In Vitro and In Silico Analysis of Plant Origin Proteinase Inhibitor Genes

Master of Philosophy
in
Plant Biochemistry and Molecular Biology

by

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ISLAMABAD, PAKISTAN.
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DECLARATION

This is to certify that this dissertation entitled **" *In Vitro* and *In Silico* Analysis of Plant Origin Proteinase Inhibitor Genes"** submitted by **Faiza Munir** is accepted in its present form by the Department of Plant Sciences, Quaid-i-Azam University, Pakistan, as satisfying the dissertation requirement for the degree of *M.Phil.* in Plant Biochemistry and Molecular Biology.

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بسم الله الرحمن الرحيم

Dedicated

To

My Loving Parents

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Faiza Munir

CONTENTS

Enhanced resistance is shown against insect pests by a number of transgenic plants expressing PIs genes. Investigations in the field of plant molecular genetics resulted in the identification of many genes in plants that help them in war against a variety of sucking and chewing insects. These defensive genes include those encoding signal transduction proteins, downstream effector genes programming various protective proteins against insect pests and other pathogens and the genes involved for gene-for-gene interactions with insects (Farmer and Ryan, 1992; McGurl *et al.*, 1994; Bogre *et al.*, 1997; Koiwa *et al.*, 1998; Rossi *et al.*, 1998; Vos *et al.*, 1998; Walling, 2000; de Bruxelles and Roberts, 2001; Kessler and Baldwin, 2002; Moran *et al.*, 2002).

One of the fundamental components of biochemical processes essential for plant growth and plant defense response is the regulation of proteinases. Plant PIs apparently amend the growth of insect pests by attenuating protein degradation. PIs comprise one of the most abundant classes of proteins in plants. It has been reported that, 1 to 10 % of the total protein content of most storage organs such as seeds and tubers are PIs, inhibiting different types of enzymes (Ryan, 1981; Pearce *et al.*, 1982). Non-storage tissues as leaves, flowers and roots also contain a large number of PIs (Brzin and Kidric, 1995; Xu *et al.*, 2001; Sin and Chye, 2004). Experimental proof is also there for the presence of these small proteins in the leaves of various plants followed by the insect attack (Ryan, 1990). The utility and functions of plant-derived PIs were recognized quite early and such transgenic tobacco plants were first reported in 1987 (Hilder *et al.*, 1987).

1.2 Origin and Classification of Proteinase Inhibitors in Plant Kingdom

Three plant families namely Leguminosae, Solanaceae and Gramineae are of great significance for the origin of major PIs studied in plant kingdom (Richardson, 1991; Mosolov *et al.*, 2001; Mosolov *et al.*, 2004). The PIs are classified into four mechanistic classes of proteolytic enzymes as serine, aspartic, cysteine and metallo-proteinase inhibitors based on the active amino acid in their reaction center (Koiwa *et al.*, 1997).

1.2.1 Serine Proteinase Inhibitors

Serine PIs have been reported from a variety of plant sources and are the most-studied class of PIs. This class is also known as Serpin family (Mello *et al.*, 2002; Gettins, 2002; Haq and Khan, 2003; Rawlings *et al.*, 2004; Christeller and Liang, 2005; Law *et al.*, 2006). All serine PIs from plants are competitive inhibitors and all of them inhibit proteinases with a similar standard mechanism (Laskowski and Kato, 1980). Serine PIs are universal throughout the plant kingdom, with trypsin inhibitors being the most common type. Two families of serine PIs that are PI-I and PI-II occur in tomato and potato. PI-I with a single reactive site inhibit chymotrypsin while PI-II type inhibit chymotrypsin and trypsin having two reactive sites. PI-II originally characterized from potato tubers (Christeller and Liang, 2005). The serine PI of Arabidopsis has been shown to play an important role in the plant immune response (Vercammen *et al.*, 2006). The core mechanism for this plant immune response is the up-regulation of complex pathways of host immune system by serine PIs (Law *et al.*, 2006).

Serine PIs have anti-nutritional effects against several *lepidopteran* insect species (Shulke and Murdock, 1983; Applebaum, 1985). Broadway and Duffey (1986) compared the effects of purified SBTI (Soybean trypsin inhibitor) and potato inhibitor II (an inhibitor of both trypsin and chymotrypsin) on the growth and digestive physiology of the larvae of *Heliothis zea* and *Spodoptera exigua* and demonstrated that growth of larvae was inhibited at levels of 10 % of the proteins in their diet. A number of serine PIs genes have been used to develop transgenic crop plants showing resistance against specific crop pests. Transgenic rice crop was developed by using soybean kunitz trypsin inhibitor. It was tested by transforming the rice protoplasts with soybean kunitz TI, the experimental results showed that 0.05 to 2.5 % of the total soluble protein content was soybean kunitz TI protein and the transgenic rice plants showed high resistance to (*Nilaparvata lugen*), brown plant hopper (Lee *et al.*, 1999). Transgenic expression of serine PIs, such as tomato and potato PI-II or cowpea trypsin inhibitor (CpTi), was found to be effective to inhibit growth and development of *lepidopteran* larvae (Hilder *et al.*, 1987; Johnson *et al.*, 1989; McManus *et al.*, 1994; Duan *et al.*, 1996). A Serine PI of Kunitz-type trypsin

inhibitor with an antifungal property and molecular weight of 20.5 kDa has been isolated from the roots of punce ginseng (*Pseudostellaria heterophylla*) (Wang and Ng, 2006).

1.2.2 Cysteine Proteinase Inhibitors

Plant cystatins or phytocystatins are the second most studied class of inhibitors. Among all the cysteine PIs in plant kingdom the rice cysteine PIs are the most studied, which are proteinaceous in nature (Abe and Arai, 1985) and highly heat stable (Abe *et al.*, 1987). Cystatins have also been reported from potato (Waldron *et al.*, 1993), ragweed (Rogers *et al.*, 1993), cowpea (Fernandes *et al.*, 1993), papaya (Song *et al.*, 1995), avacado (Kimura *et al.*, 1995), wheat (Kuroda *et al.*, 2001), sunflower (Kouzuma *et al.*, 1996), maize (Abe *et al.*, 1995; Yoza *et al.*, 2002), soybean (Misaka *et al.*, 1996) and sugarcane (Soares-Costa *et al.*, 2002). Nine cysteine proteinase inhibitor genes designated as PtCys1-PtCys9 have been recently documented in *Populus trichocarpa*, genome located on chromosome 1 (PtCys1 and PtCys2); chromosome 2 (PtCys3), chromosome 3 (PtCys4), chromosome 6 (PtCys5 and PtCys6), chromosome 9 (PtCys7), chromosome 14 (PtCys8), and chromosome 16 (PtCys9) (Margis-Pinheiro *et al.*, 2008).

1.2.3 Aspartic Proteinase Inhibitors

Wolfson and Murdock (1987) demonstrated that pepstatin, a powerful and specific inhibitor of aspartic proteinases, strongly inhibited proteolysis of the midgut enzymes of Colorado potato beetle, *Leptinotarsa decemlineata*. It has been reported that, potato tubers possess an aspartic proteinase inhibitor, cathepsin D (Mares *et al.*, 1989) that shares considerable amino acid sequence identity with the trypsin inhibitor SBTI from soybeans. Aspartic PIs have also been isolated from sunflower (Park *et al.*, 2000), barley (Kervinen *et al.*, 1999) and cardoon (*Cyanara cardunculus*) flowers named as cardosin A (Frazao *et al.*, 1999).

1.2.4 Metallo-Proteinase Inhibitors

Two families of metallo-proteinase inhibitors have been identified in plants, the metallo-carboxypeptidase inhibitor family in potato (Rancour and Ryan, 1968), and tomato plants (Graham and Ryan, 1981) and a cathepsin D inhibitor family in potatoes (Keilova and Tomasek, 1976). Metallo-carboxypeptidase inhibitor accumulates in potato leaf tissues in response to wounding along with inhibitor I and II proteins (Graham and Ryan, 1981; Hollander-Czytko *et al.,* 1985). These inhibitors accumulated in the wounded leaf tissues of potato having the power to inhibit the five major digestive enzymes i.e. trypsin, chymotrypsin, elastase, carboxypeptidase A and carboxypeptidase B of higher animals and many insects (Hollander-Czytko *et al.,* 1985).

1.3 Mode of Action

The plant's behavior under attack was recognized very first time by researchers at the US Department of Agriculture's Center for Medical, Agricultural, and Veterinary Entomology in Gainesville, Florida and their findings led to the development of genetically transformed plants with best defense strategies against insect pests. Intense investigations have been carried out for understanding the mode of toxicity of PIs (Barrett, 1986; MacPhalen and James, 1987; Greenblatt *et al.,* 1989). The release of proteinases in the insect guts depends upon the midgut protein level rather than food volume (Baker *et al.,* 1984). PIs inhibit the activity of these proteases and lessen the quantity of proteins that can be digested, accompanied with the over production of the digestive enzymes which enhances the loss of sulfur amino acids (Shulke and Murdock, 1983) as a result of which, the insects become feeble due to limited growth and eventually die. Plant defense strategies against insect pests mainly comprise of late larval development or increased attack by insect parasitoids and high level toxicity by expressing PI genes (Baldwin and Preston, 1999).

It has been reported that plants utilize a specific approach to identify an insect attack. When insects ingest parts of a plant digestion turns proteins into a peptide elicitor,

which is secreted back into the plant during subsequent feedings. The plant recognizes this elicitor and launches its defensive chemistry (Science Daily; September 1, 2007). The mode of binding of the plant PIs to the insect proteinases seems to be parallel with all the four classes of inhibitors. The enzyme active site gets effectively blocked by the binding of PI to form a complex with a very low dissociation constant 10^{7} to 10^{14} M at neutral pH values (Terra *et al.* 1996; Walker *et al.* 1998).

1.4 Regulation of Proteinase Inhibitor Genes

Plant PIs are developmentally regulated and distinct regulation patterns have been reported in response to biotic and abiotic stresses. Studies carried out in cabbage and sweet potato showed that young leaves have highest levels while older leaves have lowest levels of PIs, cabbage head also possess highest levels of PIs (Sasikaran *et al.*, 2002). It has been reported that, during seed development oryzacystatins I and II, corn cystatins, and soyacystatin are expressed (Abe *et al.*, 1987; Kondo *et al.*, 1991; Abe *et al.*, 1992; Misaka *et al.*, 1996). An expression of wheat cystatin 5 (WC5) has been reported during caryopsis development, in embryos, and in seed coverings at the maturation stage of the grain (Corr-Menguy *et al.*, 2002). During seed germination and maturation and also under cold stress a wheat cystatin, TaMDC1, expression is reported (Christova *et al.*, 2006). There are reports that, strawberry cystatin (Cyf1) is expressed in vegetative organs such as leaves and roots (Martinez *et al.*, 2005). Lievens *et al.* 2004 reported that, during nodulation of *Sesbania rostrata* a proteinase inhibitor SrPI1 belonging to Kunitz family get expressed. SrPI1 is induced in the nodule primordium at the early stages of nodule development while at the later stages its expression occur in other nodular regions including the cells neighboring the nodule meristem and the nodule parenchyma cells. SrPI1 is found to be expressed in scattered cells of the infection zone and in the uninfected cells in the young fixation zone.

1.4.1 Signal Molecules for PI gene expression

The expression of PI genes followed by insect attack is due to the local and systematic signal molecules released at the site of injury. Local signals elicit wound response at the site of injury. The mobilization of systematic signals is through the vascular system eliciting wound response in the acropetal and distal sites of injury (Wildon *et al.*, 1992; Malone and Alarcon 1995). Research conducted on tomato PI showed that the synthesis of PI proteins get switched on by the activation of proteinase inhibitor initiation factor (PIIF) as a result of wounding (Melville and Ryan, 1973; Bryant *et al.*, 1976). Koiwa *et al.* (1997) suggested that the expression of PI genes in plants get induced by the octadecanoid (OD) pathway, which catalyzes the breakdown of linolenic acid and the synthesis of Jasmonic acid (JA). Four systemic signals including systemin, electrical signals, hydraulic signals and Abscisic acid (ABA) are responsible for the translocation of wound response (Malone and Alarcon, 1995).

Over 20 defensive genes get activated in tomato plant in response to insect attack, an 18 amino acid polypeptide, systemin released from wound sites on tomato leaves that systemically regulated the expression of protective genes. The signaling molecule, systemin, is synthesized from a 200 amino acid pro-protein termed prosystemin that is encoded in 11 exons. The cDNA and gene encoding the signaling molecule, systemin, have been isolated and characterized (McGurl *et al.*, 1992; McGurl and Ryan, 1992). Systemin triggers a lipid-based signal transduction pathway in which there is a release of linolenic acid from plant membranes that is converted into Jasmonic acid, identified as an important signaling compound for the defensive genes (Ryan, 2000; Turner *et al.*, 2002). Systemin also is capable of inducing other plant defensive proteins including polyphenol oxidase (Constabel *et al.*, 1995), indicating that systemin has a role in signaling plant defensive genes other than proteinase inhibitors. A dramatic reduction of proteinase inhibitor gene expression was observed in tomato plant leaves transformed with an antisense copy of prosystemin cDNA (McGurl *et al.*, 1992). There is a strong induction of local and systemic expression of PI genes in many plants by the application of jasmonate or its methyl ester that explains the omnipresent role of jasmonate in wound

response (Wasternack and Parthier, 1997). Analysis of a Potato PI-IIK promoter by Koiwa *et al.* (1997) has revealed a G-box sequence (CACGTGG) as Jasmonate-responsive element. Stankovic and Davies (1995) suggested that the electrical action potential could induce high level PI mRNA. A *de novo* synthesis of a Bowman Birk proteinase inhibitor from rice was established to be rapidly induced in seedling leaf in response to wounding (Rakwal *et al.*, 2001).

1.5 Structural Analysis of Proteinase Inhibitor Genes

Majority of the plant-derived PIs studied so far range from 8 to 20 kDa in molecular weight but in general PIs range from 4 to 85 kDa. For-Example *Brassica campestris* trypsin inhibitor (BCTI) 8 kDa in molecular weight was isolated from *Brassica campestris* seeds. BCTI has a thermostable property and its stability is due to the presence of a disulfide bridge revealed from the structural analysis (Hung *et al.*, 2003). It has been reported that plant serine PIs have molecular mass of 39 to 43 kDa. Trypsin inhibitor of the Indian finger millet (*Eleusine coracana*) structurally consist of 122 amino acids, a trypsin binding loop and 5 disulphide bridges (Gourinath *et al.*, 2000). Winged bean, *Psophocarpus tetragonolobus* Kunitz-type double headed alpha chymotrypsin was structurally analyzed by X-ray crystallography that illustrated 12 anti-parallel beta strands joined in a form of beta trefoil having two reactive sites (Asn 38-Leu 43 and Gln 63-Phe 68) at the external loops (Ravichandaran *et al.*, 1999; Mukhopadhyay, 2000).

Structural data collected from aspartic PI cardosin A purified from cardoon *Cyanara cardunculus* showed glycolylation regions at Asn-67 and Asn-257. The sequence Arg-Gly-Asp recognizes the cardosin receptor present in a loop between two beta strands on the molecular surface (Frazao *et al.*, 1999). The 3D structure of the PIN II genes from the Solanaceae family members reveals eight sequence-repeats (the 'IP repeats') coded by the second exon in the PIN II gene (Moura and Ryan, 2001). The PIN II genes have a highly conserved architecture. The N-terminus of the signal Peptide is encoded by the first exon and the C-terminus is encoded by the second major exon. There

is a quite variable sequence of IP repeats only the cysteines constituting the four disulfide bridges and a single proline residue are conserved (Endre *et al.*, 2002). Using NMR, three dimensional structure of oryzacystatin (OC-I) was demonstrated (Nagata *et al.*, 2000). The main structure consists of amino acids from Glu 13 - Asp 97 and an alpha helix with five stranded anti-parallel beta-sheet.

1.6 Wound Inducible Proteinase Inhibitor Genes Reported in Plants

Experimental data illustrate the expression of various plant proteins induced by insect chewing. Expression of approximately 100 genes in lima bean *Phaseolus lunatus* (L.), induced by the chewing of the two-spotted spider mite, *Tetranychus urticae* (Koch) (Arimura *et al.*, 2000). There is an up or down regulation of about 80 genes in sorghum, *Sorghum bicolor* (L.), by a phloem-feeding insect, the sorghum greenbug, *Schizaphis graminum* (Rondani) (Zhu-Salzman *et al.*, 2004). Various hydrolytic enzymes in insect saliva may function as elicitors of proteinase inhibitors in plants (Miles, 1999; Felton *et al.*, 2001). Felton *et al.* (2001) identified a 78 kD glycoprotein elicitor, glucose oxidase (GOX), from the saliva of *Helicoverpa zea*. Zymogram assay indicated the active presence of soybean trypsin inhibitor and cysteine protease inhibitor (E-64) in soybean young nodules (Mashamba *et al.*, 2009). PIs have been widely tested and purified from cowpea and peanuts (Melo *et* al., 2002; Boateng *et al.*, 2005). A buckwheat inhibitor (BWI)-1 protein has been isolated from buckwheat seeds. BWI-1 has a molecular weight of 7.7 kDa and it is a member of potato inhibitor I family (Dunaevsky *et al.*, 1997).

There is a difference in transcriptional changes induced in response to insect wounding from those induced by wounding with forceps. A DNA microarray study with a set of preselected defense-related arabidopsis genes showed that caterpillar-elicited transcriptional changes differed from those of mechanical wounding. Two kinds of Kunitz-type PIs are secreted from potato tubers by wounding and water stress (Ledoigt *et al.*, 2006).

Experimental analysis has shown that defense genes in tomato plants get activated by an octadecanoic acid-based signaling pathway in response to herbivore attack or some mechanical wounding. A tomato mutant, def1, which is deficient in the octadecanoid pathway, is sensitive to insect attack. Wounding, addition of polygalacturonic acid, chitosan, and the application or overproduction of systemin in transgenic plants failed to induce PI mRNA accumulation in the mutant (Howe *et al.*, 1995).

Barley proteinase inhibitor family comprises 13 genes encoding proteins, cystatins (HvCPI-1 to HvCPI-13), these cystatins were purified as recombinant protein from *E. coli* cultures and the *in vitro* inhibitory activity of these 13 cystatins, was tested against five species of phytophagus arthropods with cysteine protease activity, mainly located in their guts. Most of the barley cystatins inhibited the enzymetic activities of the experimental arthropod species but the recombinant protein HvCPI-6 drastically reduced cathepsin-L and B-like activities in the insects and acari tested in experimental analysis (Carrillo *et al.*, 2009. Paper submitted).

1.6.1 Plant Responses to Wounding

The plants show complex responses to the wounding by insect feeding. The steady-state level of over 700 mRNAs changes in the model plant arabidopsis during defense responses (Schenk *et al.*, 2000). In tobacco over 500 mRNAs constitute the insect-responsive transcriptome. Much of the complexity of these responses is due to the expression of the genes involved in general stress responses. For-Example, in tobacco there is a down regulation of photosynthetic genes, which are not involved in defense, in response to insect attack (Hermsmeier *et al.*, 2001), most probably to let more resources to be owed to produce proteins directly involved in the resistance response.

1.7 Transgenic Plants with Proteinase Inhibitor Genes

Several Japonica rice varieties transformed with potato proteinase inhibitor II (PINII) gene, showed high-extent accumulation of PINII proteins. Wound inducible

expression of PINII gene driven by its own promoter, accompanied with first intron of rice actin 1 gene (actI). PINII gene stably inherited in each generation. Transgenic rice plants had increased resistance to a major rice insect pest pink stem borer (*Sesamia inferens*) indicated by bioassay for insect resistance performed with fifth generation transgenic rice plants (Xiaolan *et al.*, 1996). One of the key successes in agriculture is the introduction of genetically engineered insect resistant crops. The extensive use of cotton (*Gossypium hirsutum*) resistant to *lepidopteran* larvae (caterpillars) and maize (*Zea mays*) resistant to both *lepidopteran* and *coleopteran* larvae (rootworms) at a large scale in agriculture resulted in limited pesticide use and lesser production cost (Toenniessen *et al.*, 2003; Brookes and Barfoot, 2005). The tomato and potato PI families are the best studied examples of genes, systemically expressed upon wounding. In potato, proteinase inhibitor II (PI II) is a multigene family, its constitutive expression in tubers and floral buds and wound-inducible expression in leaves has been reported (Pena-Corte *et al.*, 1988; Sanchez-Serrano *et al.*, 1993).

Transgenic potato plants expressing cowpea trypsin inhibitor (CpTI) at levels up to 2 % of the leaf protein reduced the growth of the tomato moth larva (*Lacanobia oleracea*) by 45 % (Gatehouse *et al.*, 1997). CpTI gene transformed into pigeonpea induced resistance against *Helicoverpa armigera* (Lawrence *et al.*, 2001). *Solanum americanum* is a rich source of proteinase inhibitors (Brzin and Kidric, 1995), and also a suitable source for cloning PIN2 cDNAs. Two cDNA clones encoding PIN2, designated *SaPIN2a* and *SaPIN2b*, have already been isolated as a heterologous hybridization probe by screening *S. americanum* cDNA library prepared from wounded leaves using a tomato PIN2 cDNA (Graham *et al.*, 1985; Xu *et al.*, 2001). Cowpea trypsin inhibitor (CpTi) gene transformed in tobacco (Hilder *et al.*, 1987), wheat (Lawrence *et al.*, 2001), potato (Graham *et al.*, 1997), cotton (Alpteter *et al.*, 1999). Soybean Kunitz TI gene transformed in Rice (Lee *et al.*, 1999). Barley TI gene transformed in tobacco (Carbonero *et al.*, 1993, Lara *et al.*, 2000). Oryzacystatin gene transformed in rapeseed (Girard *et al.*, 1998). Bean α-amylase inhibitor gene transformed in pea against *Bruchus pisorum* (Chen *et al.*, 1999). Potato PI II transformed in birch and lettuce (Gatehouse *et al.*, 1999). *Arabidopsis thaliana* cysteine proteinase inhibitor gene transformed in White poplar (*Populus alba* L.)

conferred resistance against *Chrysomela populi* beetle (Delledonne *et al.*, 2001). Transgenic peas (*Pisum sativum*) developed by using Bean α-amylase inhibitor1 gene. Under field conditions transgenic peas showed complete resistance against pea weevil (*Bruchus Pisorum*) (Roger *et al.*, 2000). The rice Bowman Birk inhibitor is induced and up-regulated by pathogens and insects during germination of rice seeds (Lin *et al.*, 2006).

In a field experiment it was reported by Delledonne that tobacco plant expressing the serine-proteinase inhibitors KTi3, C-II and PI-IV from soybean resulted in up to 100 % mortality of first-instar cotton-leaf worms (*Spodoptera littoralis*) (Unpublished data). Promoters from wound-inducible PI-II gene, ribulose 1,5-biphosphate carboxylase small subunit gene, rice actin gene, and ubiquitin have been found effective for producing the protective proteins in transgenic plants (Ussuf *et al.*, 2001). Transgenic rice (Duan *et al.*, 1996), tobacco and tomato (Reeck. *et al.*, 1997) developed by using Potato proteinase inhibitor-II promoter (Pot PI-IIK) that is wound inducible. Insect-resistant wheat was developed by transferring the gene of barley trypsin inhibitor (BTI) that resulted in significant reduction in the survival of *Sitotroga cerealella* an argoumois grain moth. BTI accumulation in transgenic wheat was 1.1 % of total extracted protein (Altpeter *et al.*, 1999). Better insect resistance can be achieved by combined expression of defense genes with different mode of action. For-Example an increased resistance to *Heliothis obsoleta* and *Liriomyza trifolii* larvae has been reported in homozygote tomato lines over-expressing potato PI-II and Carboxypeptidase inhibitors (PCI) transgenes (Ashraf *et al.*, 2005).

A DNA construct containing a nucleic acid which encodes a serine proteinase inhibitor (BOPI) was isolated from *Brassica oleracea* having a natural insect antibiosis activity. Tobacco leaf discs were transformed with *Agrobacterium tumefaciens* containing the pBfN19/BoPI plant expression vector. The resulted transgenic tobacco plant was resistant to tobacco budworm (*Heliothis virescens*). Insect Bioassays of Transgenic Plants indicated that BOPI was effective against a wide variety of insect pests including the orders of Lepidoptera, Coleoptera, Diptera, Homoptera, Hemiptera, Thysanoptera, and Orthoptera in a wide variety of plants (Stewart *et al.*, 2005).

It has been found that, transformed suspension culture system expressing PIs facilitates in the production of recombinant proteins and may assist in protecting against recombinant protein losses resulting from extracellular proteases. Recent research illustrates a reduction in protease activity in transformed rice cell suspension cultures expressing a proteinase inhibitor. Tae-Geum Kim *et al.* (2007) obtained a synthetic serine proteinase inhibitor II gene (sPI-II) that harbored the chymotrypsin and trypsin inhibitor domains of the PI-II gene from *Nicotiana alata* by using an overlap PCR. sPI-II gene induced into a rice calli (*Oryza sativa* L. cv. Dongin) was under the control of a rice α-amylase 3D promoter induced by sugar starvation. Northern blot analysis and genomic DNA PCR amplification verified the incorporation of sPI-II gene in the transformed rice suspension cells. Experimental analysis showed that the relative protease activity of the transformed rice cell suspension culture reduced to approximately 23 %.

1.8 Fundamental Functions

The major function of plant PIs are thought to be in plant defense and the regulation of endogenous proteinases, but they may also serve as storage proteins (Mosolov *et al.,* 2001; Birk, 2003; Shewry, 2003). PIs are of interest as providing markers for studies of plant diversity and evolution (Konarev *et al.,* 2002; Lawrence and Koundal, 2002; Korsinczky *et al.,* 2004). PIs have been shown to act as defensive compounds against insects by direct assay or by expression in transgenic crop plants (Lawrence and Koundal, 2002). The activity of PIs is due to their capacity to form stable complexes with target proteases, blocking, altering or preventing access to the enzyme active site. The first convincing evidence that PIs are part of the natural defensive chemicals of plants was the demonstration that wounding of tomato and potato leaves by Colorado potato beetles induced a rapid accumulation of proteinase inhibitor I (PIN1), not only in the damaged leaves, but also in distal, undamaged leaves (Green and Ryan, 1972). The protective functions of PIN2s from tomato (Johnson *et al.,* 1989), potato (Klopfenstein *et al.,* 1997), and ornamental tobacco (Charity *et al.,* 1999) have been demonstrated by the enhanced insect resistance of transgenic plants expressing these PIN2s. A cowpea proteinase inhibitor (CpTI) was shown for the first time to confer

resistance to feeding by the tobacco budworm (*Heliothis virescens*) when the CpTI gene was expressed in transgenic tobacco (Hilder *et al.*, 1987). Several plant PIs such as soybean trypsin inhibitor, have been conveniently used for the affinity purification of their inhibited proteinases from a wide variety of sources (Richardson, 1977; Richardson, 1991).

Three isoinhibitors named as HPI-1, HPI-2, HPI-3 isolated from the latex of the rubber tree, *Hevea brasiliensis* clone RRIM 600 contribute a defensive property in the latex against pathogens and herbivores. The coding gene for the inhibitor is a representive of potato inhibitor I family and is wound inducible (Sritanyarat *et al.*, 2006). PIs have been implicated to play a role in the plant's natural defense towards fungal infections (Soares-Costa *et al.*, 2002). Plant PIs are known to confer natural as well as engineered protection against nematode attack (McPherson and Harrison, 2001; Atkinson *et al.*, 2003; Cai *et al.*, 2003). Nematode control with PIs expressed in transgenic tomato (Urwin *et al.*, 1995), *arabidopsis thaliana* (Urwin *et al.*, 2000), and rice (Vain *et al.*, 1998) has been well demonstrated. Plant PIs have also been documented as novel anti-viral agents as rice cysteine proteinase inhibitor gene induce resistance against two important potyviruses, tobacco etch virus (TEV) and potato virus Y (PVY), in transgenic tobacco plants by its constitutive expression (Campos *et al.*, 1999).

SaPIN2a, a proteinase inhibitor II from *S. americanum* is highly expressed in the phloem and could be involved in regulating proteolysis in the sieve elements (Xu *et al.*, 2001). SaPIN2a in transgenic lettuce inhibits plant endogenous protease activity further indicates that SaPIN2a regulates proteolysis and could be potentially exploited for the protection of foreign protein production in transgenic plants (Xu *et al.*, 2004). PIs also play a significant part in the modulation of apoptosis identified in soyabean (Kosslak *et al.*, 1997). Another vital function of plant PIs is the mobilization of stored plant reserves to provide aminoacids to the developing plant parts as in young nodules (Mashamba *et al.*, 2009). An important role of PIs in protein stabilization has been reported, PIs are found to accumulate relatively late during seed development and rapidly increases in the desiccation phase (Domoney *et al.*, 1995). Dehydration related stresses such as drought,

salinity and abscisic acid triggers the expression of trypsin inhibitors (TIs) in developing seeds of moong bean and lettuce (Lam *et al.*, 1999).

1.8.1 Proteinase Inhibitors as Nutraceutical Molecules

Nutraceutical properties have been reported in plant PIs. PIs present in pulses serve as anti-carcinogenic components having anti-tumoral efficacy (Murillo *et al.*, 2004). For-Example soybean serine proteinase inhibitor of Bowman-Birk inhibitor (BBI) family has been found effective against certain mammalian tumors (Kennedy, 1998). There is a major role of plant PIs in preventing breast, colon, and prostatic cancers in vegetarian population (Birk, 1993). Two recombinant wild type Bowman Birk inhibitors (BBI) from pea seeds have anti-proliferative activity on human colon cancer cells (Clemente *et al.*, 2004). BBIs also help in the control of degenerative and autoimmune diseases including multiple sclerosis, Guillain Barre Syndrome and skeletal muscle atrophy (Sweeney *et al.*, 2005). Plant PIs active towards proteases that regulate human physiological processess, e.g. cell signaling/migration, digestion, fertilization, growth, differentiation, immunological defense, wound healing and apoptosis, have great potential in therapeutic applications (Abdel-Meguid *et al.*, 2000; Leung *et al.*, 2000). Wheat α-amylase inhibitor has a significant role in the therapy of obesity and diabetes. The Nutraceutical property of wheat α-amylase inhibitor has been confirmed (Oneda *et al.*, 2004). Park *et al.* (2004) reported that (buckwheat inhibitors) BWI-1 and BWI-2a extracted from buckwheat seeds have suppressive activity against human T-acute lymphoblastic leukemia cells. Buckwheat trypsin inhibitor gene cloned and expressed in E-coli M15. The recombinant inhibitor rBTl purified with one step purification method strongly inhibited the trypsin activity and specifically hindered the proliferation of IM-9 human B lymphoblastoid cells (Zhang *et al.*, 2007).

1.9 Tomato Varieties in Pakistan

The tomato plant (*Solanum lycopersicum* L.) belongs to the Solanaceae family. Tomato is a fleshy, juicy and shiny fruit used as a vegetable worldwide. In Pakistan

tomato is widely cultivated in the plains and in hilly areas. Frost-free environment and loamy friable soil with pH 5.7-7.7 in temperate areas of Pakistan is best for tomato production. The seeds are used for the propagation, seedlings 6-10 inches in height are transplanted in the fields.

In Pakistan tomato varieties are generally categorized into two distinct types (i) determinate tomatoes (ii) indeterminate tomatoes. Determinate varieties flower and set fruit all at once followed by dropping. Determinate varieties are compact plants, they flower at the ends of shoots that determines their length. Riogrande and Roma are good examples of determinate varieties in Pakistan. Indeterminate varieties continue to grow throughout the season. Their flowers grow along the vines that do not determine their length; indeterminate varieties require support and pruning. The best example of indeterminate tomato variety in Pakistan is Moneymaker. Indeterminate varieties have high yield potential than the determinate varieties. The widely cultivated tomato varieties reported in the Punjab province are Roma, Red Top, Nagina and Pakit. In the Sindh province Roma Paylong, Sr-2, Gloriana, T-10 and Marglobe. Recent data provided by Horticultural Research Institute of Pakistan Agricultural Research Council (PARC) indicates that 23 tomato varieties were evaluated on the basis of this evaluation the results reveal that following tomato varieties CLN-657-B-GF2, Avinash-2, CL-5915-206 D4-2-5-0 and CLN-657 BG2 -274-0-0-1-5-0 gave good functioning with 2.3, 2.2, 2.1 and 1.8 g fruit weight/plant, respectively. In another study, on single plant selection basis two tomato varieties Pusa Rubi Early and Pusa Ruby Late were screened for high yield at Vegetable Research Station, Mirpurkhas (Sindh). Pusa Rubi Early gave a high yield of 3.6 kg/plant while Pusa Ruby Late yielded 2.3 kg/plant. This research data indicated the importance of these varieties in the Sindh province from agricultural point of view (http://www.parc.gov.pk/hort.html).

At Hafiz Farooq's Farm in Sehala, Islamabad and Kattha Sugral, Khushab nine tomato varieties were evaluated on farm trial under SAVERNET-Phase-II the data estimated from Islamabad showed that Punjab Chhuhara (19.5 t/ha), Moneymaker (16.8 t/ha), and Pant Bahar (15.0 t/ha) gave good performance while Moneymaker (27 t/ha), J-

15 (21.7 t/ha) and Pusa Ruby (20.2 t/ha) confirmed best at Khushab. Evaluation of eight tomato varieties was evaluated for high yield performance by the Agriculture Research Station, Mingora (Swat). The research data obtained have shown that variety 0596-0104 gave maximum yield of 22.7 t/ha while Roma-VF showed 21.5 t/ha. Post-harvest research work conducted by National Agricultural Research Centre, (NARC) Islamabad, University of Agriculture, Faisalabad, Sindh Horticulture Agriculture Research Institute, Mirpurkhas and Ayub Agriculture Research Institute, Faisalabad reported that the two tomato varieties Riogrande and Roma were high yielding with longer post-harvest life. These varieties were selected for better shelf life (http://www.pakissan.com/english/newtech/postharvesttech.shtml).

1.10 Research Objectives

The key objectives of the research work are as follows.

- *In vitro* and *in silico* analysis of a wound inducible proteinase inhibitor gene sequence from three selected Pakistani tomato varieties.
- Establishment of phylogenetic relationships for different tomato varieties.
- Comparison of proteinase inhibitor gene sequence from selected tomato varieties with already identified proteinase inhibitor gene sequences from different plant by using different bioinformatics tools.

Chapter 2

MATERIALS AND METHODS

2.1 Selection of Gene Sequence

Complete coding sequence of a wound inducible proteinase inhibitor II gene from tomato (*Solanum lycopersicum* L.) was picked from the GenBank, nucleotide database run by National Center for Biotechnology Information (NCBI) (www.ncbi.nih.gov).

2.1.1 Primer Designing

A precise pair of primers was designed by using a bioinformatics program primer 3. The primers were designed for the amplification of complete gene sequence.

PI II F:	5'	TATCCATCATGGCTGTCCAC	3'
PI II R:	5'	AACACACAACTTGATCCCCACA	3'

2.1.2 Plant Material

Solanum lycopersicum L. seeds of three different varieties were acquired from National Agriculture Research Centre (NARC) Islamabad, Pakistan.

2.1.3 Selected Pakistani Tomato Varieties

Three tomato (*Solanum lycopersicum* L.) varieties were selected for research purpose. The names and types of these varieties are mentioned (Table 1).

Table 1: Local tomato varieties selected for *in vitro* and *in silico* analysis.

S. No.	Local Name	Type
1	Riogrande	Determinate
2	Nagina	Determinate
3	Moneymaker	Indeterminate

2.1.4 Seeds Sowing and Germination Conditions

Tomato seeds were sown in small pots containing manure soil. The pots were placed in a growth chamber with preset conditions including temperature at 27 °C under cool white fluorescent light of 2000 LUX, 75 % humidity and a photoperiod regime of 16 hrs. light and 8 hrs. dark.

2.1.5 Genomic DNA Isolation

DNA was extracted from young fresh leaves. A CTAB method illustrated by Richards (1997) was followed with some alterations. Four to five fresh leaves of plant material were collected and rinsed with distilled water. The plant material was crushed into a fine paste in a pestle and mortar, by adding 1 ml of preheated (65 °C) 2X CTAB (Cetyl Trimethyl Ammonium Bromide) buffer which contained 100 mM Tris HCl (pH 8.0), 20 mM Ethylene Diamine Tetra Acetic Acid (EDTA) (pH 8.0), 1.4 M Sodium Chloride (NaCl) and 1 % β-Merceptoethanol. The CTAB/plant extract mixture was transferred to 1.5 ml autoclaved eppendorf tubes. These tubes were incubated at 65 °C on a heating block for 45 minutes. Then an equal volume of chloroform/isoamyl (24:1) was added and the tubes were inverted gently 5-8 times for mixing. Then the tubes were centrifuged at 10,000 rpm for 10 minutes to spin down cell debris. The supernatant obtained was shifted to another autoclaved eppendorf tube and again washed with an equal volume of chloroform/isoamyl (24:1) and centrifuged at 10,000 rpm for 10 minutes. The upper aqueous phase was again shifted to a new eppendorf tube, an equal

volume of cold isopropanol and 45 µl of 3 M Sodium Acetate was added and the tubes were slowly inverted several times. These tubes were left overnight in freezer at -20 °C to precipitate the DNA. Next day centrifugation was done at 13,000 rpm for 15 minutes. After centrifugation the supernatant was discarded and the DNA pellet was washed with 70 % chilled ethanol. Then the pellet was air dried for 15 minutes and re-dissolve in 30-40 µl of 0.1X TE (Tris Ethylene Diamine Tetra Acetic Acid) buffer. The DNA sample isolated in purified form was stored at -20 °C.

2.1.6 DNA Quality Confirmation

The integrity of isolated DNA was checked by agarose gel electrophoresis. The basic method of agarose gel electrophoresis provides an easiest and commonest way of analyzing DNA. Ethidium bromide is generally used for visualizing DNA in the gel. It binds strongly to the DNA by intercalating between the bases and it is a fluorescent dye. It absorbs invisible UV light and transmits the energy as visible orange light. For a minigel 25 ml of 1 % agarose gel solution was prepared in 0.5X TAE (Tris Acetate Ethylene Diamine Tetra Acetic Acid) buffer. Small 8x10 cm gels (minigels) are commonly used and give good photographs. The gel solution was poured slowly into a tray with a comb. The bubbles were pushed to the side by using an autoclaved tip. The gel was allowed to set for 30 minutes at room temperature on a flat surface. Then the gel tray was placed into the gel tank containing 0.5X TAE running buffer. The DNA samples were loaded into separate wells by using 3 µl sample and 1 µl bromophenol blue loading dye. The gel was run for 30 minutes at 80 V, when the dye covered 1/3 of the distance on the gel it was stained in ethidium bromide (10 mg/ml). Then the gel was exposed to UV transilluminator and photographed by using Wealtec Dolphin Doc Plus Gel Image System. Highly resolved intact band indicates good quality DNA and smeared band indicates DNA degradation.

2.1.7 Polymerase Chain Reaction (PCR)

2.1.7.1 PCR Optimization and Reaction Conditions

For efficient amplification and high yields of precise DNA target sequence individual reaction component concentrations, time and temperature parameters for PCR were adjusted. Annealing temperature for PCR was adjusted by using wide range of annealing temperatures from 50-65 °C. High quality amplification was observed at 55 °C, 56.9 °C and 57.8 °C by using gradient PCR.

A specific set of primers PI II F and PI II R was used to amplify a target gene sequence. PCR conditions employed for the amplification were pre-PCR denaturation at 94 °C for 5 minutes followed by 35 cycles of denaturation at 94 °C for 40 seconds, annealing at 55 °C, 56.9 °C and 57.8 °C for 40 seconds and extension at 72 °C for 45 seconds. After the last PCR cycle there was a single step final extension at 72 °C for 20 minutes to ensure that any remaining single-stranded DNA became fully extended. Then a final hold step at 4 °C was employed for the short term storage of reaction in a gradient MultiGene Thermal Cycler (Labnet).

2.1.7.2 Reaction Mixture Components

PCR reaction tubes were prepared with 25 µl reaction mixture in each tube. PCR mixture of 25 µl contained 25 pmol of each primer, 25-50 ng/µl of DNA template, 2.5 µl of 10X PCR buffer, 1.5 µl of 25 mM $MgCl_2$, 1.5 µl of 2 mM dNTPs and 1.5 U of Taq polymerase (MBI Fermentas).

2.1.7.3 PCR Product Analysis

The presence of PCR generated amplimer was checked by running the amplified products on 1.5 % agarose gel prepared in 0.5X TAE buffer. The amplified products were loaded in separate wells by using 5 µl of PCR product and 1 µl 6X loading dye (bromophenol blue). Electrophoresis was done at 80 V for 45 minutes followed by staining with ethidium bromide. Gel documentation was conducted by using Dolphin Doc Plus Gel Image System (Wealtec). The size of PCR products was determined by running a 100bp DNA ladder (Fermentas) alongside the PCR products.

2.1.8 Purification of Amplified Products

The purification of PCR products was performed with JETquick (Genomed) PCR Product Purification Spin Kit. 40 µl amplified product was taken in an autoclaved eppendorf. Then 200 µl of H1 binding solution was added and the mixture was centrifuged at 13,000 rpm for 1 minute. After centrifugation sample mixture was loaded into the cartridge having silica-based membranes and the cartridge was placed into a 2 ml wash/collection tube. The DNA was selectively bound to the highly specified silica membranes capable of adsorbing up to 20 µg of double stranded DNA per spin column. Then 500 µl of absolute ethanol containing wash buffer (also known as H2 binding solution) was added in cartridge and centrifugation was carried out at 13,000 rpm for 1 minute. Unreacted primers, dNTPs, DNA polymerases, salts and other impurities were removed in one wash. The residual eluate as well as wash tube was discarded. The cartridge was shifted to an autoclaved eppendorf and 35 µl of warm (65-70 °C) PCR TE buffer was added to the center of cartridge and then incubated at room temperature for 1 minute. DNA was eluted by centrifuging at 13,000 rpm for 2 minutes. The purified product was analyzed on 2 % agarose gel prepared in 0.5 X TE buffer which contained 1.0 mM Tris pH (8.0), 0.1 mM EDTA pH (8.0). The purified DNA sample was stored at -20 °C to be used for sequencing.

2.1.9 Sequencing PCR Reaction Set-up

Sequencing reaction was performed in 200 µl centrifuge tubes by using Dye Terminator Cycle Sequencing (DTCS) Quick start kit by Beckman and Coulter. The sequencing reaction mixture of 10 µl contained the following components.

Table 2: Reaction mixture components for dye terminator cycle sequencing.

Components	Volume
Template	2.0 µl
Primer	1.5 µl
ddH_2O	1.5 µl
Sequencing buffer	2.0 µl
DTCS Quick start master mix	3.0 µl

2.1.10 Sequencing Cycling Conditions

Following cycling conditions with MultiGene Thermal Cycler (Table 3) were used.

Table 3: Reaction conditions for sequence cycling.

Steps	Temperature	Time	
Initial denaturation	96 °C	1.0 minute	
Denaturation	96 °C	25 seconds	30 cycles
Annealing	55 °C	25 seconds	
Extension	60 °C	4.0 minutes	
Final extension	60 °C	10 minutes	

2.1.11 Concentration of DNA by Ethanol Precipitation

The samples were ethanol precipitated after thermal cycling. A stop solution was freshly prepared for precipitation. 2.5 µl of stop solution containing 1 µl Sodium Acetate (3 M, pH 5.2), 1 µl Na_2EDTA (100 mM, pH 8.0) and 0.5 µl glycogen (20 mg/ml) was added to each sequencing reaction sample in 200 µl microcentrifuge tubes. Then 70 µl of

chilled 100 % ethanol was added in each tube and the tubes were vortex briefly. The tubes were immediately centrifuged at 13,000 rpm for 20 minutes. The supernatant was carefully discarded and the DNA pellet was washed with 150 µl of 70 % ethanol (chilled). The tubes were again centrifuged at 13,000 rpm for 20 minutes. The supernatant was discarded and the transparent pellet obtained after precipitation was air dried. The pellet was re-suspended in 30 µl of sample loading solution (SLS).

2.1.12 Sample Preparation for Loading into the CEQ

The re-suspended samples were loaded to the available wells of CEQ (8800) sample plate and a drop of light mineral oil provided with the kit was over laid on each sample. Finally, a proper sequencing program was run.

2.2 Gene Sequence Analysis

Different bioinformatics softwares, databases, and tools were applied for data analysis. The sequenced data was analyzed by using "blast n" (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) that indicated the statistics of sequence similarity scores and the evidence of homology in tomato genome.

Molecular Evolutionary Genetics Analysis (MEGA3) software (http://www.megasoftware.net) was applied for deducing phylogenetic trees and exploring evolutionary distances by the statistical methods and computational tools. Various analytical techniques as pairwise and multiple gene analysis were applied. A number of identified proteinase inhibitor gene sequences from different plants having similar expression and regulation patterns related to the selected *in vitro* sequenced gene were analyzed and compared to reveal their common structure, nucleotide order and phylogenetic relationships. Clustering methods were also applied for genetic analysis.

Chapter 3

RESULTS AND DISCUSSION

3.1 Germination of Tomato Seeds

The seeds of three tomato (*Solanum lycopersicum* L.) varieties were germinated in small plastic pots containing manure soil. Young fresh leaves emerged after two weeks of sowing were used for DNA isolation.

3.2 Genomic DNA Isolation

Genomic DNA was isolated from the fresh leaves of three *Solanum lycopersicum* varieties (i) riogrande, (ii) moneymaker and (iii) nagina. The isolated genomic DNA was run on 1 % agarose gel prepared in 0.5X TAE buffer for determining the quality and quantity of genomic DNA (Figure 1).

3.3 Polymerase Chain Reaction

Isolated DNA was used as a template and the amplification of proteinase inhibitor II gene was carried out by using a precise set of primers PI II F and PI II R. The PCR amplimer of ~684bp was observed on 1.5 % agarose gel prepared in 0.5X TAE buffer by running a 100bp ladder alongside the PCR product (Figure 2).

3.3.1 PCR Optimization

By using a wide range of annealing temperatures from 50-63 °C gradient PCR was run. High quality amplification was observed at 55 °C, 56.9 °C and 57.8 °C.

Figure 1: Genomic DNA isolation from leaves of three *Solanum lycopersicum* varieties. Lane 1: riogrande, lane 2: moneymaker, lane 3: nagina.

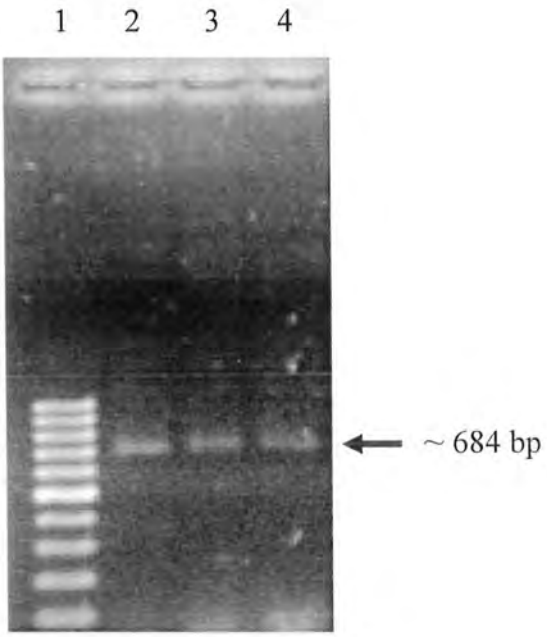

Figure 2: Gradient PCR amplification of proteinase inhibitor gene from three *Solanum lycopersicum* varieties. Lane 1: 100bp DNA ladder, lane 2: riogrande, lane 3: moneymaker, lane 4: nagina at annealing temperatures of 55, 56.9 and 57.8 °C respectively.

3.4 Proteinase Inhibitor Gene Sequencing

The amplified PCR products of selected tomato varieties were purified with JETquick (Genomed) PCR Product Purification Spin Kit. Each purified product was sequenced by using precise primers (PI II F, PI IIR) in Beckman Coulter Sequencer (CEQ 8800). High Quality sequencing results were signified by sharp peaks (Figure 3). By the dye terminator sequencing method the four bases were identified by different fluorescent labels indicated as the sharp peaks of different colors. The base sequence was determined by the interpretation of the resulted peaks. Sequenced gene of all selected tomato varieties showed homology with the selected proteinase inhibitor gene from tomato (*Solanum lycopersicum* var. cerasiforme), used for primer designing.

3.5 Sequencing Analysis

The sequencing of purified products of selected tomato varieties nagina, money maker and riogrande showed 97, 92 and 91 % homology respectively with PI-II gene of *S. lycopersicum* variety cerasiforme (Accession# AY007240). These similarity scores were obtained by using "blast n" (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi).

3.6 Phylogenetic Analysis

The phylogenetic analysis was conducted for the nucleotide sequences of these tomato varieties resulted after sequencing. *In silico* analysis was carried out for fourteen proteinase inhibitor gene sequences (Table 4) from different plants, having similar expression and regulation patterns. The phylogenetic studies revealed the assumed evolution, a historical pattern of ancestry, genetic similarities, correlations and phylogenetic relationships. The evolutionary relationships among these proteinase inhibitors of Solanaceae family were inferred by phylogenetic tree analysis. As expected most proteinase inhibitors grouped together reflecting the relatedness of these PI genes coded by closely related plants belonging to the same family.

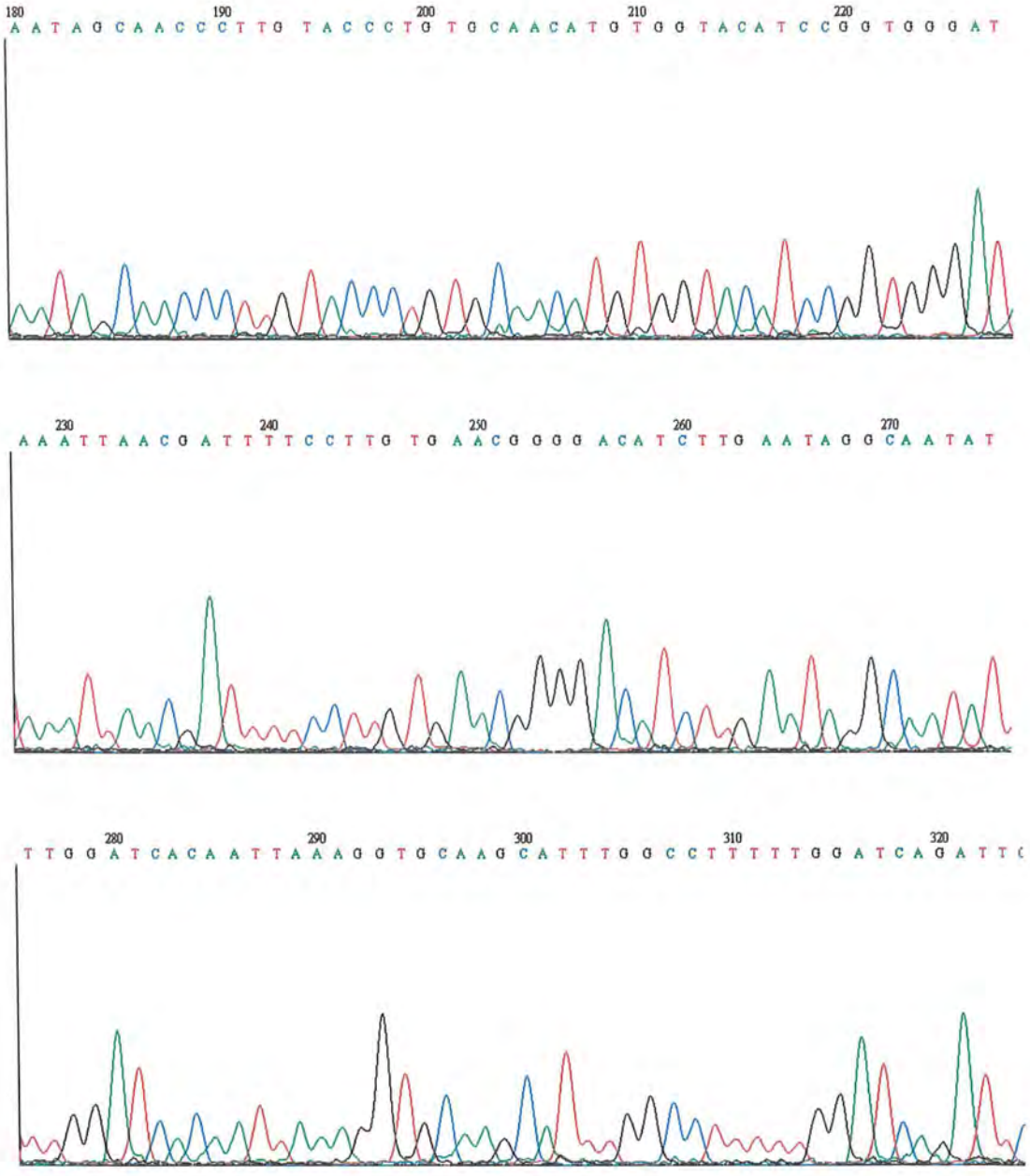

Figure 3: Sharp peaks indicating the quality of sequencing data.

Table 4: Proteinase inhibitor genes from different members of the family Solanaceae that were phylogenetically analyzed

S. No.	Genes	Origin	Accession No.	Denoted by (in phylograms)
1	Proteinase inhibitor II	*Solanum lycopersicum*	AY007240	a
2	Protease inhibitor II	*Solanum lycopersicum*	AB110700	b
3	Proteinase inhibitor II	*Lycopersicon esculentum*	AY129402	c
4	Auxin-induced proteinase inhibitor	*Lycopersicon esculentum*	L25128	d
5	Inhibitor II-chloramphenicol acetyltransferase	*Solanum tuberosum*	M15186	e
6	Proteinase inhibitor II (pin2T) gene	*Solanum tuberosum*	U45450	f
7	Proteinase inhibitor II	*Solanum tuberosum*	X04118	g
8	Proteinase inhibitor II	*Solanum tuberosum*	Z12753	h
9	Proteinase inhibitor II	*Solanum tuberosum*	Z13992	i
10	Proteinase inhibitor II	*Nicotiana tabacum*	Z29537	j
11	Proteinase inhibitor SPLTI-1	*Ipomoea batatas*	AF330701	k
12	Proteinase inhibitor SPLTI-2	*Ipomoea batatas*	AF330702	l
13	Wound-inducible proteinase inhibitor I	*Lycopersicon esculentum*	M13938	m
14	Proteinase inhibitor II	*Solanum tuberosum*	L16450	n

The gene sequences of PI genes from different plants were studied by conducting pairwise analysis for these plant origin proteinase inhibitor genes (Table 4). These pairwise similarity scores were recorded in table 5. The genetic similarity values range from 0.66-0.77 for the fourteen genes under analysis with the overall average of 0.71. These values illustrate that these genes were genetically allied with each other as there was little difference in these similarity scores. 71 % genetic resemblance on average was observed among these nucleotide sequences.

The interior branch test was applied by using neighbor joining method for phylogram construction, even the ordinary least square estimates of a given branch length were computed by MEGA software. The interior branch tests have been found to be most suitable for closely related sequences (Nei and Kumar, 2000).

3.6.1 Phylogram Analysis Based on Nucleotide Sequences of Fourteen Plant PI Genes

The phylogram revealed two major clusters denoted by C1 and C2 (Figure 4).

Cluster 1

Cluster 1 included nine PI genes Z12753 PI-II *S. tuberosum* (h), Z13992 PI-II *S. tuberosum* (i), X04118 PI-II *S. tuberosum* (g), AY129402 PI-II *L. esculentum* (c), L25128 ARPI *L. esculentum* (d), U45450 pin2T *S. tuberosum* (f), AY007240 PI-II *S. lycopersicum* (a), M13938 PI-I *L. esculentum* (m) and L16450 PI-II *S. tuberosum* (n). This cluster was further divided into two sub clusters Sc 1 and Sc 2 on the basis of genetic divergence (Figure 4). The closely related sequences lie under the same interior node and their branch length closely reproduce the observed distances between sequences (Felsenstein, 2004). G1, G2 and G3 members were most recent in origin as indicated by their reproduced branch length (Figure 4).

Sub Cluster 1:

Sub cluster 1 comprised of Z12753 PI-II *S. tuberosum* (h), Z13992 PI-II *S. tuberosum* (i), X04118 PI-II *S. tuberosum* (g), AY129402 PI-II *L. esculentum* (c) and L25128 ARPI *L. esculentum* (d). 'h', 'i' and 'g' were claded into group 1 (G 1). 37 % genetic corelation was found between Z12753 PI-II *S. tuberosum* (h) and Z13992 PI-II *S. tuberosum* (i) while X04118 PI-II *S. tuberosum* (g) showed 39 % similarity with 'h' and 'i' in G 1. 'c' and 'd' were fallen in group 2 (G 2) with 88 % genetic similarity. Phylogram illustrated that G 1 and G 2 were related genetically (55 % similarity). The member 'g' of group 1 seems to have distant origin as compared to 'h' and 'i'. It is quite possible that 'h' and 'i' were the outcome of some kind of mutation in 'g'. 'c' and 'd' were related to 'h' and 'i' in terms of evolution as observed from branch length.

Sub Cluster 2:

Four PI genes U45450 pin2T *S. tuberosum* (f), AY007240 PI-II *S. lycopersicum* (a), M13938 PI-I *L. esculentum* (m) and L16450 PI-II *S. tuberosum* (n) were fallen in this sub cluster.'a', 'm' and 'n' were claded into group 3 (G 3) with 77 % genetic relatedness. 'f' remained unresolved (Figure 4).

Cluster 2

This cluster has covered five PI genes AF330702 SPLTI-2 *I. batatas* (l), AF330701 SPLTI-1 *I. batatas* (k), M15186 IIK *S. tuberosum* (e), AB110700 PI-II *S. lycopersicum* (b) and Z29537 PI-II *S. tuberosum* (j). Bifurcation gave rise to two sub clusters Sc 1 and Sc 2 because of genetic divergence.

Sub Cluster 3:

Two members AF330702 SPLTI-2 *I. batatas* (l) and AF330701 SPLTI-1 *I. batatas* (k) were fallen in to group 4 (G 4) and these were 99 % genetically allied as

having the same taxonomic status, genes from the same plant coding for the proteinase inhibitor having a defensive chemistry.

Sub Cluster 4:

It included three representative member genes M15186 IIK *S. tuberosum* (e), AB110700 PI-II *S. lycopersicum* (b) and Z29537 PI-II *S. tuberosum* (j). Group 5 had 'b' and 'j' (45 % relatedness). 'e' showed 78 % genetic relationship with G 5. This phylogram demonstrated that G 4 and G 5 were closely associated with 92 % genetic similarity (Figure 4).

The slight genetic divergence showed in this cluster shows that very small genetic variation among the nucleotide sequences can be due to slow rates of mutations and environmental stresses. The sequences 'l' and 'k' appeared to be most recently evolved closely related members with highest similarity level. By looking at the complete cladogram, it can be easily observed which species are most closely or distantly related.

PI-I and PI-II of serine PI family are the extensively studied members of proteinase inhibitors. Seven direct ancestors of *Lycopersicon esculentum* that were *L. pennellii, L. chilense, L. hirsutum, L. parviflorum, L. peruvianum* var. humifusum, *L. cheesmanii* and *L. peruvianum* were investigated in terms of molecular and phylogenetic analysis for the wound inducible PI-I gene, it was shown by the study that PI-I was wound inducible in these seven progenitors having varied levels of accumulation in response to wounding (Lee *et al.*, 1986).

3.6.2 Phylogram Analysis of Coding Sequences in Fourteen Plant PI Genes

By using (MEGA3) software phylogram was constructed by using the data of fourteen coding sequences of plant PI genes (Table 4). The phylogram (Figure 5) was divided into two clusters C 1 and C 2 on the basis of genetic divergence (33 %).

Cluster 1

Cluster 1 had eleven gene members AY007240 PI-II *S. lycopersicum* (a), U45450 pin2T *S. tuberosum* (f), X04118 PI-II *S. tuberosum* (g), AB110700 PI-II *S. lycopersicum* (b), M15186 IIK *S. tuberosum* (e), Z12753 PI-II *S. tuberosum* (h), Z29537 PI-II *S. tuberosum* (j), AY129402 PI-II *L. esculentum* (c), L25128 ARPI *L. esculentum* (d), Z13992 PI-II *S. tuberosum* (i) and L16450 PI-II *S. tuberosum* (n). 97 % genetic similarity was found among the members of cluster 1. 'c' and 'd' remained unresolved in C 1. This cluster was subdivided into two sub clusters Sc1 and Sc 2.

Sub Cluster 1:

Sub cluster 1 had six gene members, among these there was 97 % similarity. Two groups were formed G 1 and G 2 (Figure 5). Group 1 had two members AY007240 PI-II *S. lycopersicum* (a) and U45450 pin2T *S. tuberosum* (f). They had shown 99 % similarity with each other. Two member M15186 IIK *S. tuberosum* (e) and Z12753 PI-II *S. tuberosum* (h) with 99 % similarity were claded into group 2. The member X04118 PI-II *S. tuberosum* (g) showed 54 % similarity with G 1. 73 % similarity level was found between AB110700 PI-II *S. lycopersicum* (b) and G 1. Group 1 and Group 2 had close genetic association (99 % similatity). The gene distance and branch length indicate that were the most recent members while 'j' was distant in origin at sub cluster 1 level.

Sub Cluster 2:

It included two members Z13992 PI-II *S. tuberosum* (i) and L16450 PI-II *S. tuberosum* (n) with 30 % similarity formed the group 3 (G 3). 'i' and 'n' appeared to be evolutionarily stable may be due to slow mutation rate.

Cluster 2

Cluster 2 had three gene members M13938 PI-I *L. esculentum* (m), AF330702 SPLTI-2 *I. batatas* (l) and AF330701 SPLTI-1 *I. batatas* (k). 'l' and 'k' in sub cluster 3 were claded into group 4 (G 4) with 80 % similarity and branch length depicted distant origin. The gene 'm' was unresolved but it was genetically related with the members of G4.

The overview of phylogram (Figure 5) illustrated that the coding sequences in the studied PI genes showed an overall genetic distance of 0.3 % that indicate close genetic relationship among the coding sequences in PI genes. High level stability in terms of evolution was also observed for the nucleotide sequences of exons. The gene functions depends on the stability of exons as these are translated into proteins. The changes in the exon section can alter the expression level of proteins as for-example the difference in the regulation/expression level of proteinase inhibitors from different plants against phytophagous insects.

In another study by Martinez *et al.* (2005) phylogenetic analysis was carried out for twelve cystatin genes from rice, seven from arabidopsis and seven from barley, in order to reveal the evolutionary link among these cystatin proteins, the determined amino acid sequences of these PI genes were compared by a phylogenetic tree constructed by neighbor joining method. The highest similarity scores were observed for HvCPI-4 between *Hordeum vulgare* and OC-XII protein from *Oryza sativa*. The cystatin proteins from arabidopsis were found scattered in the resulted tree that revealed that arabidopsis cystatins might not be functionally associated to those from rice and barley.

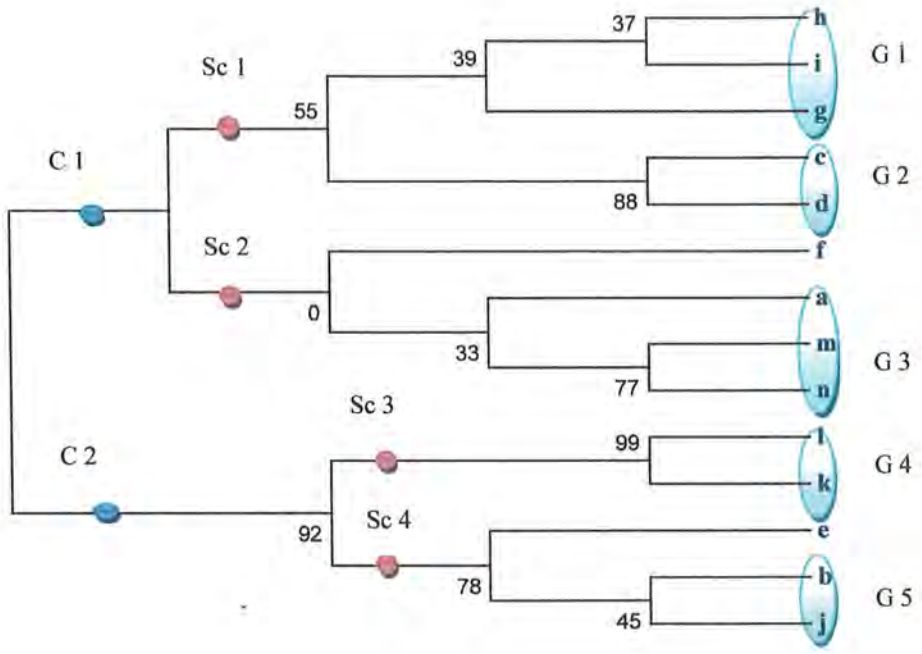

Figure 4: Phylogram by NJ method for fourteen proteinase inhibitor genes from the family Solanaceae indicating the genetic relationship among the PI genes of different Solanaceae members.

C: Cluster Sc: Sub cluster G: Group

a: AY007240 PI-II *S. lycopersicum*, **b:** AB110700 PI-II *S. lycopersicum*, **c:** AY129402 PI-II *L. esculentum*, **d:** L25128 ARPI *L. esculentum*, **e:** M15186 IIK *S. tuberosum*, **f:** U45450 pin2T *S. tuberosum*, **g:** X04118 PI-II *S. tuberosum*, **h:** Z12753 PI-II *S. tuberosum*, **i:** Z13992 PI-II *S. tuberosum*, **j:** Z29537 PI-II *S. tuberosum*, **k:** AF330701 SPLTI-1 *I. batatas*, **l:** AF330702 SPLTI-2 *I. batatas*, **m:** M13938 PI-I *L. esculentum*, **n:** L16450 PI-II *S. tuberosum.*

Table 5: Analyzed data for fourteen plant origin PI genes based on pairwise distance calculation.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. **a**	1.00													
2. **b**	0.74	1.00												
3. **c**	0.76	0.71	1.00											
4. **d**	0.74	0.70	0.66	1.00										
5. **e**	0.69	0.73	0.70	0.69	1.00									
6. **f**	0.74	0.70	0.70	0.73	0.71	1.00								
7. **g**	0.70	0.71	0.68	0.67	0.71	0.69	1.00							
8. **h**	0.74	0.71	0.70	0.68	0.69	0.75	0.70	1.00						
9. **i**	0.72	0.68	0.68	0.74	0.70	0.73	0.67	0.69	1.00					
10. **j**	0.70	0.73	0.74	0.72	0.68	0.75	0.73	0.76	0.71	1.00				
11. **k**	0.76	0.73	0.77	0.71	0.70	0.72	0.71	0.73	0.72	0.75	1.00			
12. **l**	0.74	0.73	0.73	0.73	0.70	0.74	0.74	0.72	0.74	0.72	0.67	1.00		
13. **m**	0.72	0.70	0.69	0.68	0.71	0.71	0.66	0.73	0.72	0.71	0.76	0.75	1.00	
14. **n**	0.72	0.68	0.71	0.67	0.74	0.70	0.74	0.76	0.71	0.70	0.77	0.70	0.66	1.00

a: AY007240 PI-II *S. lycopersicum*, **b:** AB110700 PI-II *S. lycopersicum*, **c:** AY129402 PI-II *L. esculentum*, **d:** L25128 ARPI *L .esculentum*, **e:** M15186 IIK *S. tuberosum*, **f:** U45450 pin2T *S. tuberosum*, **g:** X04118 PI-II *S. tuberosum*, **h:** Z12753 PI-II *S. tuberosum*, **i:** Z13992 PI-II *S. tuberosum*, **j:** Z29537 PI-II *S. tuberosum*, **k:** AF330701 SPLTI-1 *I. batatas*, **l:** AF330702 SPLTI-2 *I. batatas*, **m:** M13938 PI-I *L. esculentum*, **n:** L16450 PI-II *S. tuberosum*.

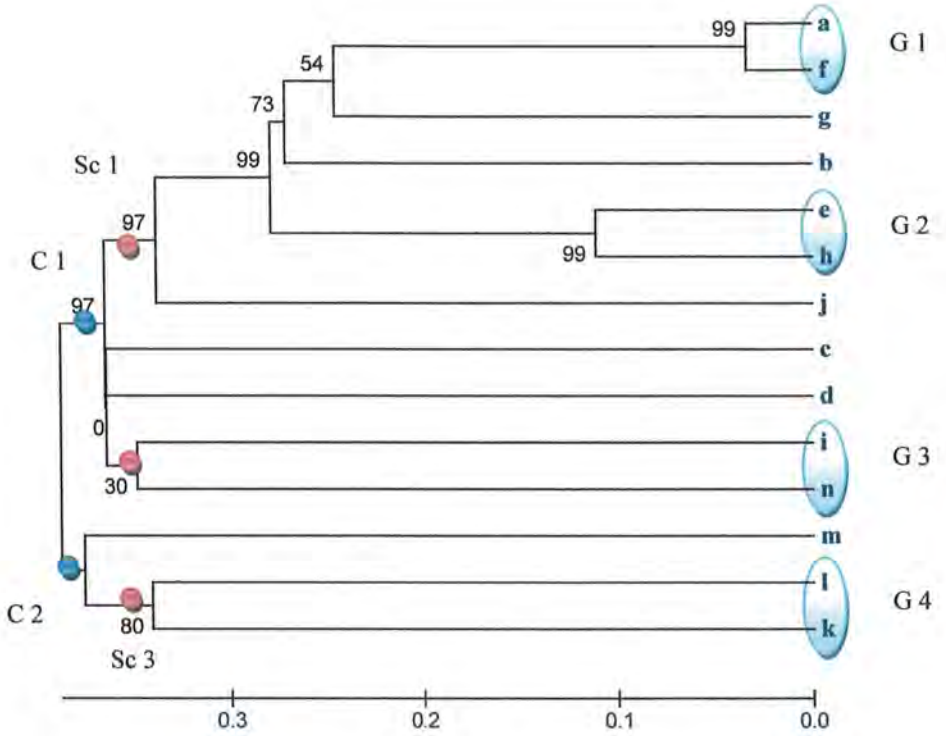

Figure 5: Phylogram tree of the family Solanaceae members based on coding sequences in proteinase inhibitor genes.

C: Cluster Sc: Sub cluster G: Group

a: AY007240 PI-II *S. lycopersicum*, **b:** AB110700 PI-II *S. lycopersicum*, **c:** AY129402 PI-II *L. esculentum*, **d:** L25128 ARPI *L. esculentum*, **e:** M15186 IIK *S. tuberosum*, **f:** U45450 pin2T *S. tuberosum*, **g:** X04118 PI-II *S. tuberosum*, **h:** Z12753 PI-II *S. tuberosum*, **i:** Z13992 PI-II *S. tuberosum*, **j:** Z29537 PI-II *S. tuberosum*, **k:** AF330701 SPLTI-1 *I. batatas*, **l:** AF330702 SPLTI-2 *I. batatas*, **m:** M13938 PI-I *L. esculentum*, **n:** L16450 PI-II *S. tuberosum.*

3.6.3 Phylogram Analysis of Non-Coding Sequences in Fourteen Plant PI Genes Cluster

This Phylogram (Figure 6) had one major cluster 'C' with four main in-groups. Cluster 'C' was subdivided into two sub clusters Sc1 and Sc 2 due to 33 % genetic divergence.

Sub Cluster 1:

It included nine representative gene members AB110700 PI-II *S. lycopersicum* (b), AY129402 PI-II *L. esculentum* (c), U45450 pin2T *S. tuberosum* (f), M13938 PI-I *L. esculentum* (m), AY007240 PI-II *S. lycopersicum* (a), M15186 IIK *S. tuberosum* (e), X04118 PI-II *S. tuberosum* (g), Z12753 PI-II *S. tuberosum* (h) and Z29537 PI-II *S. tuberosum* (j). Three groups G1, G2, G3 were formed under the Sc 1 (Figure 6). Group 1 had two member genes 'b' and 'c' with 99 % genetic similarity. 'f' remained unresolved but it showed 54 % similarity with G 1. Group 2 had three member genes 'a', 'e' and 'g' with 99 % genetic correlation. 'e' and 'g' were most recent in origin as indicated by the branch length in phylogenetic tree (Figure 6). G 1 and G 2 were closely related as phylogram showed 88 % genetic relatedness among them. 'h' and 'j' were claded into G 3 with 85 % similarity level. 'm' remained unresolved but was related to G 3 (85 % similarity). Each split in the phylogram marks the separate evolutionary event.

Sub Cluster 2:

It was characterized by two representatives Z13992 PI-II *S. tuberosum* (i) and L16450 PI-II *S. tuberosum* (n) with 53 % similarity forming the group 4 (G 4).

Outgroup

It comprised only one member L25128 ARPI *L. esculentum* (d) that remained unsolved with high gene distance value (0.33 %). This outgroup shares a common ancestor with the ingroup

On the bases of non-coding sequences the phylogram frequent variations in terms of gene distances and branch lengths the genetic distance rage from 0.09 % to 0.33 %. 'e' and 'g' seemed to be recent in origin. Nei's standard genetic distance measure assumes that genetic differences arise due to mutations and genetic drift (Nei *et al.*, 1973).

Seventeen cysteine PI proteins from different plants were phylogenetically considered by Martinez *et al.* (2005), it was reported in the study that cysteine PI from *Malus domestica* had a close phylogenetic affinity with the functionally analyzed strawberry FaCPI-1 with antifungal properties. The phytocystains have not been reported from any other cultivated fruit crop except from apple (*Malus domestica*).

3.6.4 Phylogram Analysis Based on Sequenced Data of PI gene from Selected Tomato Varieties

Phylogenetic analysis was carried out for the sequenced PI gene data for three tomato varieties (moneymaker, nagina and riogrande) and already sequenced PI-II gene picked from the gene bank (Accession# AY007240) for comparison. The phylogram (Figure 7) illustrated the phylogenetic relationship among the PI gene sequence of already sequenced PI gene 'V1' AY007240 PI-II *S. lycopersicum* var. cerasiforme and three gene sequences resulted after sequencing of PI-II gene of three tomato varieties denoted as 'V2' PI-II *S. lycopersicum* var. money maker, 'V3' PI-II *S. lycopersicum* var. nagina, 'V4' PI-II *S. lycopersicum* var. riogrande. V1, V2, V3 and V4 were genetically related with one another. The in-group (Figure 7) included V2, V3 and V1 (88 % similarity). V4 formed an out-group but it was related with the in-group members. *S. lycopersicum* var. nagina and *S. lycopersicum* var. riogrande appeared to be recent in origin as compared to *S. lycopersicum* var. moneymaker that seemed to have evolved early.

3.6.5 Phylogram Analysis Based on Coding Regions in Sequenced Data of Selected Tomato Varieties

This phylogram (Figure 8) showed that the coding regions of PI gene of all tomato varieties were closely associated. The major group included (E1) AY007240 PI-II *S. lycopersicum* var. cerasiforme (E2) PI-II *S. lycopersicum* var. moneymaker (E3) PI-II *S. lycopersicum* var. nagina (100 % genetic similarity) while (E4) PI-II *S. lycopersicum* var. riogrande was 90 % related to the major group. Phylogenetic analysis demonstrated that the coding region of PI gene in all the tomato varieties remained almost conserved with highest level of similarity observed from phylogram (Figure 8). Thus these PI genes are functionally related with each other having a major role in plant defense against a variety of competing organisms.

3.6.6 Phylogram Analysis Based on Non-Coding Regions in Sequenced Data of Selected Tomato Varieties

This phylogram (Figure 9) showed that the non-coding regions of PI-II gene of two members (N3) PI-II *S. lycopersicum* var. nagina and (N4) PI-II *S. lycopersicum* var. riogrande were closely associated genetically. (N1) AY007240 PI-II *S. lycopersicum* var. cerasiforme and (N2) PI-II *S. lycopersicum* var. moneymaker were also related with each other genetically. The clustering pattern indicates few alterations in the non-coding sequences among the PI genes in the studied tomato varieties (Figure 9).

The selected tomato varieties had the origin from different regions of Pakistan having different environmental conditions in these areas. The small scale genetic divergence reported among the tomato varieties can be due to several reasons. The genetic variations among these varieties may be induced in flax by various environmental stresses. Difference in the structural domain and genomic composition of protease genes have been reported between leguminous and cereal plants due to mutation, internal duplication and environmental influences (Wang *et al.*, 2008).

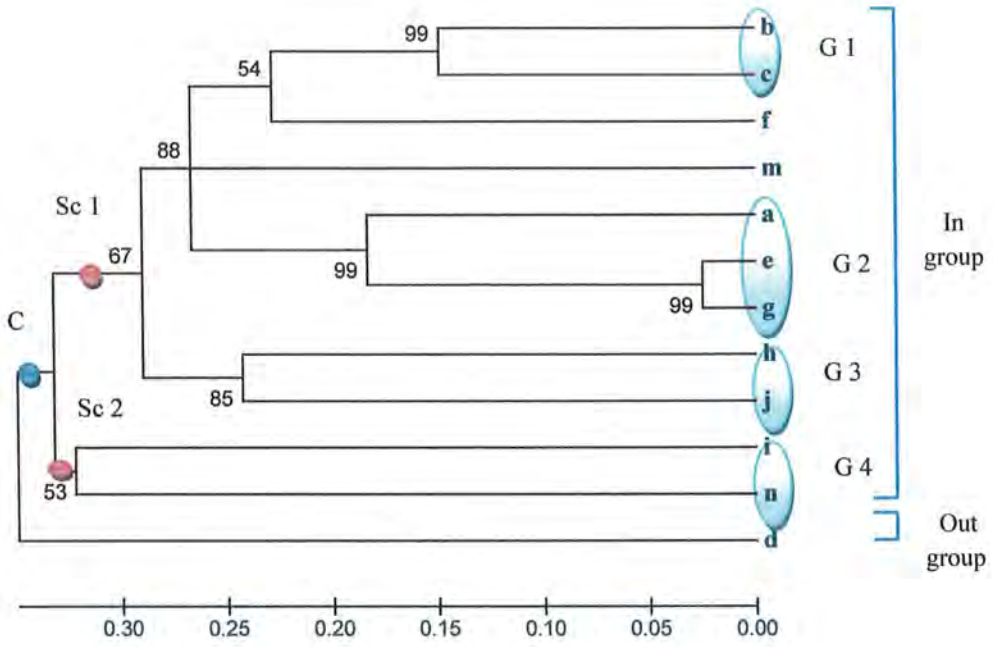

Figure 6: Phylogram by NJ method of non-coding sequences of proteinase inhibitor genes from the members of the family Solanaceae.

C: Cluster Sc: Sub cluster G: Group

a: AY007240 PI-II *S. lycopersicum*, **b:** AB110700 PI-II *S. lycopersicum*, **c:** AY129402 PI-II *L. esculentum*, **d:** L25128 ARPI *L. esculentum*, **e:** M15186 IIK *S. tuberosum*, **f:** U45450 pin2T *S. tuberosum*, **g:** X04118 PI-II *S. tuberosum*, **h:** Z12753 PI-II *S. tuberosum*, **i:** Z13992 PI-II *S. tuberosum*, **j:** Z29537 PI-II *S. tuberosum*, **m:** M13938 PI-I *L. esculentum*, **n:** L16450 PI-II *S. tuberosum.*

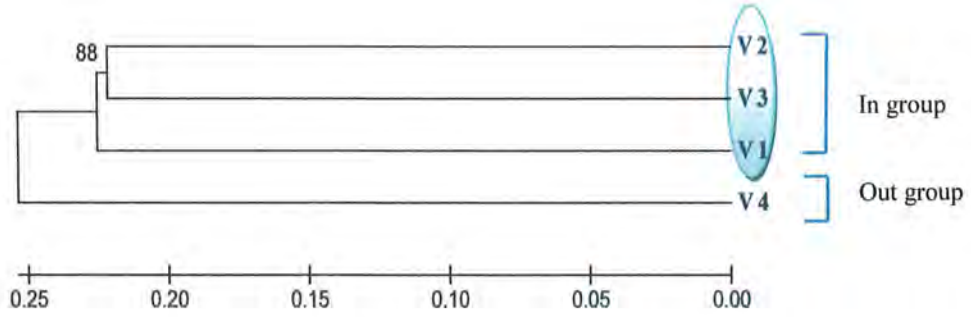

Figure 7: Phylogram by NJ method for PI gene sequences of four tomato varieties.

V1: AY007240 PI-II *S. lycopersicum* var. cerasiforme
V2: PI-II *S. lycopersicum* var. moneymaker
V3: PI-II *S. lycopersicum* var. nagina
V4: PI-II *S. lycopersicum* var. riogrande

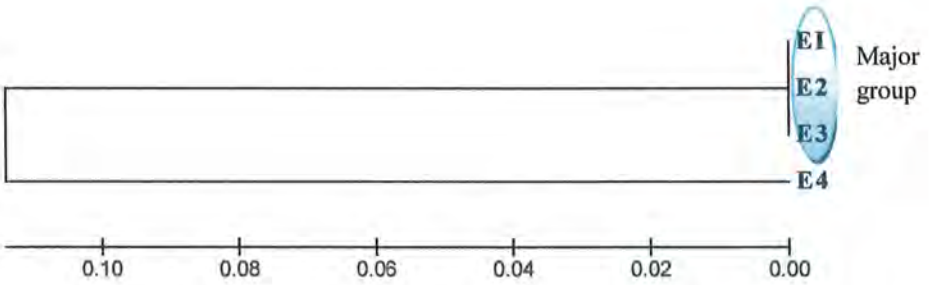

Figure 8: Phylogram by NJ method for coding sequences in PI gene of four tomato varieties.

E1: AY007240 PI-II *S. lycopersicum* var. cerasiforme
E2: PI-II *S. lycopersicum* var. moneymaker
E3: PI-II *S. lycopersicum* var. nagina
E4: PI-II *S. lycopersicum* var. riogrande

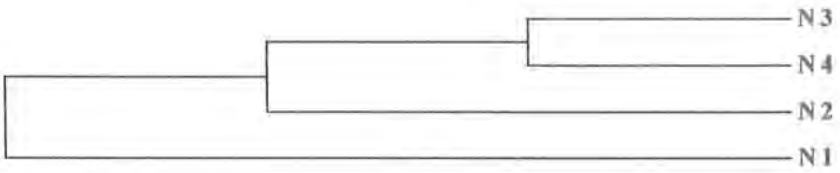

Figure 9: Phylogram by NJ method for non-coding sequences in PI gene of four tomato varieties.

N1: AY007240 PI-II *S. lycopersicum* var. cerasiforme
N2: PI-II *S. lycopersicum* var. moneymaker
N3: PI-II *S. lycopersicum* var. nagina
N4: PI-II *S. lycopersicum* var. riogrande

At a molecular level transposable elements may alter the gene structure and function through insertion, excision and transposition (Bennetzen, 2000). The phylogenetic and evolutionary studies can lead to interesting findings in a gene family as for-example the members of potato type II (pot II) proteinase inhibitor family present a good example to study gene evolution because of interesting molecular phenomenon reported in pot II PI genes as tandem duplication, domain swapping and fold circular permutation (Scanlon *et al.,* 1999; Schirra *et al.,* 2005). The molecular evolution of Bowman-Birk type proteinase inhibitor (BBI) and the structural diversity and organization of various PI genes as for the serine protease inhibitors of Kunitz-type from potato tubers and of Bowman-Birk type from sugarcane have been reported (Ishikawa *et al.,* 1994; Heibges *et al.*, 2003; Mello *et al.*, 2003). Mello *et al.* (2003) conducted phylogenetic analysis of 14 putative BBIs from sugarcane it revealed different evolution patterns for BBI inhibitors in flowering plants as depicted by tree analysis. The genomic sequences of multigene proteinase inhibitor family members from *Glycine soja* and *Glycine max* were phylogenetically analyzed that revealed evolutional proximity between these two leguminae strains (Deshimaru *et al.*, 2004).

3.7 Conclusion

The *in vitro* and *in silico* analysis of PI genes lead to significant findings, serine PIs are vital elements of chemical defense mechanism in tomato plant. Trypsin-type serine PIs are most prevalent in the family *Solanaceae*. Wounding is the major cause of plant PIs gene expression especially through the octadecanoid pathway. The sequencing similarity scores of *in vitro* analyzed PI gene from three tomato varieties *S. lycopersicum* var. riogrande, *S. lycopersicum* var. nagina and *S. lycopersicum* var. moneymaker suggest that the nucleotide order for the PI gene is common, few genetic alterations can be due to various factors as molecular transposition, excision, insertion and environmental influences as drought, cold stress, temperature and light that can lead to disparity in expression level of PI genes. *In silico* phylogenetic and evolutionary analysis conducted through bioinformatics software MEGA3 (Molecular Evolutionary Genetics Analysis) provided evolutionary insight for the sequenced gene from three tomato varieties in terms

of coding and non-coding sequences. Three tomato varieties share common ancestry, *S. lycopersicum* var. moneymaker and *S. lycopersicum* var. nagina appeared to be recent in origin while *S. lycopersicum* var. riogrande seemed to be distantly evolved member among the three studied varieties. In terms of coding regions phylogenetic analysis depicted 100 % genetic relationship between *S. lycopersicum* var. moneymaker and *S. lycopersicum* var. nagina that indicated conservative protein coding sequences, while *S. lycopersicum* var. riogrande showed 10 % genetic divergence from *S. lycopersicum* var. moneymaker and *S. lycopersicum* var. nagina varieties due to some mismatches in the sequenced nucleotide data. These results bring to light the functional similarity of PI gene product from the analyzed tomato varieties.

3.8 Future Perspectives

The sequenced data for proteinase inhibitor gene from three analyzed tomato varieties are to be submitted to the gene bank. The future aims and objectives are (i) to transform this proteinase inhibitor gene under the control of a wound inducible promoter and to develop some commercially important transgenic plants resistant against pathogens and insect attack (ii) functional analysis of the proteinase inhibitor genes at a large scale.

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