate four of its own cytochrome 450 genes to be able to break down inhibitory molecules and can therefore tolerate eating the plant (Ferry et al., 2004).

Management of Insect Resistance:

Although insects have shown a remarkable capacity to develop resistance to transgenic proteins (Sharma and Ortiz, 2000), there are means by which insect resistance can be managed. Pest control using protease inhibitors in transgenic plants will require the isolation of inhibitors that are active towards the novel insensitive proteases that are being produced (Lawrence and Koundal, 2002). This is going to require that inhibitors be developed and studied on a species by species manner before they are deployed (Leo et al., 2002) in order to avoid developing mass numbers of resistant insects.

Another tactic for insect resistance management is the expression of multiple genes in each plant. Genetic techniques like gene pyramiding would require that an insect have multiple mutations in order to be resistant to all of the genes being expressed (Babu et al., 2003). The deployment of multiple genes that possess different modes of action would cause a higher mortality rate and a lower escape rate of insects that contain genes for insensitive proteases (Hilder and Boulter, 1999).

A final method of reducing the development of insect resistance is the use of inducible or tissue-specific promoters. The use of constitutive promoters like the Cauliflower Mosaic Virus 35S promoter that express chronic levels of inhibitors proteins drives insect adaptation by enabling constant, sublethal exposure (Hilder and Boulter, 1999). Tissue-specific or induced promoters, such as the phenylalanine ammonia lyase (PHA-L) for seed-specific expression, can contribute to resistance management by expressing lethal concentrations to specific tissues when induced which would avoid continuous sub-lethal dosage induced adaptations (Sharma and Ortiz, 2000).

Effects on Non-Target Organisms, Natural Predators and Animal Biodiversity:

One of the major concerns of transgenic crops is the effects they have on non-target organisms that are integral to the structure of the agricultural ecosystem (Hilbeck, 2001). Non-target organisms are any unintended side effects of transgenic, insecticidal plants that adversely affect organisms other than the target species (Hilbeck, 2001). These organisms include pollinators, detrivorous organisms, vertebrate herbivores, and natural enemies of the insects that are both targeted and non-targeted organisms (Hilbeck, 2001).

Non-target herbivores, such as bees or Monarch butterfly larvae, can ingest the novel insecticidal compounds and as a result die (Stewart, 2000; Losey, 1999). The loss of non-target organisms can interfere with complex food web structures and the natural regulation processes of herbivores and higher trophic organisms (Hilbeck, 2001).

The higher trophic levels or 'Natural Enemies' of target and non-target insects can be heavily impacted by the introduction of transgenic plants through the indirect ingestion of inhibitory compounds, lowered nutritional quality of prey, and a decrease the number of available prey (Hilbeck, 2001). Some natural enemies' species population dynamics follow that of their prey species' in a density dependent manner. A drop in the population of available prey species can lead to either a drop in the number of natural enemies or a drastic shift in their prey selection (Sharma and Ortiz, 2000). The forced shift in trophic design can result in a loss of ecosystem biodiversity and eventually stability.

One positive influence that transgenic plants have on biodiversity in the agroecosystem is the ceasing of the use of broad spectrum pesticides (Sharma and Ortiz, 2000). The use of synthetic broad spectrum pesticides not only has high cost but it has detrimental effects on non-target organisms (Christou et al., 2006). The lack of broad spectrum pesticides will increase the number of non-target organisms and natural enemies of crop pests, which is a strong natural source of pest control (Stewart et al., 2000).

Transgene Escape and Undesired Gene Flow:

One concern that has been expressed with the use of transgenic plants is the escaping of transgenes into the wild (Ellstrand, 2001). The possibility of transgene flow from engineered crops into their wild relatives with undesirable consequences has been realized and measures are being taken to develop plants that would reduce these risks (Hilbeck, 2001). The unwelcome effects resulting from transgene escape into wild relatives include the acquired resistances to insects that are coded by novel transgenes (Sharma and Ortiz, 2000) and herbicidal resistances gained through the presence of marker genes (Tzfira and Citovsky, 2006). Gene escape can also lead to faster development of resistance in insect populations (Sharma and Ortiz, 2000).

The mechanisms by which genes can escape from a domesticated setting are through intraspecific and interspecific hybridization (Stewart et al., 2000). Intraspecific hybridization is when transgenic plants are grown in proximity to non-transgenic plants and wind borne seeds can be blown to adjacent areas where wild plants of the same species are crossed with the transgenic genes. Since most agricultural crops grown are

exotic and are not commonly found growing in the wild, this problem would be for the nearby farmers. One problem for nearby farmers who are trying to grow organic foods and are guaranteeing their food is transgene free, but in reality they are growing plants that have been crossed with transgenic crops (Stewart et al., 2000).

Interspecific hybridization is the crossing of genes between closely related species of domesticated transgenic plants to wild populations (Stewart et al., 2000). This crossing can produce transgenically resistant weeds and can cause damage to non-target organism in the wild and disrupt population dynamics. This would be done through the introduction of plants with the selective advantage of having either insect resistance or any number of transgenes that would give them an advantage over wild type plants (Snow, 2002). Crop-to-weed gene flow has created hardship through the appearance of new or more difficult weeds through hybridization. The evolution of new, more aggressive weeds has been seen in seven of the world's 13 most important crops (Ellstrand, 2001). Gene flow into the wild has also been held responsible for the extinction of wild subspecies of rice (Ellstrand, 2001) and can be a source of decreasing biodiversity in the wild.

Current regulations in an attempt to keep gene escape at a minimum is a 'buffer zone' but in reality an unmanageable buffer zone of 2000m from the fields would be necessary to avoid gene flow (Arriaga et al., 2006). Another way to keep the effects of gene escape down is through the removal of selectable markers from the genome after transformation (Ebinuma et al., 1997). The removal of genes that code for antibiotic, or herbicidal resistance can have detrimental effects in the environment as they can elicit a selective advantage in those plants expressing them (Hilbeck, 2001).

A different approach to stop the risks of gene escape was taken by Kuvshinov et al. 2001, where they used a new 'terminator' technology. RBF or recoverable block of function consists of a blocking sequence linked to the gene of interest and the recovering sequence all in one transformable construct. The blocking sequence will block a certain molecular or physiological function of the host plant if it is to hybridize with a wild type genome, which will lead to the death of the host plant or the inability for sexual reproduction (Kuvshinov et al., 2001). This technology will lead to successful elimination of wild transgenic plants and their associated potential dangers.

Safety and Health Concerns with Transgenic Plants:

Technological advances like the development of transgenic plants offers the opportunity for substantial yield increases, production cost reductions and even an increase in the quality of agricultural crops (Hareau et al., 2006). The implication of transgenic crops also has the potential to reduce worldwide demands for food associated with poverty ridden nations and population growth (Hareau et al., 2006). However, these advances have come with the aforementioned ecological and environmental hazards as well as the potential hazards to human health (Azevedo and Araujo, 2003).

Transgenic Plants and Human Health:

Public outcry against the use of transgenic crops has been over the issues of safety of new proteins to the human body. Concerns regarding transgenic foods include the allergenicity or toxicity of novel proteins, the possible gene transfer to gut microflora and the role of new food products in the diet or food processing (Kuiper et al., 2001).

In the case of newly expressed proteins in genetically modified plants or animals, the allergenic potential of the protein needs to be assessed, even in the case of proteins that are specific and well-characterized due to possible post translational modifications (Ambali et al. 2003). The considerations that are being applied towards assessing the allergenic potentials of transgenes are; if the gene expresses a common allergen or an uncommon but known allergen, or if the proteins being expressed are without any history of know allergenicity (Kuiper et al., 2001). The assessment of the safety of gene products must be done on a case-by-case basis (Ambali et al., 2003). Assessing these criteria include looking at the sequence homology to the known common allergens, using the serum from individuals with known allergies and looking for reactions with transgene products, and assessing the stability of the transgenic proteins under gastro-intestinal conditions or other harsh environments that may be encountered during processing (Kuiper et al., 2001).

The concern of genetic transfer or horizontal transfer is another potential hazard for transgenic plant use (Ambali et al., 2003). The possibility of the genetic material of ingested transgenic plant cells can be incompletely digested and has the potential to have pieces of its genetic material be introduced into the microflora of humans (Kuiper et al., 2001). One of the main concerns would be the presence of marker genes that code for antibiotics and the possible acquisition of this resistance, but it has been agreed that this transfer is too complicated and unlikely to occur (Kuiper et al., 2001).

Restrictions and Monitoring:

Since the first introduction of genetic modification there have been government restrictions and policies in all aspects of the field, from animals, human, bacteria, viruses, and of course plants (Miraglia et al., 2004). In regards to plants producing novel proteins such as protease inhibitors there has been Regulation 258/97 or the "Novel Foods Regulation". This states that (a) novel foods must be safe and not be nutritionally disadvantageous for the consumer, and (b) the consumer has the right to be informed whenever a novel food or food ingredient is no longer equivalent to an existing food or food ingredient (Miraglia et al., 2004). This act also controls the release of genetically modified products containing novel foods on the market (Miraglia et al., 2004).

A current tendency in food production is the differentiation of products on the basis of a wide variety of characteristics (Miraglia et al., 2004). One procedure that enables a food producer to differentiate between genetically modified foods from non-genetically modified foods is traceability (Kuiper et al., 2001). Traceability is "the ability for the retrieval of the history and use or location of an article of an activity through a registered identification" (Miraglia et al., 2004). Implication of traceability regulations will allow effective recall procedures that can prevent excessive economic losses as well as brand damage (Miraglia et al., 2004) or more importantly, it can be used to prevent possible hazardous human consumption.

The restrictions placed upon genetically modified crops are important and should be as stringent as possible in order to ensure that environmental and human health risks have been thoroughly evaluated and tested. These restrictions will keep environmental impacts like insect resistance and gene flow as well as human risks like allergenicity and toxicity at minimum or eliminated altogether.

Concluding Remarks:

Using transgenic plants as an additional means of pest control has the potential to be a valuable tool to increase food output and a decrease in crop losses. However, transgenics has the potential to have adverse effects on human and animal health, as well as the environment. Therefore it is the responsibility of all researchers to thoroughly develop and test this technology before it is released into massive, worldwide production. The use of *Bt* crops was too rushed and wasn't researched enough before deployment and as a result the plants are ecological hazards and are not as effective as they could be.

There is always going to be room for the improvement of molecular biology techniques that are used to transform cells, regulated gene expression and control gene mutation. Until these technologies are further along in their development or there is away to keep transgenic crop fields isolated from the environment, the deployment of these plants should be delayed in order to ensure their safety to the environment and humans.

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