Functional Characterization of Tomato Proteinase Inhibitor-II (PI-II) Gene in Transgenic *Nicotiana benthamiana*

Doctor of Philosophy

in

PLANT BIOCHEMISTRY AND MOLECULAR BIOLOGY

BY

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ISLAMABAD, PAKISTAN
2018

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A Dissertation Submitted in the Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in PLANT BIOCHEMISTRY AND MOLECULAR BIOLOGY

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2018

APPROVAL CERTIFICATE

This is to certify that the research work presented in this thesis entitled **"Functional Characterization of Tomato Proteinase Inhibitor-II (PI-II) Gene in Transgenic *Nicotiana benthamiana*."** was conducted by **Ms. Shazia Rehman** under the supervision of **Dr.Tariq Mahmood.** No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Plant Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Field of **Plant Sciences (Plant Biochemistry and Molecular Biology),** Department of Plant Sciences, Quaid-i-azam University Islamabad, Pakistan.

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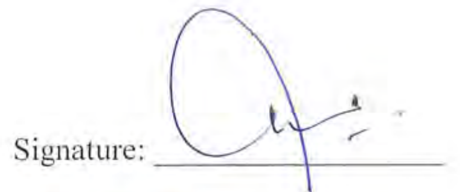
Signature: 

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Dedicated

to

Beloved parents

and sweet brothers and sisters

For their support, love and prayers

Special thanks

To

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For

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LIST OF ABBREVIATIONS

µg	Micro gram
µl	Micro liter
aa	Amino acid
ABA	Abscisic acid
BLAST	Basic local alignment search tool
bp	Base pair
cDNA	Complementary DNA
Chl	Chlorophyll
DNA	Deoxyribo nucleic acid
dNTPs	Deoxyribo nucleoside tri phosphate
EDTA	Ethylene diamine tetra acetic acid
Et	Etylene
FW	Fresh weight
GUS	β-glucuronidase
IGMAP	Interactive Genome Map for Plants
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JA	Jasmonic acid
KTI	Kunitz trypsin inhibitor
Kb	Kilobase
LB	Lauria broth
M	Molar
MCS	Multiple cloning sites
MEGA5	Molecular evolutionary genetic analysis
MeJA	Methyl jasmonic acid
ml	Milli liter

mM	Milli molar
Mol.wt	Molecular weight
mRNA	Messenger RNA
MS	Murashig and Skoog
M-MuLV	Moloney murine leukemia virus
NaCl	Sodium chloride
*Os*RGLP2	*Oryza sativa* root germin-like protein 2
PCR	Polymerase chain reaction
PI-II	Proteinase inhibitor-II
qRT-PCR	Quantative Real-time PCR
SA	Salicylic acid
SBBI	Soyabean Bowman-Brik inhibitor
SBTI	Soyabean trypsin inhibitor
SDS	Sodium dodecyl sulphate
TAE	Tris Acetate ethylene diamine tetra acetic acid
UV	Ultraviolet
WT	Wild type
X-Gal	5-bromo-4-chloro-3-indolyl-D-galactopyranoside
X-Gluc	5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid

ABSTRACT

The plant proteinase inhibitor-II (PI-II) proteins are diverse group of proteins which have been intensively investigated due to their potential role in providing plant protection against different environmental stresses. These genes have been implicated in the regulation of various physiological functions in plants such as modulation of plant growth and development, regulation of endogenous proteases, and mediating defense responses. In the present study, PI-II gene sequences from ten different plants were computationally analyzed using bioinformatics tools. The obtained results identified highly conserved domains in all analyzed sequences. Further, phylogenetic comparisons of PI-II genes representing ten different plants suggested that the high rate of retention of gene duplication and inhibitory domain multiplication may have resulted in the expansion and functional diversification of these proteins.

The induction of PI-II gene is stimulated by various physical and chemical signals like mechanical wounding and by interplay of related phytohormones. Therefore, Agrobactreium-mediated transformation was carried out in tobacco with tomato PI-II gene under the regulation of rice root germin-like protein 2 (OsRGLP2) promoter. T1 transgenic seedlings were used for expression analysis in response to wounding, abscisic acid (ABA), methyl jasmonate (MeJA) and salt stress treatments. From the results of qPCR, it was found that ABA and MeJA dependent signaling pathways are involved in stimulation of PI-II gene. The PI-II induction by ABA and MeJA indirectly indicates its defensive role against environmental biotic and abiotic stresses such as insects, pathogen, drought and salt stresses. Transgenic seedlings subjected to salt stress condition showed significant PI-II gene activity under OsRGLP2 promoter in transgenic lines. Phenotypic analysis revealed that transgenic plants had comparatively higher level of chlorophyll content, proline content and average fresh weight than wild type plants under salt stress. Moreover, relatively high GUS expression was detected in the vascular regions of leaves, stem and roots after treatment with ABA, MeJA, wounding and salt stresses. The findings of present research are useful for enlightening the role of OsRGLP2 driven PI-II gene expression to improve stress tolerance in transgenic crops.

INTRODUCTION

Proteases (also known as proteolytic enzymes or peptidases) are degradative enzymes which catalyze the hydrolysis of peptide bonds. In plants, peptidases participate in numerous physiological processes, including seed germination, photomorphogenesis, embryogenesis, flower development, programmed cell death, and hormone signaling (Fan and Wu, 2005; van der Hoorn and Kamoun, 2008). In relation with biotic and abiotic stresses, they play an important in plant defense and acclimation to changing environmental cues (van der Hoorn and Kamoun, 2008; Kidrič et al., 2014). However, uncontrolled protein degradation that is often induced by both biotic and abiotic stresses may be potentially damaging to the plant cell. Therefore, the activity of proteases has to be tightly regulated at transcriptional and translational level. The activity of proteases can be controlled by various mechanisms. The inhibitory proteins called proteinase inhibitors have been recognized as essential in modulating their activities (Habib and Fazili, 2007).

Plant proteinase inhibitors (PIs) constitute a large and complex group of proteins participating in various biotic and abiotic stress responses (Ryan, 1990; Kim et al., 2009; Srinivasan et al., 2009). They inhibit the proteolytic activity of their target proteases by forming stable inhibitor complex. They are ubiquitously present in all organisms, including microorganisms, animals and plants (Valueva and Mosolov, 2004). Plant PIs are diverse in number and in specificity toward various proteolytic enzymes. They have been extensively studied and are widely distributed among the different botanical families (e.g. Fabaceae, Solanaceae, Cucurbitaceae). Certain storage organs, such as seeds from the Leguminosae family and tubers from the Solanaceae family, are excellent sources of PIs. In plants, they are mainly synthesized in response to various stress conditions, e.g. wounding, insect, pathogens, and abiotic stresses (Hartl et al., 2010; Kidrič et al., 2014). In addition, the expression of PIs was found to vary according to the maturation stage and tissue location (Bhattacharya et al., 2007, 2009). Modern biotechnological techniques have made it possible to manipulate many defense related proteins in plants through genetic transformation. For the last few years, PIs of plant origin has been mainly focused due to their potential application in plant biotechnology for developing transgenic crops tolerant to multiple stresses.

1.1 Characteristics of plant PIs

The majority of PIs appear as proteins with a molecular mass range of typically 8-20 kDa, although proteins of 85 kDa have also been reported (Hung et al., 2003; Fan and Wu, 2005). Their general characteristics involve the presence of a high number of cysteine residues that readily form disulfide bonds. The presence of disulfide bridges seems to be essential for their activities and this makes them more resistant to extreme pHs, high temperature and proteolysis (Fan and Wu, 2005; Chye et al., 2006; Macedo and Freire, 2011). Most of plant PIs isolated from plants are active at temperatures up to 50 °C (Hamato et al., 1995). Hung et al. (2003) isolated a thermo stable trypsin inhibitor (BCTI) from seed of *Brassica campestris* with a molecular weight of 8 kDa. This stability of inhibitor at high temperature was attributed due to the presence of disulfide bridges. Proteinase inhibitor II (PI-II), a dimeric protein having a molecular weight of 21,000 Da is also a thermo stable protein (Bryant et al., 1976) containing two reactive site domains for inhibition of trypsin and chymotrypsin. An interesting feature of PI-II inhibitors is that chymotrypsin reactive site is very sensitive towards mutations, while trypsin/chymotrypsin reactive site is quite robust against mutations (Beekwilder et al., 2000).

Localization studies revealed the presence of PIs in various compartments of the plant cell. For example, a mungbean trypsin inhibitor has been localized to cytosol of cotyledonary cells (Chrispeels and Baumgartner, 1978). The soyabean trypsin inhibitor (SBTI) was found to be associated with the cell wall, cytoplasm, and nuclei of embryonic cell and cotyledonary cells. Soyabean Bowman-Brik inhibitor (SBBI) was reported to be present in nuclei, protein bodies, cytoplasm, in the intercellular space, but not in the cell wall (Horisberger and Tacchini-Vonlanthen, 1983).The wound inducible inhibitors were found to accumulate in the vacuole of tomatoes and in the leaves of potato (Holländer-Czytko et al., 1985; Wingate et al., 1991). In a study conducted by Xu et al. (2004), it was found that PIN2 protein from *Solanum americanum* was highly expressed in the phloem of stem, root and leaves, signifying the possible endogenous role of PIN2 proteins in phloem. Later Sin and Chye (2004), showed that both SaPIN2a and SaPIN2b are also localized in young floral buds and mature floral tissues. Similarly, Kunitz trypsin

inhibitor (KTI) from chickpea is located in the cell wall of different organs (Hernández-Nistal et al., 2009).

1.2 Mechanism of inhibition

Inhibitor proteins have been studied intensively for elucidating the mechanism of PIs action, as well as for studying the protein-protein association (Barrett et al., 1986; Macphalen and James, 1987; Greenblat et al., 1989). Various mechanisms have been proposed for inhibition of proteases by PIs. Proteinase inhibitors adopt different structures ranging from small molecule to macromolecular structures. PI recognizes different surfaces in the active site of target enzymes and blocks it by its exposed structural elements, like loops or protein termini. Some PI directly utilizes the mechanism of protease action to achieve inhibition. Most of protease inhibitors were shown to block the enzyme's active site by substrate like interaction in a canonical manner with active sites (Bode and Huber, 1992, 2000). These inhibitors include the Kazal, Kunitz, and Bowman-Birk family. In another mechanism (non-canonical mechanism), inhibition is achieved by interacting with the enzyme through N-terminus region of inhibitor which is disordered in solution and rearranges upon binding in the active site of an enzyme (Szyperski et al.,1992). A typical example of such mechanism is the inhibition of papain-like cysteine protease by the cystatins (Bode and Huber, 2000; Turk et al., 2000).

1.3 Proteinase inhibitor-II family

Soyabean trypsin inhibitor (SBTI) was the first well characterized plant PI that was isolated by Kuniz et al. (1942). Subsequently many PIs were identified from different families of plants including Solanaceae, Leguminosae and Gramineae (Brzin and Kidric, 1995; Jamal et al., 2013; Rehman et al., 2017). PIs are predominant in storage tissues such as seeds and tubers (up to 10 % of total protein content). So far, 48 families of plant PIs have been assigned on the basis of amino acid sequences homologies (Rawlings et al., 2012) which were further subdivided into four classes (serine-, cysteine-, aspartyl- and metallo-PIs) (Rawlings et al., 2004, 2010). Among plants PIs, serine PIs are the most studied and largest group and has been further sub divided into different classes

including Kunitz, cereal super family, Ragi A1, squash, Bowman–Birk, potato II, barley, and Thaumatin-like inhibitors (Haq et al., 2004).

PI-II proteins, classified as I20 in MEROPS database (Rawling et al., 2008), and defined as a dimeric protein with a molecular weight of ~ 21,000 Da (Tamhane et al., 2012; Rehman et al., 2017). From the survey of genomic databases and Expressed sequenced tags (ESTs) analysis, many PI-II homologous has been identified in both monocots and dicots (Barta et al., 2002). Initially members of PI-II were characterized from potato tubers (Christeller and Liang, 2005), these inhibitors have been found in wounded leaves, flowers, fruits, seed, and phloem of other Solanaceous species (Plunkett et al., 1982; Pearce et al., 1993). Atkinson et al. (1993) identified a similar homolog of this family (NaPI-II) having a low molecular mass from *Nicotiana alata* flowers (Atkinson at al., 1993). Similarly, Pearce at al. (1993) isolated six small wound-inducible PIs of this family from tobacco leaves.

1.4 Gene structure of *PI-II*

The structural studies highlighted the highly conserved structure of PI-II family genes in Solanaceous plants consisting of two exons separated by a 100-200bp intron. The one exon encodes a part of signal peptide (N-terminal part), while the other encodes the remaining part of signal peptide (C-terminus region) and the inhibitory repeat domain (Barta et al. 2002). The study of Kong and Ranganathan (2008) revealed that PI-II family also exhibits highly a conserved splicing motif and was found to be "GT……AG". This analysis also revealed that the last nucleotide of exon1 and first two nucleotide of exon 2 always encode a Gly (Glycine residue). Moreover, the conserved PI-II proteins are also characterized by the presence of a signal peptide of an endoplasmic reticulum having length of 25 aa (amino acid) followed by variable number of IRDs of 55 aa.

An analysis of these inhibitors and genes has shown that they are composed of multiple repeat units (RUs) varying between one and eight (Choi et al., 2000; Rehman et al. 2017). Each RU is composed of eight conserved cysteine polypeptides, which includes a reactive site targeting serine proteinases (Kong and Ranganthan, 2008). These domains are further characterized by being highly homologous to each other, might be due gene-duplicated elongation events (Barta et al., 2002). Inhibitors in this family have been

reported to inhibit trypsin, chymotrypsin, oryzin, subtilisin, elastase and pronase E (Antcheva et al., 1996).

Furthermore, the PI-II domains has been divided into two parts, named H and L fragment (for heavy and light fragments), that are joined by two types of linkers (Linker 1 and 2). Based on the arrangements of these structural units, each domain can be classified as H-L type (H-and L-fragment joined by Linker-1); L-H type (L-and H-fragment joined by Linker-2); H+L type (L-and H-fragment directly joined). Moreover, it was suggested that these linker sequences play an important role in stabilizing the cross repeat folding pattern of multi domain proteins (Kong and Rangathan, 2008).

1.5 Regulation and expression of *PI-II* genes

Plants have sophisticated mechanisms to protect themselves from changing environmental factors, which adversely influence their growth, survival rate and reproduction. These include both abiotic and biotic factors. These stresses in general occur in multitude and plant induces different pathways under the control of various stress hormones to survive unfavorable situations. Phytohormones like Jasmonic acid (JA), salicylic acid (SA), ethylene (ET) and abscisic acid (ABA) are induced during adaptive responses to abiotic and biotic stresses (Anderson et al. 2004). The inhibitory proteins such as PI-I and PI-II are particularly known to be induced by injuries such as insect damages or other mechanical damages. In tomato, it has been shown that proteinase inhibitor initiation factors (PIIF) triggered by wounding or injury switch on cascade of events that ultimately lead to the synthesis of PIs in plants (Bhattarcharjee et al., 2012; Rehman et al., 2017).

Wounding of tomato or potato leaves result not only in local accumulation of these two inhibitors, but also in systemic response throughout the plant in which unwounded leaves also produced inhibitors (Ryan, 1992). PI genes in plants can be activated by the oligosaccharide fragments and by an 18-aminoacid polypeptide called systemin. The oligosaccharides are relatively immobile in plants and involved in localized signaling only (Ryan, 1992, Doares et al., 1995). However, systemin is mobile and can induce the systemic wound responses (reviewed in Sun et al., 2011). The activation of PI genes by wounding, oligosaccharides and systemin has been proposed to occur via lipid-derived pathway known as octadecanoid (OD) pathway in which linoleic

acid is released in response to signals and ultimately converted to JA to induce the transcriptional activation of defensive genes (Habib and Fazili, 2007; Larrieu and Vernoux, 2016).

The Systemin signaling pathway is initiated upon binding of systemin with a cell surface receptor called SR160, a 160-kDal transmembrane protein. This binding of systemin to cell surface is associated with several rapid signaling events that include triggering of mitogen-activated protein kinase (MAPK) and a phospholipase, opening of ion channels and increase in intracellular Ca^{2+} concentration. As a result, linoleic acid is liberated from the plasma membrane and production of JA is started through octadecanoid pathway (reviewed in Bhattarcharjee et al., 2012; Rehman et al., 2017).

The critical role of ABA and JA in production of inhibitors has been convincingly demonstrated in many studies (Peña-Cortés et al., 1995; Rehman et al. 2017). These plants were shown to accumulate proteinase inhibitors when leaves are wounded. The results of Herde et al. (1996) and Falco et al. (2001) showed that endogenous ABA level increases in response to wounding, electric current application and to local burning, which in turn can mediate the PI gene expression through OD pathway (Peña-Cortés et al., 1995). SA has been proposed to inhibit the proteinase inhibitor synthesis induced by JA in tomato (Doares et al., 1995). Similarly, phytohoromone auxin prevents the wound-induce Pin 2 gene expression (Kernan and Thornburg, 1989).

Like wounding, the electric current applicantion can elevate the ABA and JA levels in plant and subsequently PI-II gene expression (Herdi et al., 1996; Bhattarcharjee et al., 2012). Widon et al. (1992) reported that wounding of young tomato cotyledons resulted in the transmission of an electric potential out of cotyledone and throughout the plant. In another report, it was observed that application of electric current has triggered the PI-2 gene expression in pepper (Kim et al., 2001).

1.6 Endogenous role

There are some evidences that plant PIs can play key role in in several important endogenous processes in cell that include, cell proteolysis by regulating protease activities, programmed cell death and macromolecular trafficking (Fan et al., 2005; Tamhane et al. 2012). Many seeds and seedlings contain proteinase inhibitors which in certain cases might control the onset of proteolytic activity during germination (Shewry,

2003). The expression analysis of PI-II (PIN 2) from nightshade plants from phloem tissue of stem, roots and leaves suggesting its novel endogenous functions (Xu et al. 2001; Sin and Chye, 2004).

Sin et al. (2006) exploited RNAi-based gene silencing strategy to show that SaPIN2a and SaPIN2b are important for seed development and 80 % of total seeds are aborted with the silencing of both genes. Contrary to these observations for *S. american*, Harlt et al. (2010) detected only 0.7 to 2.8 % seed obortion after silencing SPI2a and SPI2b gene of *Solanum nigrum*. Liu etal. (2006) have shown that SaPIN2b is also constitutively expressed in glandular trichome and could play vital role in trichome based defense and glandular trichome development. Furthermore, tobacco plants overexpressed with SaPIN2b showed increased glandular trichome density and branching.

From some studies, it was found that silencing or overexpression of *PI* genes has resulted in impaired growth and leaf development. For example, transformants with silenced-NaTPI gene showed earlier flowering, had faster growth rate, and produced more seed capsule when compared with TPI producing genotypes (Zavala et al. 2004). Similarly, plants over-expressed with SaPIN2a gene showed significant lower height than the WT plants (Xie et al. 2007). In another study, Bezzi et al. (2010) observed that silencing of TPI expression in *N. attenuata* resulted in higher levels of nectar and H_2O_2 levels which in turn reduce nectar removal by native insect flower visitor. This suggested the positive role of PIs in processing and secretion of nectar proteins.

1.7 Tolerance against multiple stresses

Plant PIs have been suggested to play significant role in plant responses to phytopathogenic microbes, insects and fungal attack and also to certain abiotic stresses (Tamhane et al. 2009; Li et al. 2015; Rehman et al. 2017).

1.7.1 Protection against insect

Several plant PIs have been implicated in transgenic research for their defensive role against the wide range of insects including lepidopterans, coleopterans and dipterans. These inhibitors act on the digestive enzymes of insects by forming stable complexes to inhibit the enzymatic reactions, resulting in critical shortage of essential amino acids vital for their growth and development (Lawrence and Koundal, 2002; War et al., 2012). The defensive functions of many PIs against insects in transgenic plants have been well

demonstrated in several studies. The insecticidal potential of PIs was first investigated by Hilder et al. (1987) who successfully generated transgenic plants by transferring cowpea serine PIs (*CpTI*) in tobacco for improving the resistance against *Heliothis virescens*. Subsequently, genes encoding inhibitory proteins from different species have been introduced into many important crops by genetic engineering. For instance, the inhibitory effect of PI-II genes derived from potato and tomato against *Manduca sexta* on defences was evaluated by Johnson et al. (1990). Similarly, Tamhane at al. (2007) reported that CaPI-7 gene from *Capsicum annum* suppress the growth and development of *Helicoverpa armigera*. In another report, resistance to *H. armigera* and *Spodoptera litura* was enhanced in transgenic plants expressing SaPIN2 (Luo et al., 2009). Transgenic Chinese cabbage with potato PI-II gene showed resistance against multiple herbivore insects (Majeed et al., 2011; Zhang et al., 2012).

Furthermore, development of recombinant PIs with strong inhibitory effects against specific herbivores has clearly confirmed the potential of these proteins to prevent crop damage caused by insects. For example, in some reports it was observed that combination of inhibitory proteins along with a sublethal dose of Bt toxin have more marked effect on the growth and development of insects (Zhu et al., 2007). Abdeen at al. (2005) reported increased resistance against *Heliothis obsolete* and *Liriomyza trifolii* by combined expression of potato PI-II and carboxypeptidase inhbitors (PCI) in transgenic plants. Likewise, Dunse et al. (2010) studied the molecular basis of resistance against *Helicoverpa* larvae using combination of inhibitors from *Solanum tuberosum* (*Stpi1A*) and *Nicotiana alata* (*NaPI*).

1.7.2 Fungal and bacterial resistance

PIs derived from plants are also helpful in suppressing the fungal diseases by inhibiting the proteases that display important roles in metabolic and infection processes (Paiva et al., 2013; Rehman et al., 2017). The first indirect evidence for the involvement of plant PIs in protecting the plants against the fungal infections came from a study conducted by Mosolov et al. (1976) in which they demonstrated that inhibitors of trypsin and chymotrypsin are capable of suppressing the proteases secreted by *Fusarium solani*. Similarly, reduced protease activity of proteases from *Alternaria* and *Fusarium* species was observed with PIs derived from barley and buckweed seeds (Pekkarinen et al., 2007;

Dunaevsky et al., 1997, 1998). PIs of faba bean were shown to have significant antifungal activity against variety of fungal strains (*Vicia faba*) (Ye et al., 2001). Kim et al. (2005) showed inhibitory effect of potato PIs that provide protection against *Candida albicans* and *Rhizoctonia solani*. An increased proteinase inhibitory activity in potato and tomato plants infected with *Phytophthora infestans* was noted by Valueva et al. (2003). In a related study, Charity et al. (2005) enhanced resistance against *Pseudomonas solanacearum* (bacterial pathogen) and *B. cinerea* (fungal pathogen) by over-expressing NaPI gene in transgenic plants. Vanjildorj et al., 2009 demonstrated antibacterial activity against bacterial soft rot disease by developing transgenic Chinese cabbage line harboring AHL-lactonase gene from Bacillus sp. GH02 fused with PinII signal peptide from potato. Majeed et al. (2011) reported that PI-II from potato also elicits antifungal and insecticidal effects against *Rhizoctonia solani* and *Helicoverpa armigera* respectively.

1.7.3 Resistance against nematode and viruses

The potential of proteinase inhibitors in conferring the nematode resistance has been demonstrated by various groups (Atkinson et al., 2003; Cai et al., 2003). Trypsin inhibitors from cow pea (CpTI) control a very broad spectrum of nematods like, *Globodera pallida*, *G. tabaccum* and *Meloidogyne incognita* (Williamson and Hussey, 1996). Genes encoding cysteine proteinase inhibitors have been cloned and analysed for their effectiveness in controlling the nematode attack in several crops like tomato (Urwin et al., 1995), rice (Vain et al., 1998) *Arabidopsis thaliana* (Urwin et al., 2000), potato in fields (Urwin et al., 2001, 2003; Lilley et al., 2004), banana (Atkinson et al., 2004), and plantain (Roderick et al., 2012). Similarly resistance to cereal cyst nematode (*Heterodera avenae*) infestation was conferred in transgenic wheat expressing the potato inhibitor (Pin 2) (Vishnudasan et al., 2005). Resistance against the two potyviruses, tobacco etch virus (TEV) and potato virus Y (PVY) was enhanced after expressing the rice cysteine PI in transgenic tobacco plants (Gutierrez-Campos et al., 1999). Expression of cysteine proteinase inhibitors from *Celosia cristata* suppressed TMV-induced hypersensitive cell death in *Nicotiana glutinosa* (Gholizadeh et al., 2005). Recently, a serine proteinase inhibitor (*CmSPI*) from *Cucumis metuliferus* (horned melon) was introduced in both *C. metuliferus* and *N. benthamian* to confer the resistance against Potyvirus (Potato virus Y) (Lin et al., 2015).

1.7.4 Role of PIs under abiotic stresses

There has been large number of evidences that supports the potential effect of plant proteinase inhibitors in transgenic approaches for the improvement of crop resistant against abiotic stresses (Benchaban et al., 2010; Li et al., 2015; Rehman et al., 2017). Numerous independent studies have revealed that expression of plant PIs was increased in response to multiple abiotic factors (Fujita et al., 2006; Rehman et al., 2017). Abiotic stresses can execute its influences on the activities proteases and their inhibitors at different levels. Conconi et al., (1996) reported that PIs are induced in response to UV-C radiations and subsequent studies were made under drought, high salinity and at high or low temperature (Dombrowski et al., 2003; Fujita et al., 2004; Huang et al., 2007). These studies suggest that they have a role in modulating the abiotic stress tolerance. Induction of cystatin expression in barley, chestnuts and rice OCPI (*Oryza sativa* chymotrypsin inhibitor like -1) by cold, salt and drought stresses has been reported, as well as by exogenous ABA treatment (Huang et al., 2007). Salt and drought stresses have been shown to result in accumulation of PI containing Kunitz motif in radish and Arabidopsis respectively (Lopez et al., 1994; Gosti et al., 1995). The expression of PI-II gene (CaPI-2) from red pepper was reported to be also effected by high salt stress (Kim et al., 2001). Dombrowski (2003) showed that tomato serine PI-II and other wound-inducible genes also responded to salt stress. Similarly, Srinivasan et al. (2009) noted that expression of trypsin PI gene (NtPI) was strongly induced in tobacco by multiple abiotic factors including high salt, pH and drought. Heat shock tolerance was enhanced in transgenic Arabidopsis overexpressing the phytocytatine gene (Je et al., 2014). Recently, over expression of another chymotrypsin inhibitor gene (OCPI2) from rice was found to enhance the salt tolerance in transgenic Arabidopsis (Tiwari et al., 2015).

In addition, some plant PIs have been exploited for enhancing heavy metal tolerance and phytoremediation of contaminated soil. For example, Shitan et al. (2007) observed that BBIs in yeast are involved in cadmium toletance, while expression of wheat BBIs was found to increase in wounded leaves and aluminium stress (Snowden et al., 1995). Furthermore Liu et al. (2006) demonstrated that *Sa*PIN2b on the basis of trichome-based defense can be used as potential candidate for the removal of heavy metal

and molecular farming. However, PI genes exact role and mechanisms in conferring heavy metal tolerance are yet to be defined from other plants.

1.8 Medical significance

In the last few years, the interest in plant PIs have increased due to their potential use in medicines and biotechnology. A great deal of work has been done in this context to find the possible candidates of PIs having therapeutic importance from plant. The investigation to search for plant PIs to combat several clinical disorders like allergy, inflammatory disorders, started in early 1950's (Vogel et al., 1968). A number of PIs that regulate human physiological processess have been exploited to treat human diseases like cancer, pancreatitis, emphysema, thrombosis, AIDS, hepatitis, muscular dystrophy, inflammatory and allergic disorders (Scarpi et al., 2004; Rachel and Sirisha, 2014). Seeds like rice, maize and beans all are known to contain high level of PIs (Birk, 1974, 1975). Reports have indicated that risk of breast, colon and prostate cancer rates can be reduced with higher intake of seeds (Correa, 1981). Similar reports of decreased rate of colorectal and breast cancers formation among individuals with higher intake of PIs from plant sources (Blondell, 1988) are also available.

Although soybean and soybean products are rich in several anti-carcinogenic natural products like isoflavonoids, saponins and several other polyphenolic compounds (Messina and Barnes, 1999), but the main component is the trypsin and chymotrypsin inhibitor (Bowman-Birk inhibitor) that are more effective than the other anticancer compounds (Kennedy, 1995, 1998). Bowman-Birk inhibitor from soybean has been reported to inhibit the growth of human colorectal adenocarcinoma HT29 cells *in vitro*, suggesting a possible application of this protein as anti-carcinogenic drugs (Clemente et al., 2005), while the BBI isolated from chickpea prevents the malignant transformation of cells (Yavelow et al., 1985). A member of Bowman-Birk PI family, the Black-Eyed Pea Trypsin/Chymotrypsin Inhibitor (BTCI) derived from *Vigna unguiculata* seeds has been reported to induce lysosome membrane permeabilization and apoptosis in human breast cancer cell MCF7 (Joanitti et al., 2010). Another reported cereal BBI has been shown to induce apoptosis in human colon cancer cells (Caccialupi et al., 2010). In addition to these, the potato PIs, especially PI- 1 and PI-2 (Huang et al., 1997; Liu et al., 2001) have been shown to be potent anti-carcinogenic agents.

Similarly, PIs having activities against HIV were also identified. These inhibitors work by blocking a protease in HIV. For example, a Bowman-Birk TI isolated from the seeds of Hokkaido large black soybeans (Ho and Ng, 2008) and a TI from *Clausena lansium* (Lour) Skeels (Ng et al., 2003) inhibited HIV-1 reverse transcriptase activity. The similar HIV-1 reverse transcriptase inhibitory activities were also found in *B. variegates* inhibitor (Fang et al. 2010) and black soybean inhibitor (Ye and Ng, 2009). Trypsin inhibitor isolated from *Peltophorum dubium* seeds and soybean kunitz type trypsin inhibitors have been reported to have anti-proliferative effect on human leukemia cells (JURKAT) and induce apoptosis in the particular cells (Troncoso et al., 2007). These advantages made PIs an ideal choice in medical field for development of drugs to treat number of human diseases.

1.9 Objectives

The overall aim of the present study was to analyze the expression of a tomato proteinase inhibitor-II gene under the control of a wound-inducible promoter in transgenic *Nicotiana benthamiana.* To achieve this, the following objectives were set up;

- To characterize the *PI-II* gene sequences from selected plants using various bio-computational tools.
- To evaluate the *PI-II* gene induction under the control of wound-inducible *Os*RGLP2 promoter in response to signaling molecules such as ABA and MeJA in transgenic plants.
- To investigate the *PI-II* gene activity in transgenic plants under salt stress.
- To analyze the *GUS* expression of transgenic plants in response to wounding, ABA, MeJA and salt stresses.

Material and Methods

The material and methods section was comprised of two parts;

- Computational analysis
- Functional analysis

2.1 Computational analysis

2.1.1 Sequence analysis

Amino acid sequences of *PI-II* genes for *Arabidopsis thaliana, Brachypodium distachyon, Brassica rapa, Solanum lycopersicum, Oryza sativa, Vitis vinifera, Solanum tuberosum, Zea mays, Sorghum bilocolor*, and *Populus tricocarpa,* were retrieved from the Plant Ensemble (http://plants.ensembl.org/index.html) and Plaza 3.0 (http://bioinformatics.psb.ugent.be/plaza/). Alignment of all protein sequences was done using Clustal W (Thompson et al., 1994) program. Conserved motif analysis was performed using Multiple Em for Motif Elicitation (MEME) server (http://meme.nbcr.net/meme/cgi-bin/meme.cgi) (Bailey et al., 2015). Subcellular localization of *PI-II* genes was predicted using CELLO server v.2.5 (Yu et al., 2006) and WoLF PSORT (Horton et al., 2007).

2.1.2 Prediction of chromosomal locations of *PI-II* genes

IGMAP (Interactive Genome Map for Plants) database was used to determine the location of PI-*II* genes on chromosomes of selected plants. IGMAP is a novel toolbox dedicated to plant gene identification, mapping and clustering (Priya et al., 2015).

2.1.3 Phylogenetic analysis

The Molecular Evolutionary Genetic Analysis (MEGA 6) software (Tamura et al., 2013) was used to evaluate the phylogenetic relationship among deduced amino acid sequences using an NJ (Neighbor-joining) phylogram with 1000 replicates.

2.2 Functional analysis

For functional analysis following methodology was used.

2.2.1 Selection of plant material

The green house grown tobacco species *Nicotiana benthamiana* was arranged from Copenhagen University Denmark which was later used as experimental material for *Agrobacterium*-mediated transformation.

2.2.2 Transformation

The modified pCAMBIA1391Z vector having *Os*RGLP2 promoter (Mahmood et al., 2013) was used to ligate or insert the *Solanum lycopersicum PI-II* gene (Accession #: AY007240) downstream to *Os*RGLP2 promoter, which was further used for tobacco transformation. This part was divided into following steps

- Expression vector designing
- *Agrobacterium*-mediated transformation of plant
- Expression analysis under various stresses

2.2.2.1 Expression vector designing

The full length 684-bp *PI-II* gene was amplified from tomato and ligated directly into T/A cloning vector (pTZ57R/T) according to the manufacturer instructions (MBI, Fermentas). The vector harbors ampicillin resistant gene and *lac*Z complementary system for screening by X-Gal and IPTG. For ligation, 30 μl of ligation mixture containing PCR product (4 μl), 10X ligation buffer (3 μl), PEG 4000 (3 μl), vector (3 μl) and T4 DNA ligase (1 μl) was prepared and incubated at 22 °C for overnight.

2.2.2.1.1 Preparation of electro competent cells of DH5α

For preparation of electrocompetent cells, starter culture of DH5α (an *E.coli* strain) was prepared by inoculating a single colony of DH5α in 3 ml of liquid Lauria Broth (LB) media (Appendix-1) in a test tube following the incubation at 37 °C with overnight constant shaking at 250 rpm. The culture was poured in 1 L LB media and again incubated at 37 °C with 250 rpm shaking until the O.D reached at 0.4-0.5. The bacterial cells were pelleted by centrifugation at 4000 rpm for 15 minutes at 4 °C (Centurion Scientific, K3 Series) in 50 ml falcon tubes. The resultant pellet was washed with ice cold 15 % glycerol and again centrifuged (4000 rpm for 15 minutes) with subsequent resuspension of pellet in ice cold 15 % glycerol. This step of washing was repeated at least three times. After final washing, the glycerol was decanted and pellet was resuspended in residual 15 % glycerol. The suspension cells were aliquoted in to 50-100 μl in pre-chilled eppendorf tubes and were immediately frozen at -80 °C for further use. To check the viability of cells, 10 and 100 μl of competent cells were spread on solid LB plates following the incubation at 37 °C for 16 hours.

2.2.2.1.2 Electroporation

For electroporation, 50 µl of electrocompetent cells (DH5α) along with 2 µl ligated mixture were taken in a cold cuvette. The electroporation was done at specific voltage (2.5 KV/0.2 cm) in Porator (Bio-Rad Cell Porator). The electroporated cells were immediately resuspended in 1 ml liquid LB media in eppendorf tubes following its incubation at 37 °C with continuous shaking at 250 rpm for 1 hour. The transformation mixture was spread on LB agar plates with XGal/IPTG and ampicillin (50 mg/L) and incubated at 37 °C for overnight. White and blue colonies were observed after 16-20 hours incubation.

2.2.2.1.3 Plasmid isolation and confirmation

For plasmid isolation, a single colony was picked up after 16-20 hours incubation, cultured in test tubes by adding 3 ml liquid LB media along with ampicillin (50 mg/L) and shaked at 250 rpm for 12-16 hours at 37 °C. Plasmid was isolated from the cultures (Appendix-2) and confirmed by PCR with PI-II primers. PCR bands were later visualized on 1.5 % agarose gel. Glycerol stock of the PCR positive clones were prepared and preserved at -80 °C for long term storage.

2.2.2.1.4 Sequencing of targeted region of *PI-II* gene

The confirmed clone containing targeted region of *P-II* gene was purified and sent to Macrogen (Seoul, Korea) for sequencing with *PI-II* gene specific primers.

PI-II (Forward): 5′ TATCCATCATGGCTGTCCAC **3′**

PI-II (Reverse): 5′ AACACACAACTTGATCCCCACA **3′**

The obtained sequence data was compared with sequences from National Center of Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) to find out the similarity.

2.2.2.1.5 Restriction digestion of plasmid

The *PI-II* gene in T/A cloning vector and p1391Z_*Os*RGLP2 vector were digested with *Eco*R1 restriction enzyme and eluted from low melting point 1 % agarose gel in TAE buffer (Appendix-3) using DNA elution kit (Fermentas). The following reaction setup was used for the plasmid digestion.

1. Nano pure water 15 µL
2. Tango buffer 2 µL

3. Plasmids 1 μL (each)
4. Enzyme (*Eco*R1) 1 μL

The reaction mixture was incubated at 22 °C for overnight and digested products were confirmed by running on 1.5 % agarose gel.

2.2.2.1.6 Ligation of *PI-II* gene in p1391Z_*Os*RGLP2 vector

The recombinant plasmid was constructed by ligating the digested and eluted *PI-II* gene insert and p1391Z_*Os*RGLP2 plasmid. Ligation reaction was carried out by including the digested p1391Z_*Os*RGLP2 plasmid (2 μL), eluted *PI-II* insert (6 μL), 10 X ligation buffer (1 μL) and T4 ligase (1 μL). The ligation mixture was incubated for overnight at 22 °C. The designed construct was named as p1391Z_*Os*RGLP2::PI-II vector.

2.2.2.1.7 Transformation of *Agrobacterium* with recombinant vector

The *Agrobacterium* strain (EHA101) was transformed with ligated recombinant vector (p1391Z_*Os*RGLP2::PI-II) through electroporation. Electrocompetent cells of *Agrobacterium* were prepared following the same procedure as done for DH5α except the incubation temperature was 28 °C and incubation time was 24-48 hours. Transformation mixture (100 μL) was spread on LB kanamycin (50 mg/L) plates and incubated at 28 °C for two days for screening. The transformed colonies were later confirmed by colony PCR and restriction digestion (with *Eco*R1) after isolation of plasmid from the cultures of transformed cells.

2.2.2.2 *Agrobacterium*-mediated transformation of *Nicotiana benthamiana*

The tobacco plants were transformed with *Agrobacterium* by following the protocol developed by Horsch et al. (1985).

2.2.2.2.1 Preparation of leaf discs

The leaves from one month old tobacco plants were cut and surface sterilized with 70 % ethanol for 1 minute and 1.5 % hypoclorite with 2-3 drops of Tween-20 for 15 minutes followed by the three times washing with sterilized water. After drying, small leaf discs were prepared by cutting the leaves into small pieces without the midrib and were placed upside down on MS media (Table 2.1). Nearly 10-15 leaf discs were placed on a single plate, sealed with parafilm and incubated for 48 hours with photoperiod of 16/8 hours.

Table 2.1: MS media composition, used for co-cultivation and rooting.

Sr.No	Components	Concentration
1	MS	4.43 g/L
2	Sucrose	30 g/L
3	Bacto agar	9 g/L

2.2.2.2.2 Infection and co-cultivation

A single colony of recombinant *Agrobacterium* strain EHA101 was picked up and was grown in 25 ml liquid LB media containing 50 mg/L kanamycin by incubating at 28 °C with constant shaking at 250 rpm for 36-48 hours. The *Agrobacterium* suspension was poured in tubes and was centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded and pellet was resuspended in hormone free 5 ml MS liquid media. The suspension was poured in petridishes and sterilized explants on MS media were immediately transferred to petridishes. The petridishes were sealed with parafilm and were placed in growth room at 22 °C with 16 hour light and 8 hour darkness for two days.

2.2.2.2.3 Selection

After co-cultivation, the infected leaf discs were shifted to selection media (Table 2.2) i.e MSH (MS-Hormonal) media having timitin (200 mg/L) and hygromycin (100 mg/L). The pH of the media was adjusted to 5.8. Both timitin and hygromycin were added after autoclaving. Timitin was used to inhibit the Agrobacterial over growth and hygromycin was used to select the transformants. After every 14 days, the leaf discs were subcultured on fresh selection media.

2.2.2.2.4 Regeneration and shooting

Callus started to develop in approximately 15 to 20 days from cut edges of the putative transformed leaf discs. After 14-28 days, a cluster of adventitious shoots emerged which were later shifted to large containers for proper growth having same selection media under same growing conditions.

2.2.2.2.5 Rooting of transformed plants

When shoots become large having two to three internodes, they were cut off from the explant and shifted to large containers having hormone free solid MS media for proper rooting. These plants were then shifted to the soil and maintained in the green house under controlled conditions of 16:8 light/dark cycles at 27 °C. These T_0 plants were then self-pollinated to harvest T_1 transgenic *N. benthamiana* seeds.

2.2.2.2.6 Confirmation of transgenic plants

Transgenic tobacco plants were selected by growing on selection media and later confirmed by PCR and *GUS* assay.

Table 2.2: MSH media used for selection.

Sr.No	Components	Concentration
1	MS	4.43 g/L
2	Sucrose	30 g/L
3	BAP	1 mg/L
4	NAA	0.1 mg/L
5	Bacto agar	9 g/L
6	Timitin	200mg/L
7	Hygromycin	100mg/L

2.2.2.2.6.1 Molecular Analysis

The extraction of genomic DNA from leaves of transgenic plants was carried out with DNeasy Plant Mini Kit. The obtained DNA was used for PCR using *PI-II* and hygromycin resistant gene primers. A PCR reaction volume of 25 µl was prepared with addition of 45 ng/µl of genomic DNA, 1µl (25pmole) of forward and reverse primer, 2.5 µl of 10X PCR buffer, 1.5 µl (2.0 mM) dNTPs, 1.5 µl (25 mM) $MgCl_2$, and 1.5 U Taq polymerase.

The sequences of primers used are given below;

PI-II (Forward): 5′ TATCCATCATGGCTGTCCAC **3′**

PI-II (Reverse): 5′ AACACACAACTTGATCCCCACA **3′**

Hygro (Forward): 5′ GCTCCATACAAGCCAACCAC **3′**

Hygro (Reverse): 5′ CGAAAAGTTCGACAGCGTCTC **3′**

Gradient Multigene Thermal Cycler (Labnet) was used to run amplification reaction by following the conditions given below

PAD (Pre Amplification Denaturation) at 94 °C for 5 mins

Denaturing at 94 °C for 40 sec,
Amplification at 55 °C for 40 sec, } 35 cycles
Extension at 72 °C for 45 sec,

Final extension at 72 °C for 20 min.

2.2.2.2.6.2 *GUS* staining assay

β-Glucuronidase activity was determined in the young leaves and flowers collected from the 2 months old plants following the method of Jefferson (1989). For this purpose, the fragments of tissues were incubated overnight at 37 °C in a 2 mM X-Gluc in 50 mM sodium phosphate buffer, pH 7.0. The photo documentation was done using Leica microscope (20X and 40X magnifications).

2.2.2.2.7 *In vitro* germination and selection of transgenic seeds

Seeds from T_0 transgenic plants (TL1, TL2 and TL3) and wild type (WT) were collected and sterilized with 3 % (v/v) clorox solution by vigorous shaking for 5 minutes. After removing clorox, seeds were washed for 3-5 times with autoclaved distilled water. The sterilized seeds were sown on selection media containing hygromycin (50 mg/ml) and

placed in a growth room with 16 hours light/8 hours dark cycle at 25 °C. T_1 progeny was selected on the basis of hygromycin resistance.

2.2.2.3 Expression analysis under various stresses

Expression analysis was carried out by exposing the T_1 transgenic seedlings and WT to various stress treatments and analyzing the expression through RT-PCR and *GUS* assay.

2.2.2.3.1 Stress experiments

For stress analysis, the effect of wounding, different signaling molecules like ABA, MeJA, and salt stress (NaCl) were studied. The details of applied stresses are mentioned in Table 2.3.

2.2.2.3.1.1 Application of signaling molecules

The signaling molecules namely ABA and MeJA were applied on plants. For ABA and MeJA stress treatments, ABA and MeJA solutions (10 μM, 50 μM, and 100 μM), were prepared by dissolving in 96 % ethanol (2 mL) and subsequent dilutions were made with distilled water before use. For each treatment, 15 days old transgenic seedlings (TL1, TL2, and TL3) and WT (control) were sprayed with each of these signaling molecules. After 24 hours, all treated samples were harvested, frozen in liquid nitrogen and stored at -80 °C for RNA extraction and further use. For tissue specific expression, *GUS* histochemical assay was carried out as described by Jefferson (1989). Microscopic study of seedlings was done using Leica microscope at 20X and 40X magnifications. For each stress, each experimental trial was repeated three times.

2.2.2.3.1.2 Salt stress

In order to treat the seedlings with NaCl, plants were submerged in MS basal salt solution containing 100 mM, 200 mM, and 300 mM NaCl concentrations. For this three independent transgenic lines (TL1, TL2, and TL3) and WT (control) were used. Samples were taken after 24 hours and relative expression of *PI-II* gene was measured by performing RT-PCR after RNA extraction and cDNA synthesis. Salt stressed seedlings were also subjected to *GUS* staining assay.

2.2.2.3.1.2.1 Phenotypic analysis

For phenotypic analysis under salt stress, 10 days old seedlings of transgenic lines (TL1, TL2 and TL3) along with WT were shifted to solid MS media having NaCl of

concentrations (100 mM, 200 mM, and 300 mM NaCl). Phenotypic analysis was carried out after 14 days of stress treatment.

2.2.2.3.1.2.1.1 Average fresh weight calculation

Average fresh weight of all NaCl treated seedlings was measured at 14 days after stress treatment.

2.2.2.3.1.2.1.2 Estimation of chlorophyll content

The chlorophyll content in WT and transgenic seedlings was determined following the method of Arnon (1949). The chlorophyll content extraction was done with 80 % acetone by using 0.2 g NaCl seedlings. The absorbances of chlorophyll extracted from stressed samples were measured at wavelength 645 and 663 nm. The concentration of total chlorophyll was calculated using the following formula and results were expressed in μg per g of fresh tissue.

Total chlorophyll= $[\{(20.2 \times A645) + (8.02 \times A663)\}/1000 \times W] \times V$. Where V= extracted volume (ml); W=fresh weight of the material (g).

2.2.2.3.1.2.1.3 Analysis of proline contents

Proline contents of transgenics and WT tobacco seedlings were measured spectrophotometrically according to Bates et al. (1973). For this purpose, plant tissues (0.3 g) were homogenized with 3 % (w/v) sulphosalycyclic acid and then centrifuged at 1000 rpm for 5 minutes. Supernatant (2 ml) was incubated for 1 hour at 100 °C after adding 2 ml of acid-ninhydrin and 2 ml of glacial acetic acid. After cooling, the mixture was extracted with 2 ml of toluene, and again centrifuged at 1000 rpm for 5 minutes. Upper aqueous phase (containing proline) was collected and its absorbance was read at 520 nm using toluene as blank. Proline concentration was quantified by referring to a standard curve of proline.

2.2.2.3.1.3 Wounding stress

Wounding stress was given with the help of forceps for 12, 24, and 36 hours to the plants growing on MS media. After wounding, leaves were harvested at indicated time points for histochemical staining. Unwounded WT plants were used as negative control. *GUS* activity was observed at the cut edges and wounded areas using Leica microscope.

Table 2.3: Concentration of applied hormones and salt used for stress treatment.

Stress	ABA (μM)	MeJA (μM)	Salt (mM)
Concentrations	10	10	100
	50	50	200
	100	100	300

2.2.2.3.2 RNA extraction

Total RNA was isolated from ABA, MeJA and NaCl treated samples using Trizole reagent (Invitrogen). Approximately 100 mg of plant tissue was grounded with pestle and mortar in the presence of liquid nitrogen. Then 1 ml of Trizole reagent was added to powdered tissue and material was transferred to eppendorf tubes. The mixture was rotated on daisy wheel rotor at room temperature for 5 minutes. Afterwards centrifugation was carried out at 4 °C for 10 minutes at 12,000 rpm. The supernatant was shifted to new eppendorf tubes and 200 µl of chloroform was added. The tubes were shaken vigorously by hands for 15 seconds and then incubated at room temperature for 2 to 3 minutes. Again centrifugation of the samples was done at 12,000 rpm for 15 minutes at 4 °C and upper aqueous phase was transferred to new eppendorf tubes. Thereafter, 500 µl of isopropanol was added, mixed properly and incubated at room temperature for 10 minutes. Tubes were centrifuged at 4 °C for 10 minutes at 12,000 rpm, the RNA in the form of white or brown pellet was precipitated at the bottom of eppendorf tube and aqueous phase was poured out. The pellet was washed with 1 ml of 75 % ethanol, vortexed briefly and subjected to centrifugation at 9000 rpm for 5 minutes at 4 °C. After removing the ethanol, the pellet was allowed to air dry in fume hood for 10 minutes. RNA pellet was resuspended in 35 µl of RNase-free water and incubated at 60 °C for 10 minutes. RNA was stored at -80 °C for further use.

2.2.2.3.3 Removal of genomic DNA from RNA preparations

RNA samples were subjected to Turbo DNA-free DNase treatment to remove the contaminating DNA according to the manufacturer's protocol. The reaction mixture was consisting of the following components;

1. RNA 10 µg
2. 10 X Turbo DNase buffer 5 µl
3. Turbo DNase 1 µl

The total reaction mixture volume was made up to 50 µl by adding nuclease free water and incubated at 37 °C for 30 minutes. Then 5 µl of DNase inactivating reagent was added to the mixture and incubated at room temperature for 2 minutes after mixing. The mixture was centrifuged at 10,000 rpm for 2 minutes and purified RNA was shifted to new RNase-free eppendorf tube.

2.2.2.3.4 Quantity and quality confirmation

Total RNA quantification was done through NanoDrop 2000 (Thermo Scientific). For this 1 µl of each sample was loaded and readings were obtained using ND-1000, Ver. 3.8 software. Later RNA quality and integrity was checked on 2 % agarose gel.

2.2.2.3.5 cDNA synthesis

To synthesize first strand cDNA, 1 µg of DNase-treated RNA along with oligo (dT) primers were used. The total volume was made up to 20 µl by adding nuclease free water and incubated at 70 °C for 10 minutes. Master mixture with reverse transcriptase was prepared by adding 10X RT buffer (2 µl), 10 mM dNTPs (3 µl), and M-MuLV reverse transcriptase (1 µl). The total reaction mixture was incubated for 1 hour at 42 °C followed by 70 °C for 10 minutes to stop the reaction. To check the contamination of genomic DNA, a control was run without the reverse transcriptase enzyme. Quantity and quality of cDNA was confirmed through Nanodrop method and PCR using housekeeping gene (actin) primers. Finally cDNA was stored at -20 °C until further use.

2.2.2.3.6 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was done in Stratagene Mx3005P QPCR Systems having 96 well reaction plates using the following reagents.

1. cDNA (1:10) dilution — 4 µl,
2. 1x EvaGreen master mix — 4 µl
3. Primers (forward and reverse) — 0.6 µl (5 µM)
4. Nuclease free water — 10.8 µl

RT-PCR reaction was performed by using the gene specific primers that can amplify the short region of 151 bp.

2PI-II (Forward): 5′-TTCGGGATATGCCCACGTTC**-3′**

2PI-II (Reverse): 5′-AGGTGCAAGCATTTGGCCTT**-3′**

N. benthamian actin primers were used as an internal control.

Nb actin (Forward): 5′-GATGAAGATACTCACAGAAAGA**-3′**

Nb actin (Reverse): 5′-GTGGTTTCATGAATGCCAGCA**-3′**

The PCR programme was as followed: pre-denaturing at 95 °C for 10 minutes, 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 56 °C for 1 minute, extension at 72 °C for 10 seconds.

2.2.2.3.7 Data analysis

The relative expression level of PI-II gene was calculated according to mathematical model $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001). There was a selection of an internal control and calibrator for $2^{-\Delta\Delta Ct}$. The cycle threshold values (CT) were determined automatically and all reactions were conducted in triplicate.

2.2.2.3.8 Statistical analysis

All the data were subjected to analysis of variance (ANOVA) using a PROC GLM procedure of SAS 9.4.

Results and Discussion

The result and discussion section was divided into two parts;

- *In silico* analysis of *PI-II* gene sequences
- Functional analysis

3.1 *In silico* analysis of *PI-II* genes

In silico analysis for *PI-II* genes was carried out based on the following approaches;

- Identification and sequence analysis of *PI-II* genes
- Phylogenetic analysis

3.1.1 Identification and sequence analysis of *PI-II* gene

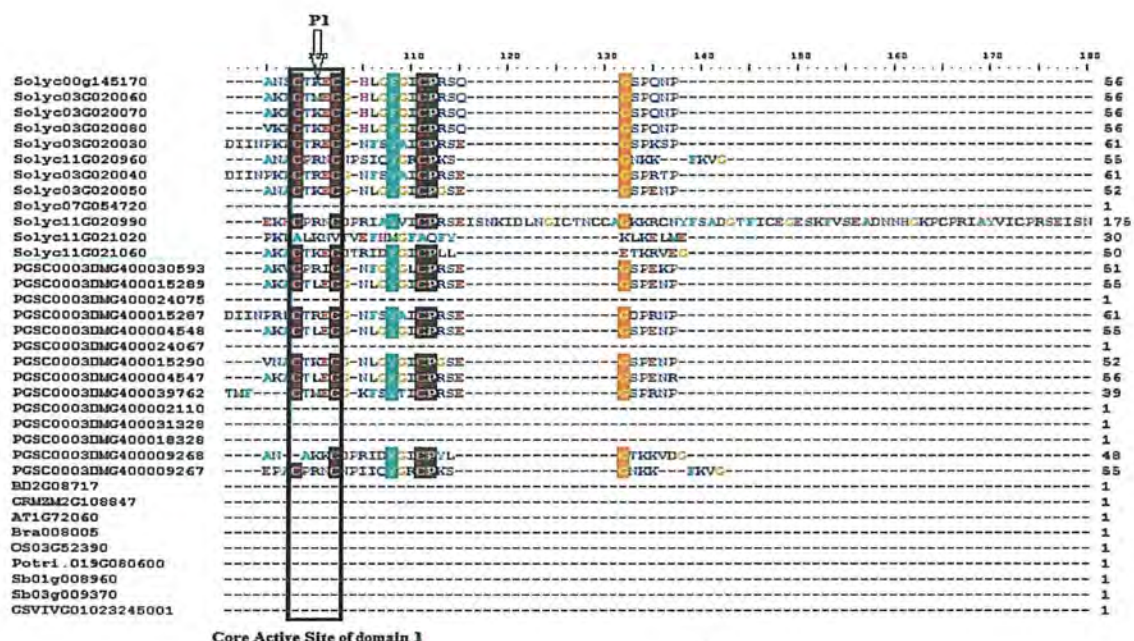
A total of 35 *PI-II* coding sequences were identified from genome database of 10 plant species that contain 14 sequences of *Solanum tuberosum*, 12 sequences of *Solanum lycoperiscum*, 2 sequences each of *Sorgum bilocolor* and 1 sequence each of *Arabidopsis thaliana, Brachypodium distachyon, Brassica rapa, Vitis vinifera, Oryza sativa, Populus tricocarpa*, and *Zea mays*. The species name, along with gene identification numbers, aminoacids (aa) lengths, Isoelectric point (pI), locus position, and molecular weight (Mol.wt.) are given in Table 3.1.

3.1.1.1 Multiple sequence alignment

The analysis of multiple sequence alignment of all PI-II protein sequences showed presence of distinct regions with high sequence similarity (Figure 1). Such sequence variations are considered to be important to target different proteinases having broad range of substrate specificity. The specificity of inhibitory protein is usually determined by the key role of P1 residue which is often critical for substrate recognition. The inhibitors having trypsin like activity prefers Arginine or Lysine at P1 position, while chymotrypsin inhibitors have hydrophobic P1 residues (Leucine, Isoleucine, Tryptophan, or Phenylalanine). In the current study of sequence alignment, it was observed that potato and tomato has two highly conserved PI-II regions namely domain 1 and domain 2. In contrast, single PI-II domain was present in all non-Solanaceous plant. In a similar analysis, Santamaria et al. (2014) reported single inhibitory domain for Angiosperm, whereas pseudofern were shown to have two domains. Moreover, both domains may have either antitrypsin or antichymotrypsin potential due to the presence of arginine (R),

Table 3.1: The gene identification numbers for *PI-II* genes from selected plants along with Locus position, No. of introns, length of amino acids (aa), Molecular weight (Mol.wt.), isoelectric point (pI) and subcellular localization.

Genus	Gene ID	Locus position	No. of introns	Length of amino acid (aa)	Mol.wt. (Dalton)	pI	Subcellular localization
Solanum tuberosum	PGSC0003DMG400030593	Chr02: 41576324-41577018	1	145	15688.8	4.97	Extracellular
	PGSC0003DMG400015289	Chr03: 50043509-50044247	1	205	21860.0	6.75	Extracellular
	PGSC0003DMG400024075	Chr03: 2777493-2778861	1	83	8977.3	4.36	Extracellular
	PGSC0003DMG400015287	Chr03: 50085156-50086034	1	207	22784.2	8.34	Extracellular
	PGSC0003DMG400004548	Chr03: 49930746-49931538	1	205	21860.0	6.75	Extracellular
	PGSC0003DMG400024067	Chr03: 2851935-2853380	1	81	8917.2	4.24	Extracellular
	PGSC0003DMG400015290	Chr03: 50010387-50011368	1	200	21514.4	4.96	Extracellular
	PGSC0003DMG400004547	Chr03: 49956094-49956945	1	154	16660.2	6.77	Extracellular
	PGSC0003DMG400039762	Chr03: 50089248-50089808	0	186	20390.5	8.25	Extracellular
	PGSC0003DMG400002110	Chr06: 44785867-44789318	1	84	8980.4	4.25	Extracellular
	PGSC0003DMG400031328	Chr06: 46964292-46964851	1	81	8704.3	9.35	Extracellular
	PGSC0003DMG400018328	Chr07: 48432313-48433228	1	77	8327.7	8.11	Extracellular
	PGSC0003DMG400009268	Chr11: 14406781-14408214	1	174	19726.0	8.65	Extracellular
	PGSC0003DMG400009267	Chr11: 14382374-14383072	1	146	15745.2	8.95	Extracellular
Solanum lycopersicum	Solyc00g145170.1	Ch00: 17251047-17251697	2	142	15408.6	6.48	Extracellular
	Solyc03g020030.2	Ch03: 6903189-6903918	1	207	22653.2	8.60	Extracellular
	Solyc03g020040.2	Ch03: 6911347-6912078	1	207	22682.2	8.53	Extracellular
	Solyc03g020050.2	Ch03: 6918091-6918859	1	201	21370.2	4.90	Extracellular
	Solyc03g020060.2	Ch03: 6921071-6921808	1	206	22018.4	7.31	Extracellular
	Solyc03g020070.2	Ch03: 6927490-6928190	2	159	17144.7	8.12	Extracellular
	Solyc03g020080.2	Ch03: 6933651-6934514	2	159	17304.9	7.85	Extracellular
	Solyc07g054720.1	Ch07: 60269248-60269891	1	77	8343.6	7.46	Extracellular
	Solyc11g020990.1	Ch11: 3167991-13169189	1	360	39279.7	7.55	Extracellular
	Solyc11g020960.1	Ch11: 12996643-12997161	1	146	15636.8	8.58	Extracellular
	Solyc11g021020.1	Ch11: 13195204-13195581	0	125	13985.9	5.20	Extracellular
	Solyc11g021060.1	Ch11: 13312553-13313384	1	223	24697.3	5.32	Extracellular
Brachypodium distachyon	Bradi2g08717	Bd2: 7080644-7081324	1	79	8329.7	7.51	Extracellular
Zea mays	GRMZM2G108847	Chr3: 33851323-33851858	1	78	8091.4	5.45	Extracellular
Arabidopsis thialana	AT1G72060	Chr 1: 27118447-27119011	1	81	8667.3	8.47	Extracellular
Oryza sativa	LOC_Os03g52390	Chr3: 30078324-30078929	1	82	8637.2	6.49	Extracellular
Brassica rapa	Bra008005	A02: 12473639-12473967	1	81	8739.4	6.67	Extracellular
Populus tricocarpa	Potri.019G080600	Chr19:11422165-11422594	1	78	7976.4	7.51	Extracellular
Sorghum bilocolor	Sb01g008960	Chr01: 7706303-7706685	1	91	9626.3	7.42	Extracellular
	Sb03g009370	Chr03: 10102734-10103654	2	77	8058.3	5.54	Extracellular
Vitis vinifera	GSVIVG01023245001	Chr12: 20686937-20688126	1	78	8328.8	4.75	Extracellular



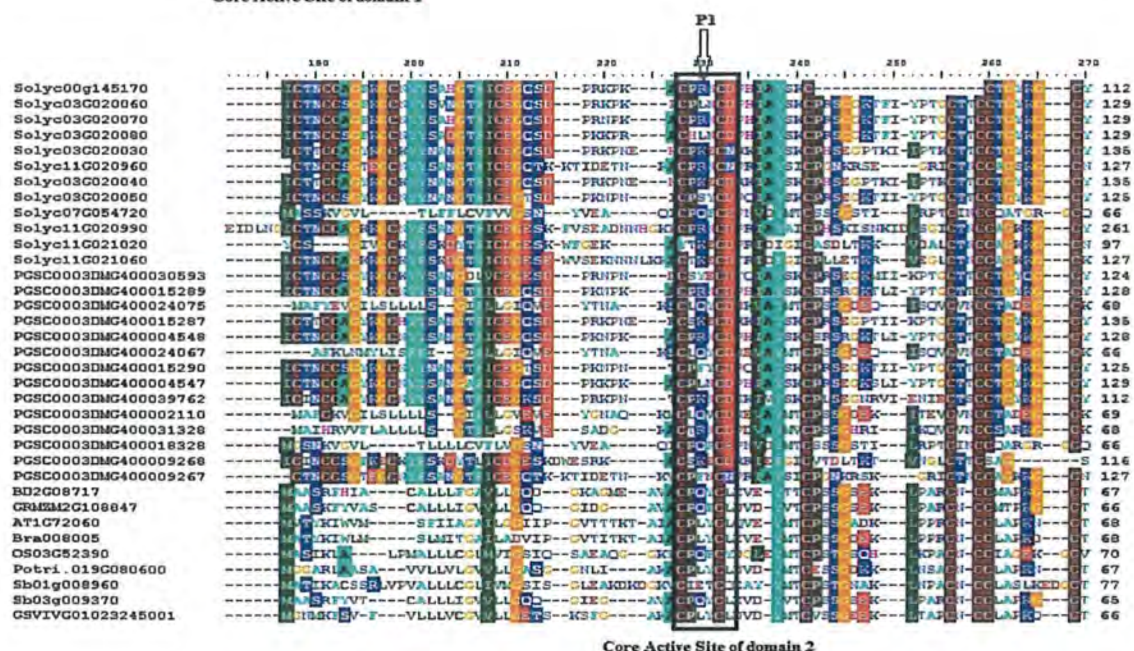


Figure 1: Comparison of PI-II domain sequences. Peptide sequence information for selected plants was taken from the Plaza 3.0 online tool (http://bioinformatics.psb.ugent.be/plaza/) and Plant Ensemble (http://plants.ensembl.org/index.html). The core active sites of domain 1 and domain 2 are boxed. The reactive site residue P1 is marked by arrow. The presence of Lys (K) or Arg (R) at the P1 site indicates trypsin inhibitory site, and Leu (L) indicates chymotrypsin inhibitory site.

leucin (L) or lysine (K) residues at P1 position (Figure 2). Beekwilder et al. (2000) also reported the importance of P1 residue in PI-II domain for trypsin and chymotrypsin inhibition.

The comparative analysis of PI-II amino acid sequences via MEME program showed a highly conserved motif having cysteine residues along with the two Glycine residues (Gly-26 and Gly-34) and a proline residue (Pro-6) (Figure 2), which are probably thought to have important role in stabilizing the 3D structure of proteins (Kong and Ranganathan, 2008). From structural analysis, it was observed that each PI-II protein is double-headed and contain two reaction centres, one present at each head. Moreover, two conserved cysteine residues of the five amino acid residues array are present in each head which are responsible for forming two disulphide bonds by pairing with the two counterpart cysteine residues present in another domain. The three internal amino acid residues amongst two cysteine residues frequently vary among homologs and can be modified by genetic engineering (Beekwilder et al., 2000; Li et al., 2011). The present PI-II proteins amino acid sequence analysis in different plants also showed such variants in their active sites. For instance, the first reaction centre of domain 1 in potato PI-II contain CPRNC, CTLEC, CTMEC, CTREC, and CPRIC amino acid sequences, while the second reaction centre were CSKEC, CPKNC, CPRNC, CPFYC, and CPLNC. On the other hand, the active site variants of domain 1 in tomato PI-II were CPRNC, CTKEC, CTMEC, and CTREC, while active site of domain 2 were CPQFC, CPSYC, CPLNC, CPKEC and CPRNC. The similar active site variants were identified in CaPIs for trypsin inhibitors (CPRDC, CPRYC, CPKNC, and CPRNC) and two types of cysteine inhibitor sites (CTPNC and CTLNC) (Mishra et al., 2012). This sequence diversity in PI genes possibly contributes in recruiting a diverse pool of PI proteins to counter different biotic stresses (Mishra et al., 2012).

3.1.1.2 Subcellular localization

Subcellular localization studies of PI-II proteins via CELLO and PSORT servers showed that most of PI-II proteins are extracellular in location (Table 3.1), suggesting their possible defensive role in response to insect or pathogens attacks. It has been found that inhibitory proteins are located in different parts of plant cells such as cytoplasm, cell

Figure 2: Sequence Logo representation of conserved motif in PI-II amino acid sequences.

wall, nuclei, and vacuoles (Kidric et al., 2014; Rehman et al., 2107). For example, wound-inducible PIs of tomato and potato showed vacuolar localization (reviewed in Rehman et al., 2017). However, the nightshade inhibitory proteins (PI-II) were reported to be present in the phloem of roots, floral tissues leaves and stem (Xu et al., 2001; Sin and Chye, 2004). In tomatoes, serine PIs were found to be associated with the cell wall of endosperm and secretary cells of root cap (Narwaez-Vasquez et al., 1993) which may provide protection to growing root meristems against pathogens and insects. Similarly, Gruden et al. (1997) showed that the potato cysteine proteinase inhibitor (PCPI) accumulated in the vacuole of stems after JA application suggesting their possible role in providing plant protection against the attacks of pathogens and insects.

3.1.1.3 Chromosomal localization of *PI-II* genes

From IGMAP database, total 12 tomato *PI-II* genes were identified, out of which eleven genes were shown to be localized on three different chromosomes (Chr 03, Chr 07 and Chr 11), while single gene copy (i.e Solyc00g145170.1) was located on Chr 00. Furthermore, six gene copies were assembled at single locus in the form of cluster on chromosome 3. Similarly, four gene copies were organized as cluster at single locus on chromosome 11 (Figure 3a). On the other hand, survey of potato genome identified 14 *PI-II* like genes on five different chromosomes (Chr 02, Chr 03, Chr 06, Chr 07 and Chr 11). Three distinct gene clusters were assembled on same chromosomes 3 and a single cluster was observed on chromosome 11 (Figure 3b). Two gene copies of potato *PI-II* (i.e PGSC0003DMG400018328 and PGSC0003DMG400030593) were shown to be located independently on chromosome 7 and chromosome 2 respectively. Two such genes were recognized on Chr00 and Chr07 in tomato.

This study also showed that there is a single exon in most of these sequences. However, Solyc11g021020.1 (in tomato) and PGSC0003DMG400039762 (in potato) were shown to be transcribed from single exon due to lack of intron. Furthermore, two introns were found in *PI-II* like genes (Solyc03g020080.2, Solyc03g020070.2, and Solyc00g145170.1) of tomato and sorghum (Sb03g009370) (Table 3.1). Additionally, Gly residues were recognized in the last nucleotide of exon 1 and first two nucleotide of exon 2. As a result of these, the reported PI-II genes displayed high sequence similarity

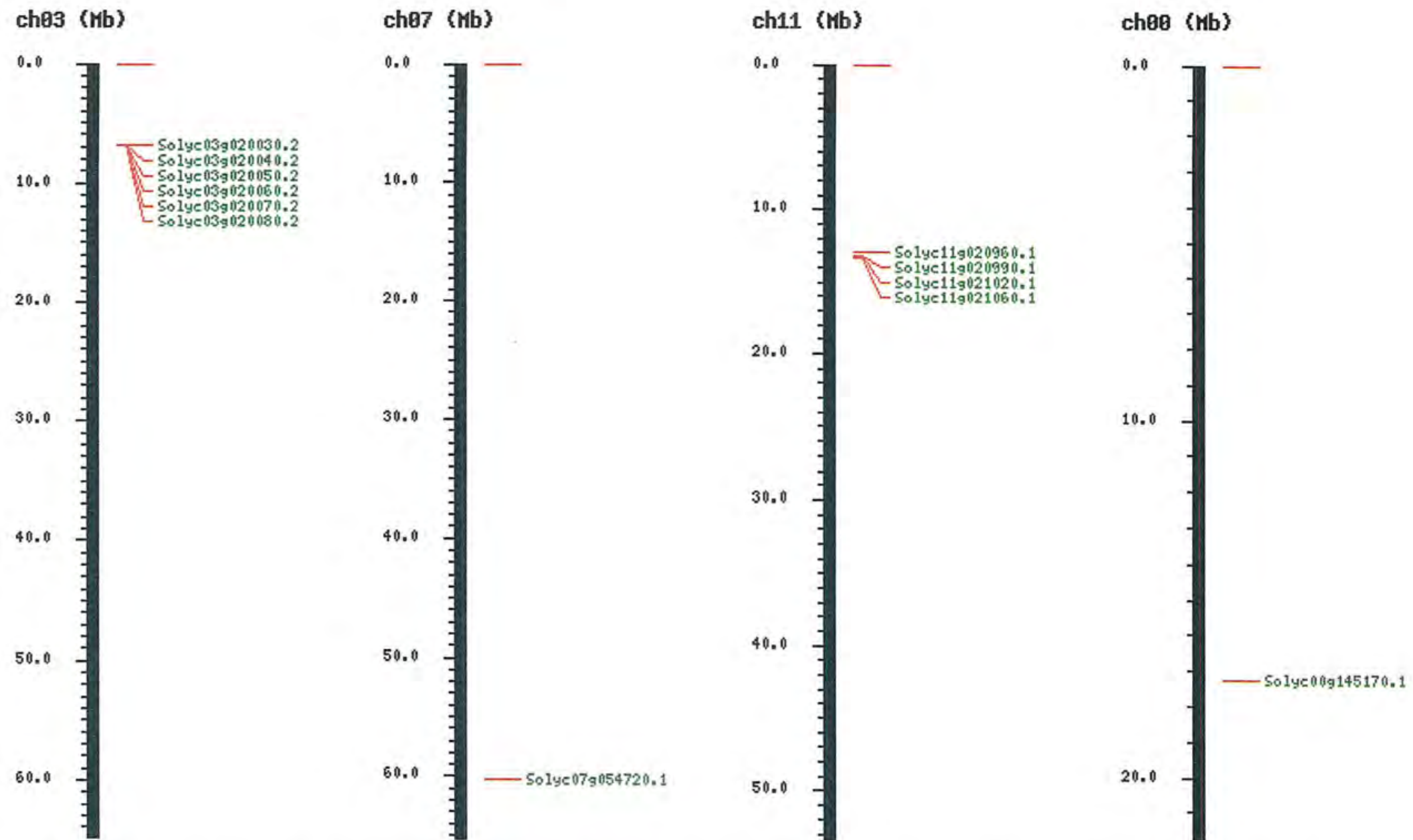


Figure 3a: Chromosomal position of *PI-II* genes in tomato according to the information available in IGMAP (Interactive Genome Map for Plants) online database (http://nipgr.res.in/igmap.html).

chr02 (Mb)
0.0
10.0
20.0
30.0
40.0
50.0
60.0
PGSC0003DMG400030593

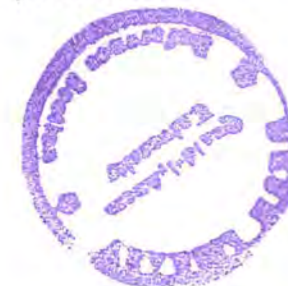
chr03 (Mb)
0.0
PGSC0003DMG400024075
PGSC0003DMG400024067
10.0
PGSC0003DMG400004548
PGSC0003DMG400004547
20.0
30.0
PGSC0003DMG400039762
PGSC0003DMG400015287
PGSC0003DMG400015289
PGSC0003DMG400015290
40.0

chr06 (Mb)
0.0
10.0
20.0
30.0
PGSC0003DMG400031328
40.0
PGSC0003DMG400002110
50.0

chr07 (Mb)
0.0
10.0
20.0
30.0
40.0
PGSC0003DMG400018328
50.0

chr11 (Mb)
0.0
10.0
PGSC0003DMG400009268
PGSC0003DMG400009267
20.0
30.0
40.0

Figure 3b: Chromosomal position of *PI-II* genes in potato according to the information available in IGMAP (Interactive Genome Map for Plants) online database (http://nipgr.res.in/igmap.html).



due to conservation of exon/intron organization and Gly residues which is in accordance with earlier finding of PI-II sequence analysis (Barta et al. 2002; Kong and Ranganathan (2008). Previously, potato inhibitors 2 (PIN2) or Kunitz-type proteinase inhibitors (KPI) organization was observed on potato chromosomes III as mixed clusters (Heibges et al., 2003; Odeny et al., 2010). In the current study, the clustering of majority of PI genes at the same chromosomal loci is an indication of their common ancestry from which they have evolved by a series of duplication events. Duplication of PI-II family gene from a single ancestor has been studied mainly in Solanaceous plant species (Kong and Ranganathan, 2008; Rehman et al., 2017). However, no duplication in PI-II genes was found in non-Solanaceous plants as they have single or two gene copies (Table 3.1).

3.1.2 Phylogenetic analysis

The neighbor-joining phylogenetic tree construction based on 35 PI-II gene sequences showed two distinct clusters (Cluster 1 and Cluster 2) having well supported bootstrap values (Figure 4). This analysis showed that most of the members from Solanaceous family (i.e potato and tomato) are found in Cluster 1, while all non-Solanaceous plants are arranged in Cluster 2. This phylogentic analysis also revealed that multi domain PI-II proteins in Solanaceae might have evolved from ancestral single domain PI-II proteins of non Solanaceous plants through duplication process. Munir et al. (2013) carried out comparative phylogenetic analysis based on 30 PI genes of different plants and reported similar clustering pattern for the studied sequences. Furthermore, it was also observed that separation of genes from same plant family into different clades may be due to differences in their coding products. In the current phylogram, genes from same plant family (Solanaceae) might have separated into different clusters due to differences in their sizes (amino acid lengths).

Phylogenetic studies are the important to give significant insights into the evolutionary realatioship among the investigated species. Previously, Lee et al. (1986) studied the common evolutionary origin for wound inducible *PI-I* genes of 7 direct *Lycopersicum* ancestors. In a similar report, Kong and Ranganathan (2008) phylogenetically analyzed Pot-II family genes from different plants and observed seven clades based on repeat numbers and species. The similar evolutionary association among CaPIs was depicted on the basis of inhibitory repeat domain (IRD) sequences (Tamhane

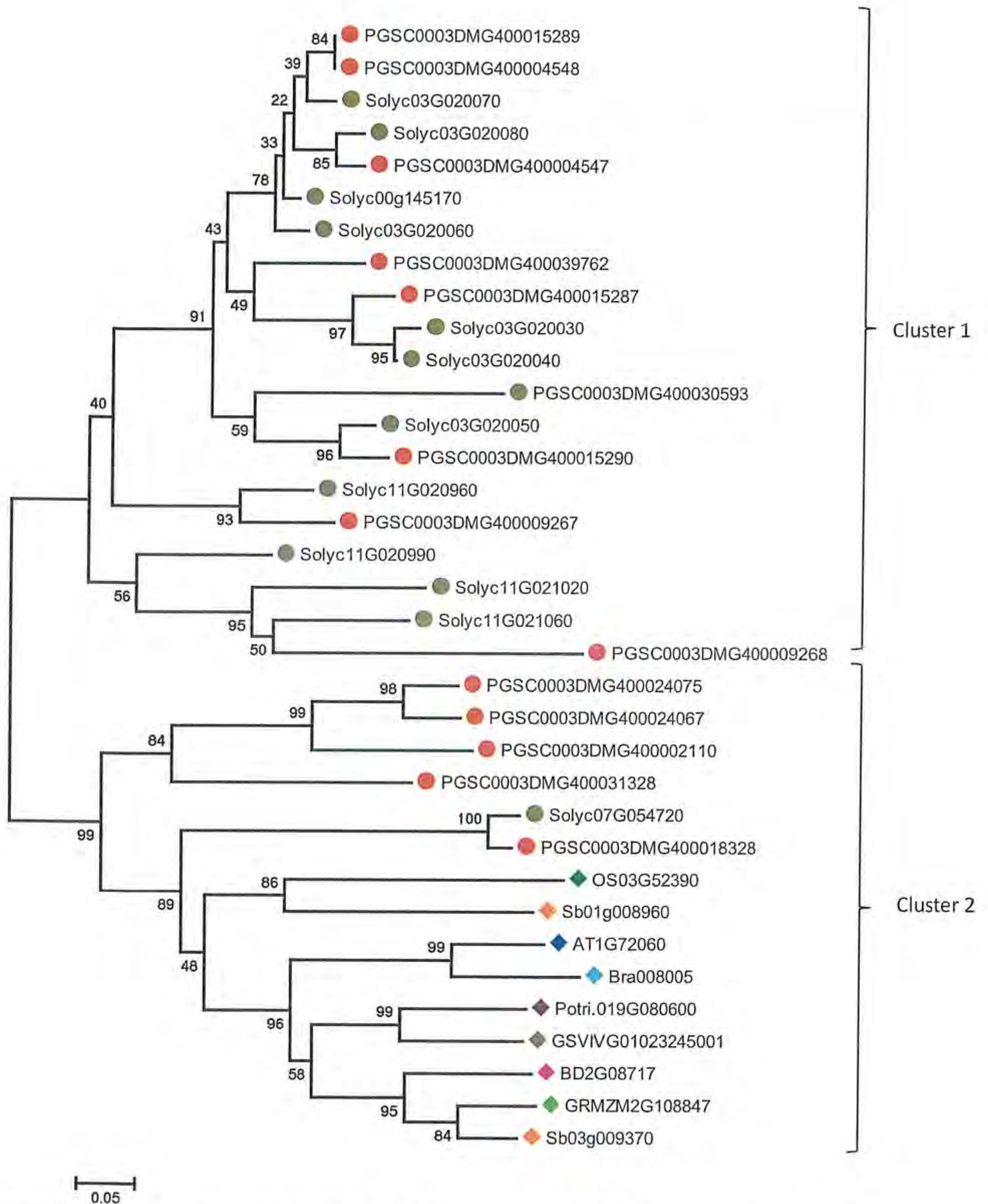


Figure 4: Phylogenetic clustering of *PI-II* genes representing different plant species by MEGA 6.0.

et al., 2009; Mishra et al., 2012). In another study, common evolutionary relationship for both monocots and eudicots was reported by Santamaria et al. (2014) using PI-II proteins of different plants. The present phylogram also indicated the occurrence of genes in both monocot and dicots suggesting their evolutionary background.

3.2 Functional analysis

This section includes three parts

- Vector designing
- *Agrobacterium*-mediated transformation of plants
- Analysis of transgenic plants

3.2.1 Vector designing

3.2.1.1 Cloning of *PI-II* gene

For construct preparation, the *PI-II* product cloned in T/A cloning vector (Figure 5) was transformed into *E. coli* strain (DH5α) through electroporation. The positive clones (white colonies) were selected on the basis of blue-white screening and ampicillin resistance.

3.2.1.2 Confirmation of cloning of *PI-II* by PCR

For confirmation of cloned gene, plasmid from culture was isolated after growing the white colonies in LB media containing 50 mg/L ampicillin and incubating at 37 °C with 250 rpms shaking. Plasmid was confirmed by PCR (Figure 6).

3.2.1.3 Sequencing of *PI-II* clone

The confirmed *PI-II* clone was isolated for sequencing that resulted in ~684 bp long fragment (Text Box 3.1). The sequence was found to show maximum similarity (98 %) with already reported *PI-II* gene of *Solanum lycopersicum* in NCBI genome database which also confirmed the cloned target gene.

3.2.1.4 Construction of recombinant vector

PI-II gene was ligated in an expression vector to investigate its functional analysis in plants. For this, p1391Z_*Os*RGLP2 vector (Mahmood et al., 2013) and *PI-II* cloned vector were treated with *Eco*R1 restriction enzyme followed by the ligation of digested and eluted product of *PI-II* gene and digested p1391Z_*Os*RGLP2 vector. *PI-II* gene was ligated downstream to the *Os*RGLP2 promoter at *Eco*R1 restriction site. This vector possesses kanamycin resistance gene for bacterial selection and hygromycin resistant

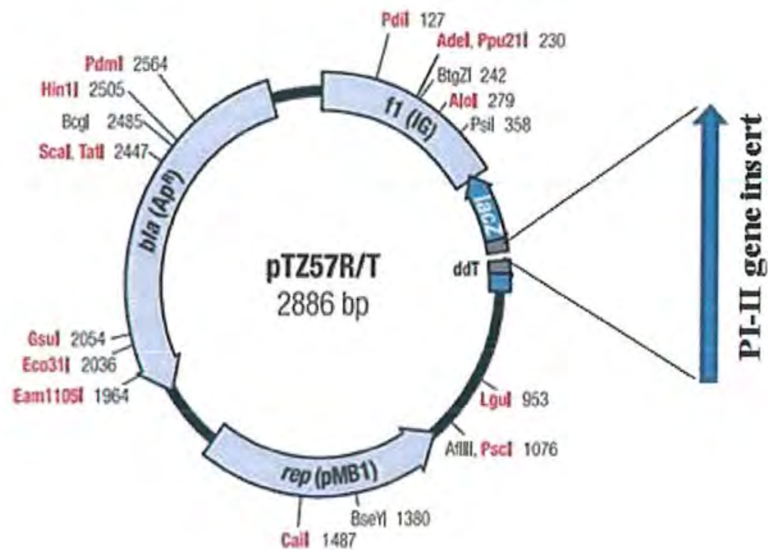


Figure 5: Overview of T/A cloning vector showing the insertion of *PI-II* gene.

Figure 6: PCR confirmation of cloning of *PI-II* gene (T/A clones). L1: 1kb ladder (Fermentas), lane 1-3: amplified products of *PI-II* genes from plasmid.

Text Box 3.1: Sequence of cloned *PI-II* gene

TATCCATCATGGCTGTCCACAAGGAAGTTAATTTTGTCGCTTACCTACTAATTGTTCTTGGTAAGATTTTCC
TTTAGTCTCTTTTTTTTTTTTTTAAAAAAAAGAGAAAAATATTGGTTTATACATACACAAGTAGTTTTATATTT
TTCCTTATATTATATTTGTTGTAGGAATGTTTCTATATGTTGATGCCAAGGCTTGTACTAGAGAATGTGGTA
ATCTTGGGTTCGGGATATGCCCACGTTCAGAAGGAAGTCCGCTAAATCCCATATGCATCAATTGTTGCTCA
GGCTATAAGGGTTGTAATTATTATAATTCTTTCGGAAAATTTATTTGTGAAGGAGAATCTGATCCAAAAAG
GCCAAATGCTTGCACCTTTAATTGTGATCCAAATATTGCCTATTCAAGATGTCCCCGTTCACAAGGAAAAT
CGTTAATTTATCCCACCGGATGTACCACGTGTTGCACAGGGTACAAGGGTTGCTATTATTTTGGTAAAGAT
GGAAAGTTTGTATGTGAAGGAGAGAGTGATGAACCCAAGGCAAATATGTACCCTGTAATGTGACTCTAG
ACTTGTCCATCTTCTGGATTGCCCAAAATTAAGTAATTAATGTATGAAATAAAAGGATGCACACTTATATA
ATGACATGCTAATCATTATAATGTGGGGATCAAGTTGTGTGTT

gene for screening the transgenic plants. *GUS* reporter gene was also present downstream to the multiple cloning sites (MCS) (Figure 7).

3.2.1.5 Transformation and confirmation of recombinant vector

The recombinant vector was electroporated into *Agrobacterium* strain (EHA101) and subjected to colony PCR with the help of *PI-II* gene specific primers along with *Os*RGLP2 promoter specific primers that resulted in the amplification of products ~684 bp and ~1100 bp respectively (Figure 8). Restriction digestion with *Eco*R1 further confirmed the correct transformation of recombinant vector.

3.2.2 *Agrobacterium*-mediated transformation of plants

Young leaves of tobacco were sterilized and incubated on solid MS media for 48 hours. Sterilized leaf discs were infected with *Agrobacterium* suspension having O.D of 0.2 for 10 minutes and later explants were co-cultured on solid MS media for 2 days at 22 °C in dark. After co-culturing, the infected leaf discs were maintained on solid selection MSH media (Table 2.2, section 2.2.2.2.3) with timitin (200 mg/L) and hygromycin (100 mg/L). At selection stage, it was observed that tobacco plants started to regenerate in the form of small calli from cut edges of explants within two to three weeks shown in close view in figure 9, while bunch of shoots initiated approximately two weeks later from each callus. Afterwards, there was regular shifting of regenerated plants to the fresh selection media after every 10 days. Later shoots were excised individually from each callus and shifted to large containers for proper growth. Regenerated shoots were later shifted to hormone free MS media for rooting when at least two to three nodes were produced. Rooting was observed after 15 days of transfer to rooting media. Transgenic plants were shifted to the soil when roots become well established to obtain T_0 plants (Figure 10), that were maintained in the green house under controlled conditions of 16:8 light/dark cycles at 27 °C. Total thirty transgenic plants were shifted to the soil for maturity and 10 plants could survive in the soil. Flowering initiated after two months in these transgenic plants growing in soil. T_0 seeds were collected and stored at 4 °C till further analysis.

3.2.2.1 Confirmation of transgenic plants

To verify the insertion of transgene, PCR was carried out using extracted DNA as template from transgenic plants. Both PI-II and hygromycin gene primers sets resulted in

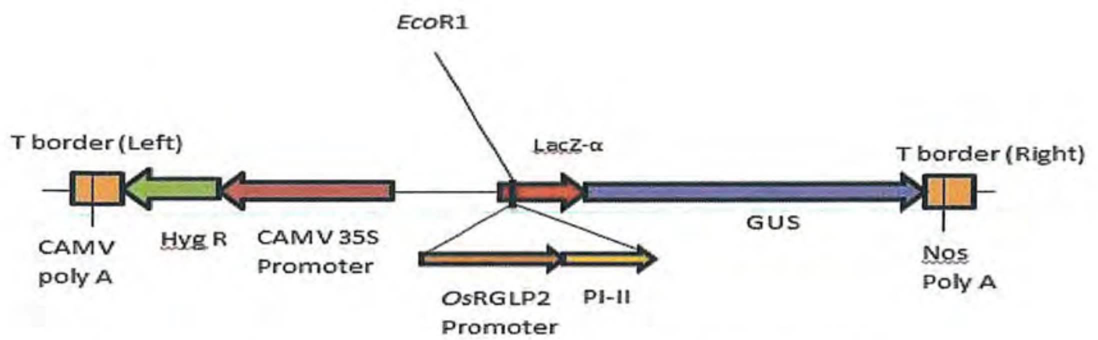


Figure 7: A schematic representation of pCAMBIA1391Z_*Os*RGLP2::PI-II construct. The proteinase inhibitor gene (*PI-II*) was cloned in pCAMBIA1391Z_*Os*RGLP2 at *Eco*R1 restriction site.

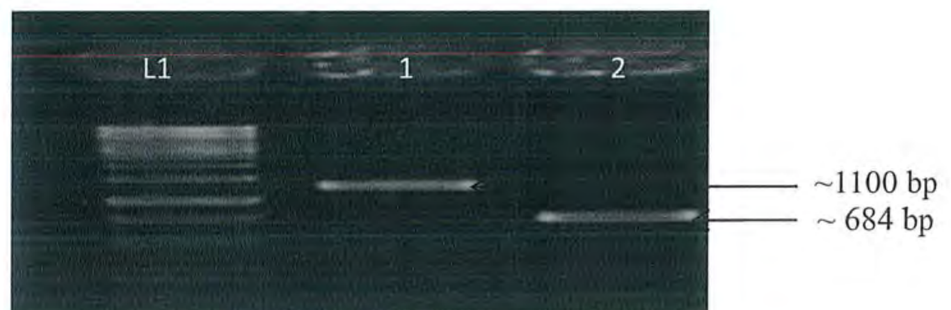


Figure 8: PCR confirmation p1391Z_*Os*RGLP2::PI-II construct from plasmid with *PI-II* gene along with *Os*RGLP2 promoter primers. L1: 1kb ladder (Fermentas), lane 1: amplified products of *Os*RGLP2 promoter from plasmid, lane 2: amplified product of *PI-II* gene from plasmid.

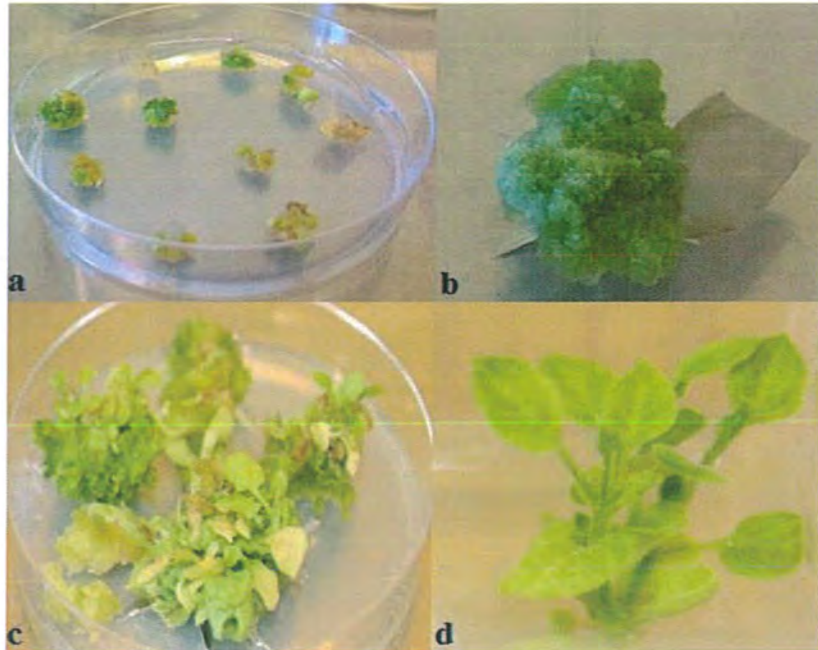


Figure 9: Regeneration of trasgenic tobaccco plants from leaf discs growing on selection media. a) Calli emerging from cut edges of leaf discs, b) 14 days old callus, c) Regeneration of shoots from calli, d) 28 days old shoot growing on selection media.

Figure 10: Three months old transgenic plants at flowering stage growing in green house.

successful amplification products with approximately 684 bp and 700 bp in sizes respectively (Figure 11 and Figure 12).

3.2.2.2 *GUS* histochemical assay

The transgenic plants were subjected to *GUS* staining assay that revealed positive results, indicated by blue staining in different parts of transgenic tissues. *GUS* expression was observed in leaves, midrib portion and flowers (Figure 13). In the leaves, the *GUS* expression was prominent at the cut site of petiole and at the site of wounding which led to support that *Os*RGLP2 promoter is a robust promoter that is capable of driving the expression of downstream genes under various stresses, including wounding, salt, dehydration and pathogenic infection in transgenic plants (Mahmood et al., 2013; Munir et al., 2016). In flowers, *GUS* staining was evident in stigma and at the base of pistils, while no *GUS* expression was observed in control un-transgenic leaves. The present results corroborates well with the previous studies in which high *GUS* expression was revealed in transgenic plants at mechanical wounding sites of leaves (Peña-Cortés et al., 1995; Koiwa et al., 1997; Mahmood et al., 2013; Munir et al., 2016) and in flowering parts (Xu et al., 2001; Sin and Chye, 2004). Based on this information, pCAMBIA_*Os*RGLP2::PI-II construct was further analyzed in T_1 transgenic seedlings in response to wounding, ABA, MeJA and salt stresses, the results of which have been discussed in the later part of this chapter.

3.2.2.3 Procurement of transgenic seeds

T_0 seeds from transgenic plant and wild types (WT) were sterilized and selected on hygromycin selection media. WT and hygromycin resistant transgenic T_1 seedlings were shifted to hygromycin free MS media (Figure 14), which were later subjected to stress treatments, RNA isolation and RT-PCR analysis.

3.2.3 Analysis of transgenic plants

In the present study, the activity of *PI-II* was checked in WT and three T_1 transgenic lines (TL1, TL2 and TL3) in response to ABA and MeJA and NaCl treatments by analyzing the transcript level of transgene. Later, *GUS* histochemical assay and microscopic study was carried out for a single transgenic line (TL1) in response to wounding, ABA, MeJA and salt stress treatments.

Figure 11: PCR amplification of *PI-II* gene from transgenic plants. L1 and L2: 1 kb plus ladder (Fermentas), lane 1 to 8: amplified products of *PI-II* gene, lane 9: negative control, lane 10: positive control.

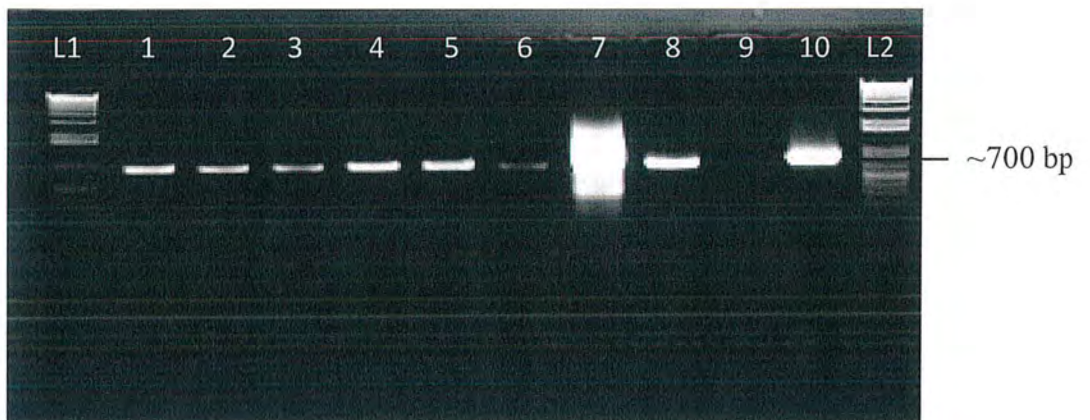


Figure 12: PCR amplification of hygromycin gene from transgenic plants. L1 and L2: 1 kb plus ladder (Fermentas), lane 1 to 8: amplified products of hygromycin gene, lane 9: negative control, lane 10: positive control.

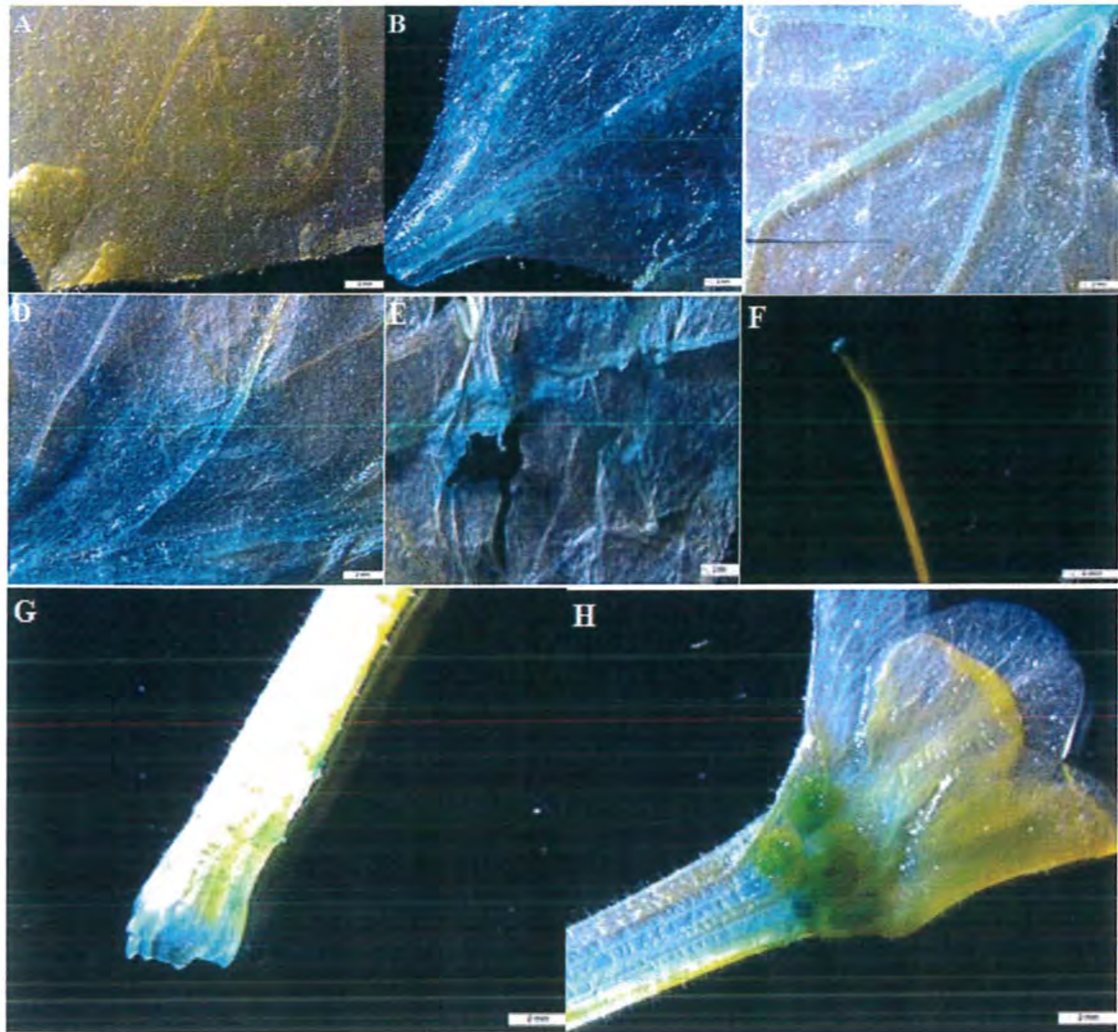


Figure 13: *GUS* expression in different parts of transgenic plants. (A) No *GUS* expression in control (WT) (B) *GUS* expression at the cut site of petiole, (C) *GUS* expression in the midrib portion, (D) *GUS* expression at the site of wounding, (E) *GUS* expression at the cut site (F) *GUS* expression in stigma, (G) *GUS* expression at the base of pistil, (H) *GUS* expression in complete flower. Bars indicate 2 mm.

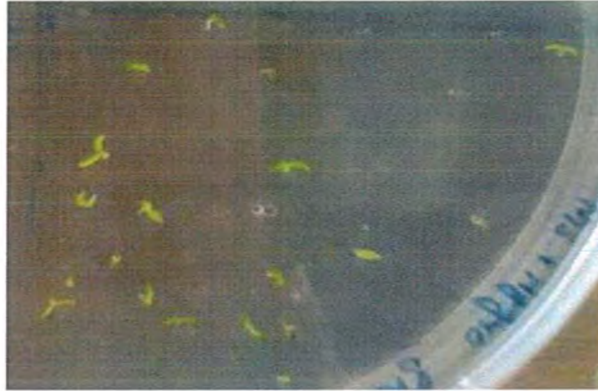


Figure 14: Hygromycin resistant T_1 transgenic seedlings growing on selection media.

3.2.3.1 Induction of *PI-II* in response to ABA

The relative quantification data showed that *PI-II* gene expression was triggered significantly at higher level in three independent transgenic lines (TL1, TL2 and TL3) than WT under three different ABA concentrations (10 μM, 50 μM, and 100 μM) after 24 hours (Figure 15). After 10 μM ABA treatment, *PI-II* gene was initially induced at very low level in all treated plants. The transcript level was found to increase and differ significantly ($P<0.01$) between transgenic lines versus WT plants with an increase in ABA concentrations. However, transgenic lines displayed comparatively higher expression than WT (Figure 15). When treated with 100 μM ABA, TL1, TL2, TL3 showed higher fold change of 9.5, 8.4 and 5.2 respectively when compared with WT. Moreover, the transcript level of TL1 lines was highest among all the other transgenic lines and control plants at all concentrations. These results correlate well with earlier studies in which an increase in *PI-II* mRNA transcript level was reported in potato leaves and stem after applying 100 μM ABA to leaves and this spray treatment on potato leaves resulted in local and systemic induction of *PI-II* mRNA accumulation (Pena-Cortes et al., 1989, 1995). The present data indicated that *PI-II* gene is responsive to ABA application which suggested that ABA might play an important role in the induction of *PI-II* gene under abiotic stress conditions.

The plant hormone, ABA serves as significant signaling molecule that is critical for growth and development of plants, and provides adaptations to wide range of stresses like drought, salinity and cold (Shinozaki and Yamaguchi-Shinozaki, 2000; Sah et al., 2016). The induction of wound inducible *PI-II* in response to ABA was detected in potato, tobacco and tomato (Sanchez-Serrano et al., 1991; Pena-Cortes and Willmitzer, 1995) in which endogenous levels of ABA was found to increase after wounding implying the essential role of ABA for the induction of *PI-II* genes. Further studies indicated that spraying or exogenous application of ABA on plants leaves induces the systemic pattern of *PI-II* mRNA accumulation in the absence of wounding (Peña-Cortés et al., 1988; Peña-Cortés and Willmitzer, 1995). These studies were further demonstrated by several investigations on ABA deficient mutants to evaluate the involvement of ABA on expression of wound-inducible genes. For example, the exogenous application of ABA was able to induce the *PI-II* induction in ABA deficient mutants of tomato (*sitiens*)

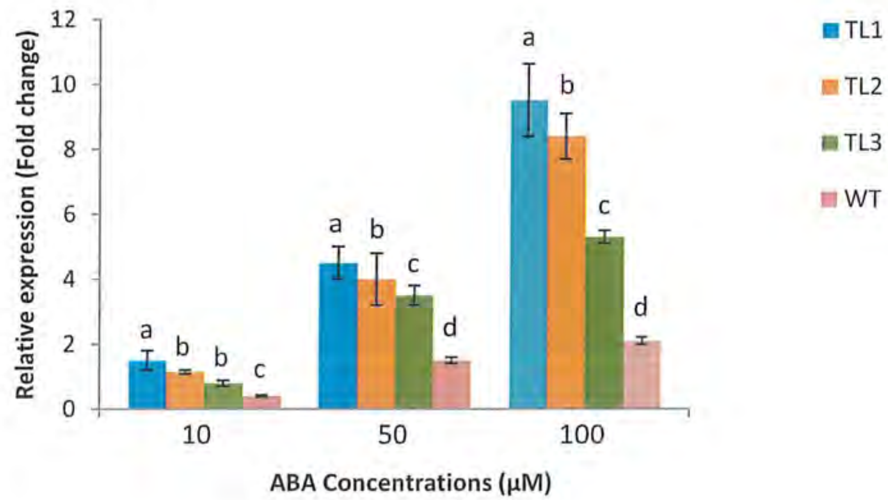


Figure 15: Expression profile of *PI-II* gene in transgenic lines and WT in response to ABA treatment with different concentrations. The data is the means ± SE of three replicates (n=3). The letters on each bar within each treatment indicate the significant differences at P<0.01, and bars sharing a common letter are not significantly different.

and potato (*droopy*), however no activation of *PI-II* gene was observed with wounding in mutant potato or tomato plants deficient in the synthesis of ABA (Pena-Cortes et al., 1989). Similarly, ABA deficient mutants (*abi1* allele) of *Arabidopsis* prevent the accumulation of *PI-II* transcripts in transgenic tomato plants by blocking the ABA signal transduction pathway (Carrera and Prat, 1998). However, experiment using ABA deficient *flacca* mutants revealed that expression of *PI-II* gene was not dependent of exogenous ABA (Chao et al., 1999).

The up-regulation of *PI* genes in response to exogenous ABA treatment under constitutive promoters has been observed in many reports. Earlier, Xu et al. (1993) studied the systemic induction of potato PI-II promoter fused to *GUS* reporter gene in transgenic rice plants by applying 100 μM ABA after 24 hours. Similarly, Kim et al. (2001) observed the effect of exogenous ABA treatment (50 μM) on the expression of *CaPI-2* in pepper and demonstrated elevated *CaPI-2* expression after 12 hours. In another report, transgenic tobacco plants overexpressing the *Nicotiana benthamaiana* trypsin inhibitor gene (*NtPI*) under transcriptional control of *CAM*35S promoter showed steady and rapid mRNA expression within 3 hours of ABA (100 μM) treatment and kept increasing till 24 hours (Srinivasan et al., 2009). It is notable that, there is no previous report for the investigation of *PI-II* gene under the regulation of a wound inducible plant origin promoter (*Os*RGLP2) in response to ABA treatment in transgenic plants. However, the efficacy of GLPs promoter in response to different signaling molecules including auxin, gibberellin (Berna et al., 1997; 1999; Yin et al., 2009), SA, MeJA, and ABA (Tabuchi et al., 2003; Bai et al., 2014) has been demonstrated in numerous studies. In accordance with the previous results, target gene expression analysis in the present study was up-regulated under *Os*RGLP2 promoter in response to ABA stress treatment.

3.2.3.1.1 *GUS* expression analysis in response to ABA treatment

The results of *GUS* staining experiment in TL1 line also revealed higher expression pattern in response to ABA treatment (100μM) (Figure 16). Microscopic examination of histochemically stained leaves of T_1 transgenic line (TL1) showed strong *GUS* expression in midrib, veins and mesophyll cells, while slight *GUS* expression was observed in stomatal guards cells (Figure 17). Moreover, strong *GUS* expression was found in vascular bundle and in the cortical cells of stem (Figure 18). In roots *GUS* activity was

noted in vascular tissues, and root hairs (Figure 19). Similar *GUS* activity was observed by Singh et al. (2009) with chymotrypsin protease inhibitor (OCPI2) promoter fused to *GUS* reporter gene transformed in calli of rice plants following treatment with ABA (10 μM) via histochemical and flourimetric analysis. In that study, the results of *GUS* staining revealed high expression in the vascular bundles and epidermal layers of roots, while in shoots expression was seen predominately in vascular bundles. In the same study, analysis of flourimetric *GUS* assay showed constitutive *GUS* expression both in root and shoot tissues in response to 100 μM ABA treatment. The results of the present study clearly indicated that *GUS* gene showed strong induction under *Os*RGLP2 promoter in roots, shoots and also in leaves in response to ABA. Similar to these results, Yang et al. (2013) analyzed the regulation of the GLP13 promoter-GUS gene construct via expression analysis in *Arabidopsis thaliana* and *Nicotiana tobacum* and revealed higher expression in vascular tissues mainly in phloem tissues. In another report, Sassaki et al. (2014) observed transient *GUS* expression in cotyledons, shoot apical meristem, and leaf vasculature with *Eg*GLP-GUS construct.

3.2.3.2 Induction of *PI-II* in response to MeJA

Jasmonates (JA/MeJA) are vital signaling molecules that are wide spread in plant kingdom and involved in the activation of many defensive genes including *PI* and pathogenesis-related (PR) genes (Turner et al., 2002) as well as in processes related to plant growth and development. In order to verify the role of MeJA in the induction of *PI-II* gene, the relative expression of target gene was analyzed in selected transgenic lines (TL1, TL2 and TL3) and WT by qPCR. The results showed significant difference in expression level of *PI-II* gene in transformants and WT following the exogenous MeJA treatment at certain particular concentrations (Figure 20).

Overall, the transcript levels of transgene were transiently induced by MeJA and vary significantly with concentrations of MeJA in transgenics and WT ($P<0.01$). After increasing the concentration from 10 μM to 50 μM MeJA, the expression levels of all lines gradually increased that reached to the maximum level at 50 μM MeJA and then declines at 100 μM MeJA. Compared to WT, a significant higher expression level was noted at 50 μM MeJA in all transgenic lines that was about 8.2 folds for TL1, 7.8 folds for TL2 and 4.5 folds for TL3, while 10 μM MeJA and 100 μM MeJA treatments resulted

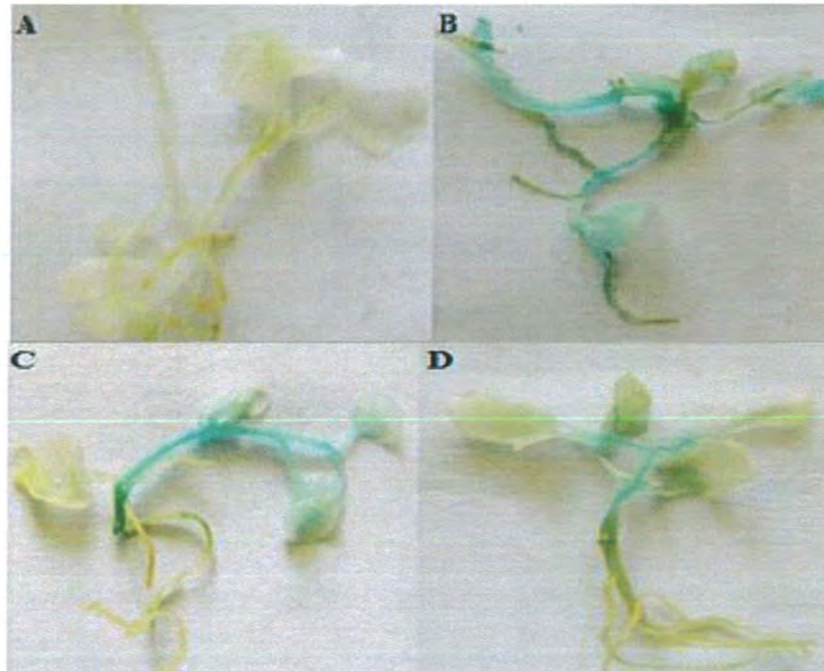


Figure 16: *GUS* expression in response to ABA treatment at different concentrations after 24 hours. (A) Untreated WT seedling as control, (B) Transgenic seedling at 100 μM ABA, (C) Transgenic seedling at 50 μM ABA, (D) Transgenic seedling at 10 μM ABA.

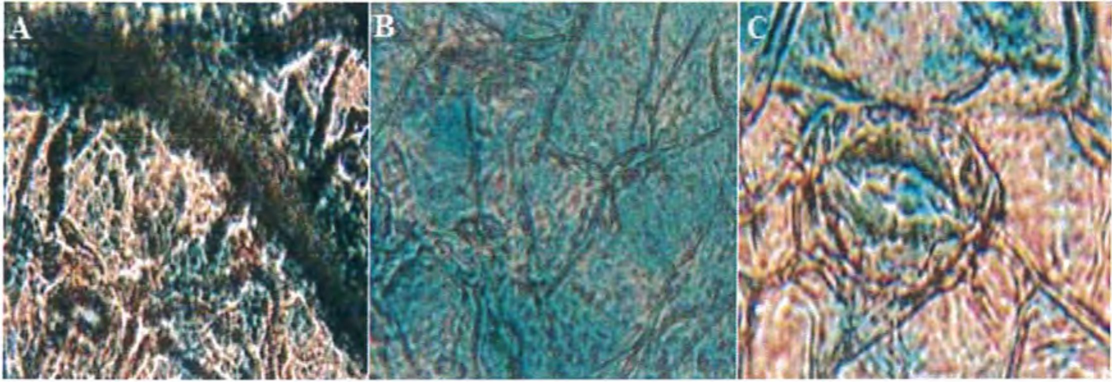


Figure 17: *GUS* expression in the leaf tissues under ABA stress. A) *GUS* expression in vascular bundles, B) *GUS* expression in mesophyll cells, C) *GUS* expression in stomatal guard cells.

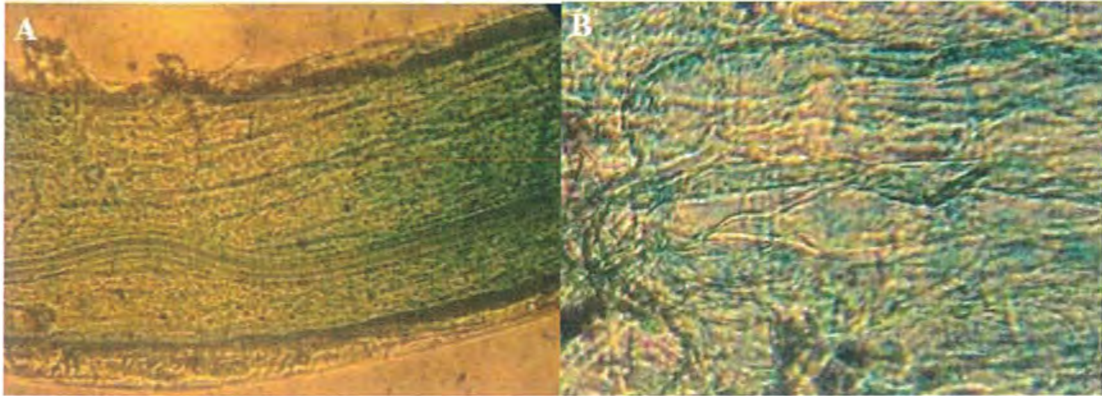


Figure 18: *GUS* expression in the stem under ABA stress. A) *GUS* expression in vascular bundles, B) *GUS* expression in cortical cells.

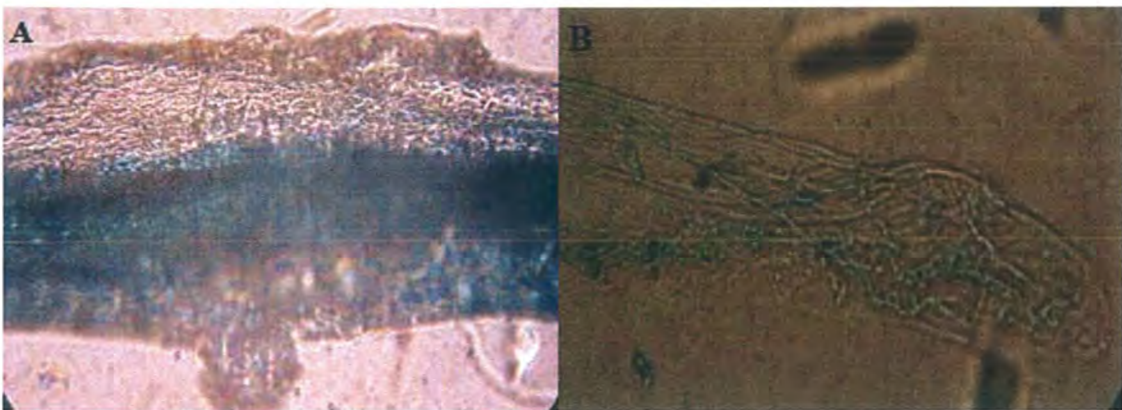


Figure 19: *GUS* expression in the root under ABA stress. A) *GUS* expression in vascular bundles, B) *GUS* expression in root hairs.

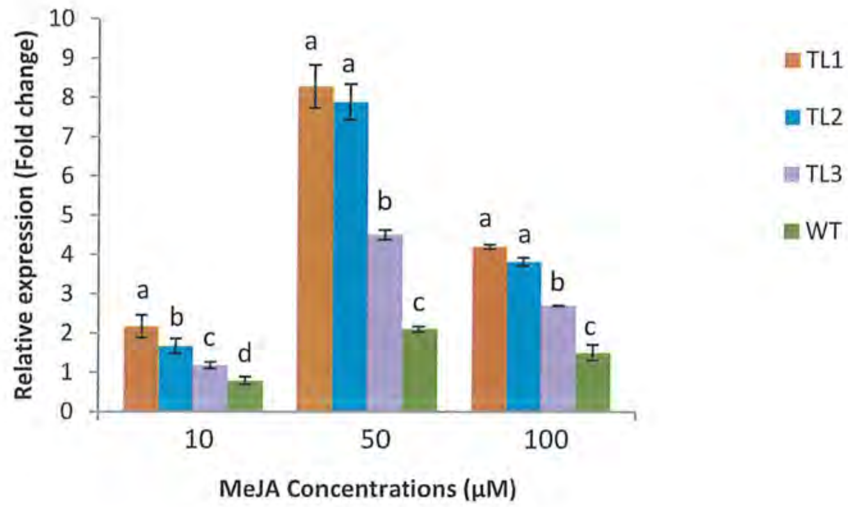


Figure 20: Expression analysis of *PI-II* gene in transgenic lines and WT in response to MeJA treatment with different concentrations. The data is the means ± SE of three replicates (n=3). The letters on each bar within each treatment indicate the significant differences at P<0.01, and bars sharing a common letter are not significantly different.

in decreased *PI-II* activity (Figure 20). However, transcript levels of all transgenic lines were comparatively higher at 100 μM MeJA than at 10 μM MeJA. Moreover, among all transgenic lines tested, TL1 was highly responsive to MeJA treatment at all concentrations. This transcriptional activation of *PI-II* gene in response to MeJA treatment clearly indicates the positive role of MeJA in the regulation of stress responsive genes.

The potential role of JA or MeJA in regulating the expression of *PI* genes has been the subject of intense research which has been reviewed in many studies (Doares et al., 1995; Sun et al., 2011). Earlier studies concluded that MeJA or JA application strongly induced the wound-inducible *PI-I* and *PI-II* in potato, tobacco and alfalfa (Farmer et al., 1992; Peña-Cortés et al., 1995). Mutant analysis and traditional grafting experiments have indicated that JA is the long distance mobile signaling molecule required for expression of defense related gene. In a report, the two tomato mutants (JL1 and JL5) were shown to be deficient in the systemic induction of *PI-I* and *PI-II*; however these mutants were able to induce the PI synthesis in response to MeJA application application (Lightner et al., 1993).

In further studies, it was observed that treatment with MeJA or intermediates of JA biosynthetic pathway lead to the plant responses similar to those caused by ABA treatments in both tomato and potato leaves (Hildmann et al., 1992; Peña-Cortés et al., 1992). In case of tomato serine *PI-II (TPI-2),* treatment with JA and α-Linolenic acid (LA) resulted in significant up-regulation of *TPI-2 (*Zhang et al., 2004). In another study Moura and Ryan (2001) demonstrated that wounded *Capsicum annuum* plants exposed to MeJA vapours, accumulated high *PI* transcript after 12 hours. Likewise, the exogenous application of JA (40 μM) on barley has been shown to trigger the proteinase inhibitory activity after 24 hours (Casaretto et al., 2004). In another report, 100 μM MeJA treatment has elevated the transcript level of trypsin inhibitor (*NtPI*) in tobacco leaves after 24 hours (Srinivasan et al., 2009). Moreover, jasmonate-treated plants have been shown to confer broad resistance against biotic attackers such as insects, nematodes and necrotrophic pathogens (Brader et al., 2001; Tierranegra-García et al., 2011; Yamada et al., 2012). Even it was found that plants growing from seeds previously exposed to JA were more tolerant towards herbivory (Worrall et al., 2012). This increased resistance

was ascribed due to enhanced induction of PIs and other plant toxins. All these reports strongly support that MeJA is a key regulator in plant stress responses.

3.2.3.2.1 *GUS* expression analysis in response to MeJA treatment

The histochemical *GUS* assay in TL1 also revealed strong expression at 50 μM MeJA which decreases following the treatment with 10 μM MeJA and 100 μM MeJA (Figure 21). The similar response was observe in transgenic *Solanum brevidens* plants expressing the Pin2-*GUS* construct in which 50 μM MeJA treatment resulted in the induction of *GUS* activity in the leaves of transgenic plants after 12 hours which steadily increases through 24 or 48 hours (Liu et al., 1996). Microscopic observation showed widespread *GUS* activity in leaves, stem and roots in response to MeJA application in transgenic plants. *GUS* staining revealed diffused expression in leaves and was mainly observed in the vascular bundles, mesophyll cells, and stomata guard cells (Figure 22). In stem a more uniform and stable *GUS* expression was detected in both cortical and vascular regions (Figure 23). Moreover, outer region of vascular bundles was found to have more *GUS* expression. Similarly in roots, *GUS* activity was predominately associated with the root hairs, root cells and vascular tissues (Figure 24). The intensity of *GUS* expression in vascular tissue was comparatively more than any other parts in roots. In a previous report, Liu et al. (1996) detected *GUS* expression only in leaves of transgenic *Solanum brevidens* plants using Pin2-GUS construct after MeJA treatment. Similarly, analysis of *Sa*PIN2b promoter fused with *GUS* gene showed *GUS* activities in leaves of transgenic tobacco and nightshade plants (Liu et al., 2006). However, in the present results, *GUS* activities were observed in all parts of plants which showed that *Os*RGLP2 promoter was strongly induced in response to MeJA treatment.

3.2.3.3 Response of T_1 progenies to salt stress

Tolerance to salt stress in transgenic plants was evaluated by measuring the transcript levels of transgene through qPCR and by analyzing their phenotypic responses at different concentrations of salt stress treatments.

3.2.3.3.1 Expression analysis

The quantitative real time analysis revealed that level of transgene expression was significantly varied ($P<0.01$) among transgenic lines and WT in response to different

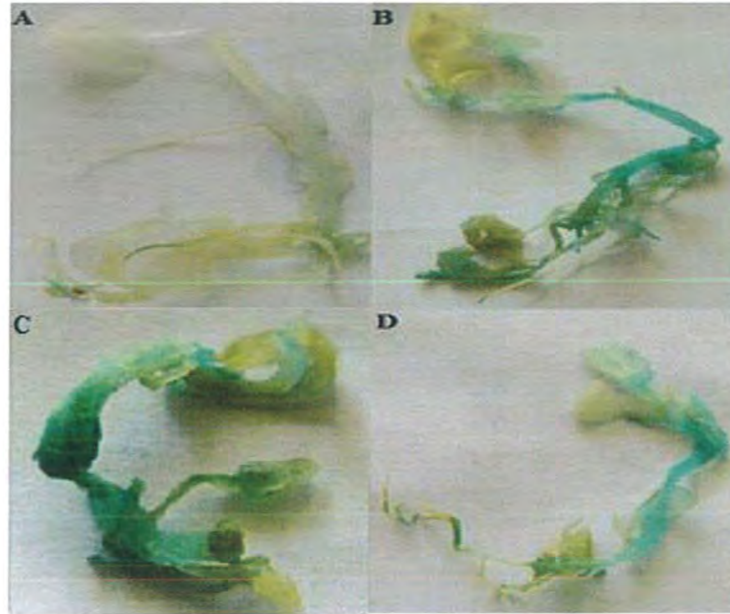


Figure 21: *GUS* expression in response to MeJA treatment at different concentrations after 24 hours. (A) Untreated WT seedling as control, (B) Transgenic seedling at 100 μM MeJA, (C) Transgenic seedling at 50 μM MeJA, (D) Transgenic seedling at 10 μM MeJA.

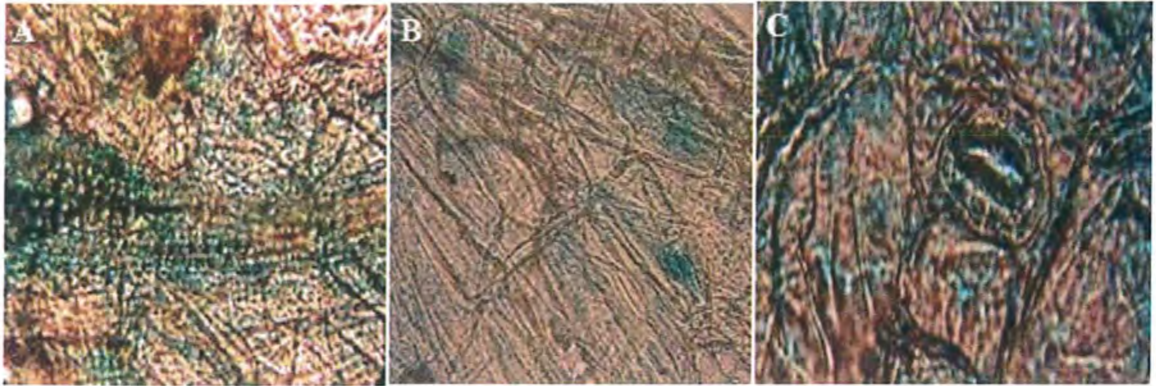


Figure 22: *GUS* expression in the leaf tissues in response to MeJA treatment. A) *GUS* expression in vascular bundles, B) *GUS* expression in mesophyll cells, C) *GUS* expression in stomatal guard cells.

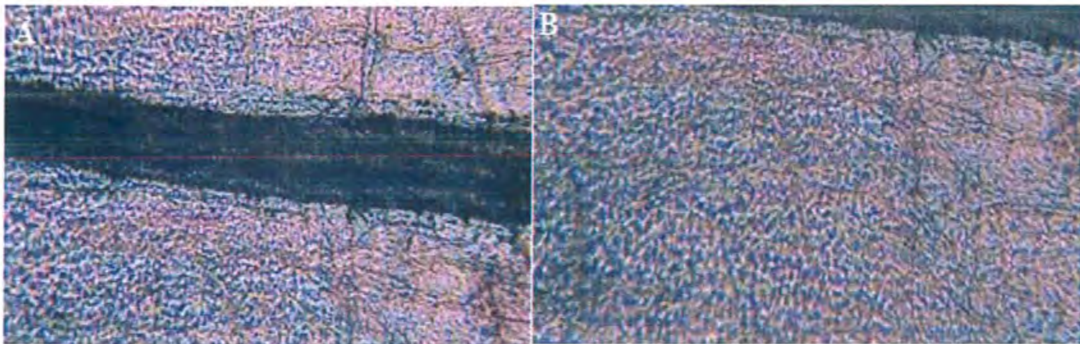


Figure 23: *GUS* expression in the stem in response to MeJA treatment. A) *GUS* expression in vascular bundles, B) *GUS* expression in cortical cells.

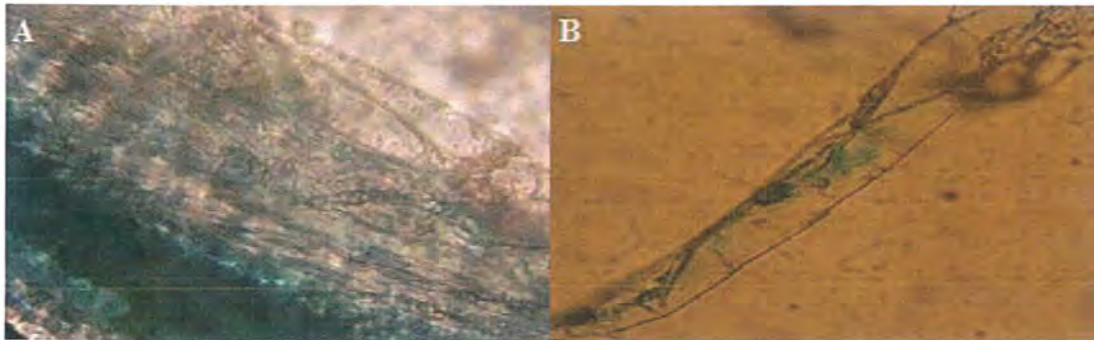


Figure 24: *GUS* expression in the root in response to MeJA treatment. A) *GUS* expression in vascular bundles, B) *GUS* expression in root hairs.

concentrations of NaCl (Figure 25). After 24 hours of salt treatment, comparatively higher induction of 7.4, 5.6 and 5.1 folds were observed in TL1, TL2 and TL3 respectively at 100 mM NaCl. However, expression level of PI-II gene was found to decrease at concentration higher than 100 mM NaCl. In addition, TL1 showed significantly higher expression over the other transgenic lines and control plants under different levels of salt treatments. Similar moderate level of up-regulation of PI gene was observed in three transgenic lines expressing *NtPI* using *CaMV35*S promoter at elevated salt stress condition (Srinivasan et al. 2009). In a similar study, a cysteine PI gene (JcCPI) from *Jatropha curcas* under control of 2XCaMV35 promoter was strongly induced by salt stress in three transgenic tobacco lines (Li et al. 2015). In an earlier study, Kim et al. (2001) reported a significant up-regulation of a serine PI (*CaPI-2*) in red pepper under salt stress. In another report, Shan et al. (2008) showed that salt tolerance in wheat has improved using a Bowman-Birk type PI. Furthermore, transgenic Arabidopsis plants over expressing a rice OCPI2 gene were found to be more tolerant to salt stress as compared to wild type (Tiwari et al. 2015). The present results correspond well with previous reports, thus indicating the positive role of PI genes in conferring resistance against salt stress conditions. Since OsRGLP2 promoter contain several putative stress responsive regulatory regions (Mahmood et al. 2013; Munir et al. 2016), which might play important role in up-regulating stress responsive genes including PI-II gene.

3.2.3.3.2 *GUS* expression analysis in response to salt stress

In case of salt stress, *GUS* activity was checked under salt stress in TL1 and WT plants at indicated concentrations, where too much salt stress beyond 200 mM resulted in decreased *GUS* activity in transgenic line (Figure 26), while no *GUS* activity was observed for WT plants. The leaves of transgenic plants showed diffused *GUS* expression in midrib (central cylinder), veins, and mesophyll cells by microscopic analysis (Figure 27). Previously, strong *GUS* activity was detected in the leaves of transgenic rice plants expressing OCPI1 promoter::GUS construct (Huang et al., 2007). In stem, an intense *GUS* expression was observed in both vascular region and cortical region (Figure 28) suggesting the importance of these parts during salt stress (Singh et al., 2009). In roots *GUS* activity was observed in central cylinder and root hairs (Figure 29) but low in cortical region which may be due to fact that xylem and phloem serve as passage for food

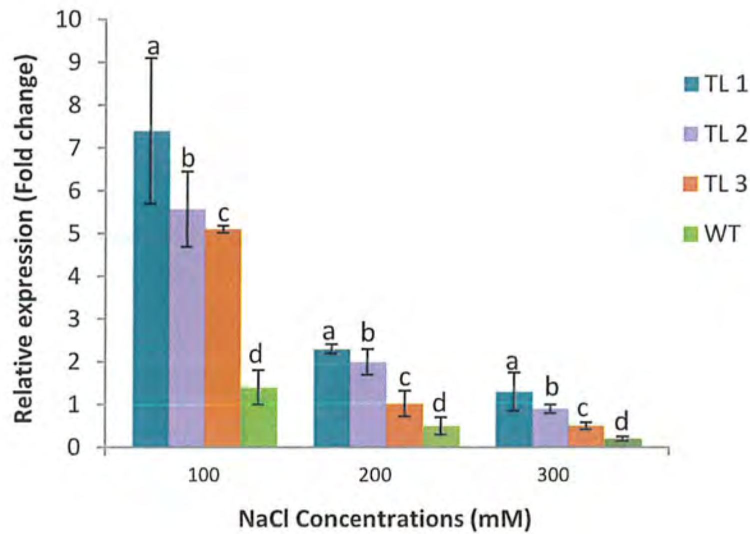


Figure 25: Expression profile of *PI-II* gene in transgenic lines under salt stress. The data presents is the means ± SE of three replicates (n=3). The letters on each bar within each treatment indicate the significant differences at $P<0.01$.

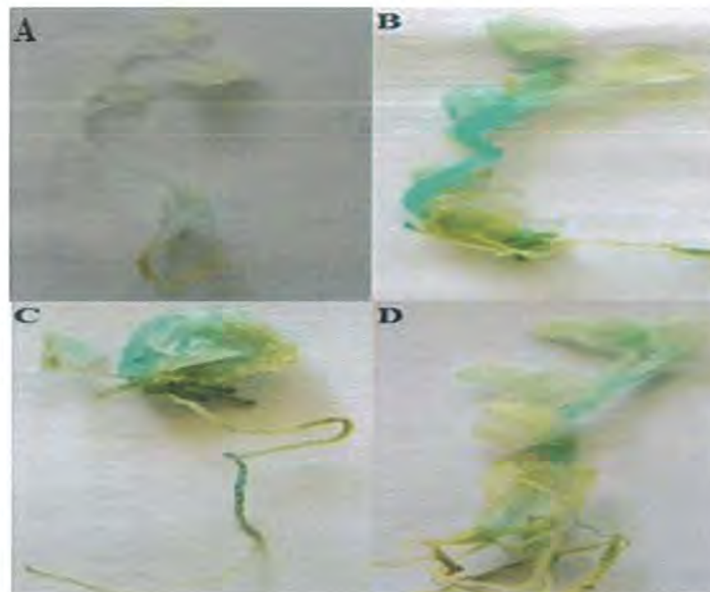


Figure 26: *GUS* expression under salt stress after 24 hours. Transgenic T_1 seedlings were submerged in MS basal salt solution at different concentrations (100 mM NaCl, 200 mM NaCl and 300 mM NaCl). (A) Untreated WT seedling as control, (B) Transgenic seedlings at 100 mM NaCl, (C) Transgenic seedlings at 200 mM NaCl, (D) Transgenic seedlings at 300 mM NaCl.

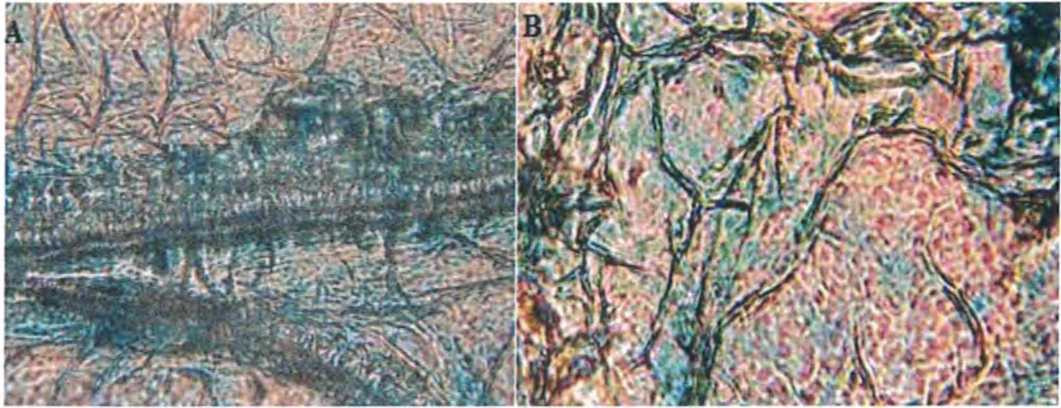


Figure 27: *GUS* expression in the leaf tissues under salt stress. A) *GUS* expression in vascular bundles, B) *GUS* expression in mesophyll cells.

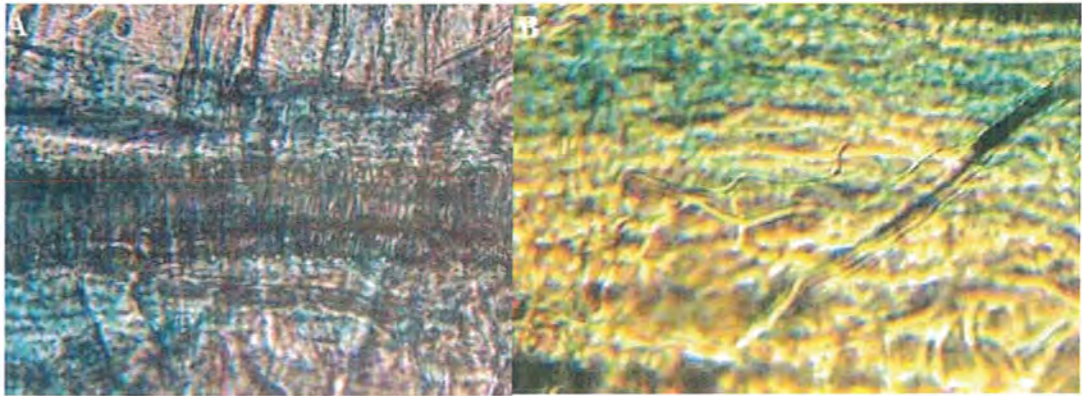


Figure 28: *GUS* expression in the stem under salt stress. A) *GUS* expression in vascular bundles, B) *GUS* expression in the cortical cells.

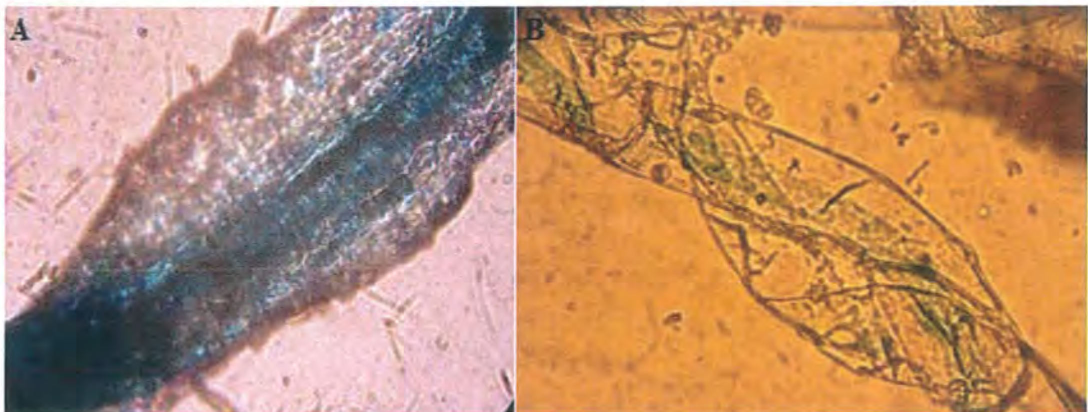
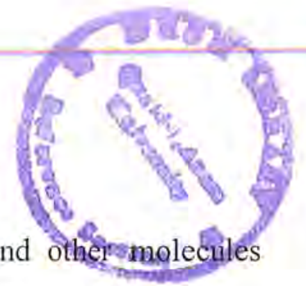


Figure 29: *GUS* expression in the root under salt stress. A) *GUS* expression in vascular bundles, B) *GUS* expression in root hairs.



and water transport and root hairs control the inflow of water and other molecules (Turgeon and Wolf, 2009). Such shoot and root specific expression was also noted for *Oryza sativa* chymotrypsin protease inhibitor (OCPI2) promoter fused to *GUS* gene (Singh et al., 2009). The transgenic rice plants expressing *GUS* gene driven by OCPI2 promoter showed strong *GUS* expression in epidermal layer and vascular bundles of root, while in shoots the *GUS* expression was mainly found to be associated with vascular bundles only (Singh et al., 2009). In the present results, it was observed that salt stress has significantly activated the *Os*RGLP2 promoter activity that induces the *GUS* expression in stem, roots and also to some extent in leaves.

3.2.3.3.3 Phenotypic analysis

It is well known that salt stress induces several biochemical and physiological responses in plants and affects almost all functions including growth and development (Nemoto and Sasakum, 2002). In present study, transgenic lines and WT displayed prominent phenotypic differences after exposure to different salt stress treatments (Figure 30). It was observed that the presence of salt has severely affected the overall growth of WT at all concentrations compared with transgenic lines. After three weeks of transfer to 100 mM NaCl, *PI-II* expressing transgenic plants exhibited better growth with significantly increased seedlings height when compared with WT. Under 200 and 300 mM treatments, there was chlorosis and stunted phenotypes in both non-transgenic controls and transgenics (Figure 30). As compared to WT seedlings, most of transgenic seedling showed higher tolerance at elevated concentrations (200 and 300 mM NaCl). However, growth response of transgenics was better at lower concentration. Previously, the same phenotypic effect was illustrated in transgenic plants transformed with a tobacco PI (NtPI) gene (Srinivasan et al. 2009) and a rice chymotrypsin proteinase inhibitor (OCPI2) gene (Tiwari et al. 2015).

Salinity tolerance in transgenic plants can be manifested by measuring chlorophyll content as it can be used to show good correlation with effect of salinity levels (Srivastava et al., 1988; Ali et al., 2004). There was significant difference in chlorophyll content between WT and transgenics ($P<0.01$; Figure 31) after exposing the seedlings to different concentrations of NaCl, which indicated the better metabolic status of transgenic lines over the non-transgenic control under salt stress. At low NaCl

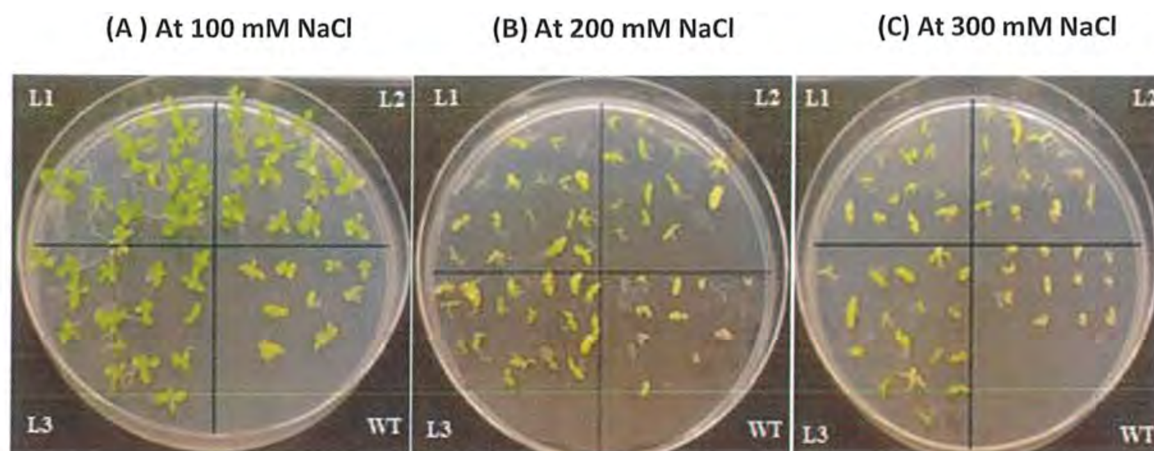


Figure 30: Phenotypic response of transgenic seedlings and wild type under salt stress. (A) Growth response of transgenic lines and WT at 100 mM NaCl. (B) Growth response of transgenic lines and WT at 200 mM NaCl. (C) Growth response of transgenic lines and WT at 300 mM NaCl.

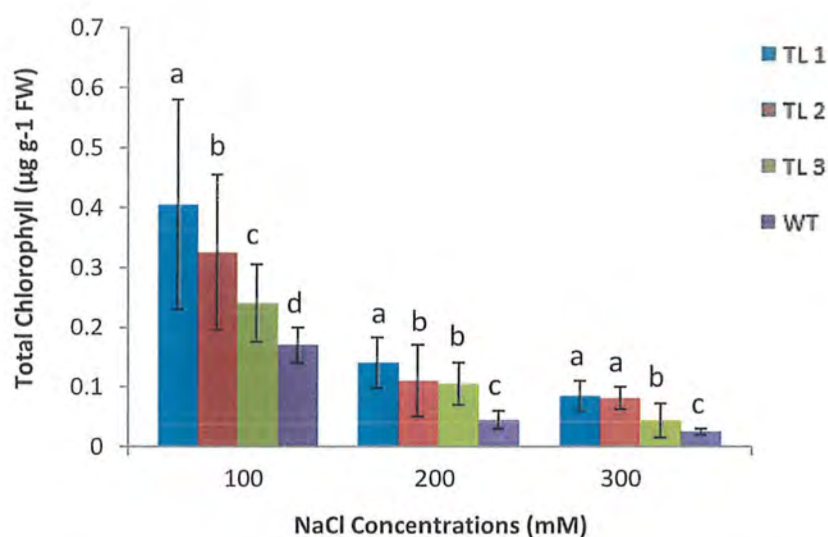


Figure 31: Chlorophyll content of wild type plants and transgenic lines under salt stress after 14 days of stress treatment. The data is the means ± SE of three replicates (n=3). The letters on each bar within each treatment indicate the significant differences at P<0.01, and bars sharing a common letter are not significantly different.

concentration (100 mM NaCl), the highest chlorophyll content was shown by TL1 as compared to the other transgenic lines and WT plants (Figure 31). Furthermore, TL2 and TL3 did not show any significant difference in total chlorophyll content in presence of 200 mM NaCl. Likewise, no notable variation for total chlorophyll content was observed in TL1 and TL2 at 300 mM NaCl. Additionally, treatment with elevated NaCl concentrations (200 and 300 mM) resulted in chlorophyll reduction in both transgenic and WT seedlings. However, chlorophyll reduction in transgenic seedlings was comparatively less than the WT as transgenic lines remained green and withstand the salinity stress for a longer period at concentration higher than the 100 mM NaCl. Overall, WT seedlings showed up to 84 % reduction in chlorophyll content with an increase in NaCl concentration (100 to 300 mM). However, an average decline of 63 %, 73 % and 77 % in chlorophyll content was noted for TL1, TL2 and TL3 respectively (Figure 31). In a related study, an increase in chlorophyll content was demonstrated in transgenic *Arabidopsis* plants overexpressing a novel cystatin gene (*MpCYS5*) from *Malus prunifolia* with treatment of 200 Mm NaCl concentration (Tan et al. 2015). Similar response was observed by Jamussi et al. (2014) in transgenic plants overexpressed with stress responsive gene *VvRD22* when compared with WT in presence of 400 Mm NaCl concentration.

With regard to fresh weight, significant difference was observed in average fresh weight of WT and transgenic plants at all NaCl concentrations ($P<0.01$; Figure 32), showing over all enhanced growth performance of transgenic lines compared to WT plants under salt stress conditions. Notably, among the transgenic lines tested, the difference in average fresh weight between TL1 and TL2 was non-significant at 100 mM NaCl treatment. Similarly, TL2 and TL3 also showed negligible difference in average fresh weight upon exposure to 300 mM NaCl concentrations. Moreover, the average fresh weight gradually decreased after increasing NaCl concentrations (200 and 300 mM NaCl). However, average fresh weight in WT plants was greatly reduced as compared to transgenic plants (Figure 32). Between 100 to 300 mM NaCl, there was 48 %, 50 %, 44 % and 61 % reduction in average fresh weight shown by TL1, TL2, TL3 and WT respectively. In a similar study conducted by Srinivasan et al. (2009), a comparative higher fresh weight was shown by transgenic plants constitutively expressing the NtPI

gene than WT plants under osmotic stress. In another report, an increased in salt tolerance was demonstrated for transgenic Arabidopsis plants by overexpressing two cysteine proteinase inhibitor (*AtCYSa* and *AtCYSb*) having better growth and average fresh weight of transgenics in comparison to WT (Zhang et al. 2008).

Moreover, accumulation of proline has been suggested a common phenomenon in plant growing under various stresses such as drought and salt (Zhang et al., 2013; Zhang et al., 2015). Therefore, an increase in proline content under salt stress can be a vital criterion for evaluating plant salt tolerance. In present study, overall proline content in PI-II expressing transgenic plants was significantly ($P<0.01$) higher than the WT at all NaCl concentrations tested (Figure 33), thus documenting better osmotic adjustment in PI-II expressing transgenic plants under salt stress. After 100 mM NaCl treatment proline content gradually increases and then declines at elevated concentrations. Overall, there was gradual decreased in proline content both in transgenic line and WT at 200 and 300 mM NaCl, but proline content in WT plants was comparatively lower than transgenics after increasing NaCl concentrations (Figure 33). In presence of elevated concentrations (100 to 300 mM NaCl), the average decline in proline content was 31.14 %, 38.6 %, 53.3 % and 59.21 % in TL1, TL2, TL3 and WT respectively. At 300 mM NaCl, the total proline content of TL1, TL2 and TL3 were 80.25 %, 73.27 % and 59.74 % respectively, with respect to WT. These results of present study suggested that transgenic lines were comparatively more tolerant than control plants under salt stress conditions. Similarly, Tiwari et al. (2015) reported higher proline content in transgenic plants overexpressing *OCPI2* gene at high salt concentrations. In general, protein degradation and recycling are observed in plants that are subjected to abiotic stress conditions (Ingram and Bartels, 1996; Sahi et al., 2006). Protein degradation can be controlled by curbing proteases and PIs are said to inhibit this protease activity. Transgenic plants constitutively expressing the PIs have advantage of increased proteinase inhibitory activity and enhanced stress tolerance (Huang et al., 2007; Srinivasan et al., 2009). The current study showed that transgenic plants constitutively *PI-II* gene under *Os*RGLP2 promoter might resulted in increased *PI-II* activity under salt stress which ultimately leads to enhance salt tolerance in transgenic plants.

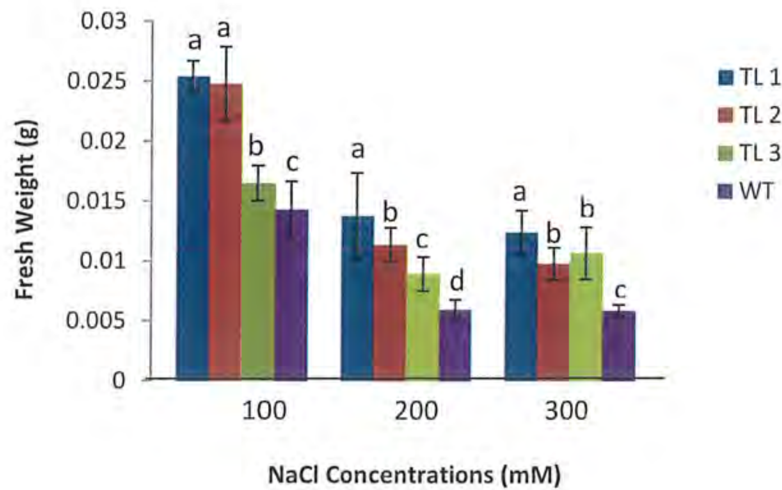


Figure 32: Average fresh weight of WT and transgenic lines under salt stress after 14 days of stress treatment. The data is the means ± SE of three replicates (n=3). The letters on each bar within each treatment indicate the significant differences at P<0.01, and bars sharing a common letter are not significantly different.

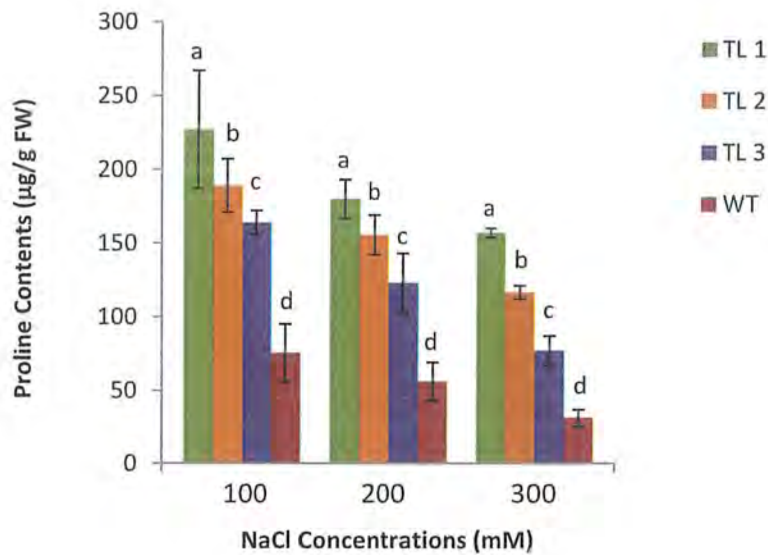


Figure 33: Proline content of wild type plants and transgenic lines under salt stress after 14 days of stress treatment. The data is the means ± SE of three replicates (n=3). The letters on each bar within each treatment indicate the significant differences at P<0.01.

3.2.3.4 *GUS* expression analysis in response to wounding stress

Plants have evolved wound related defense system to cope with various forms of physical damages and biotic factors. Since the wound-inducibility of *Os*RGLP2 promoter is already known (Mahmood et al., 2013; Munir et al., 2016), therefore, induction of *Os*RGLP2 promoter was also investigated in 15 days old T_1 transgenic tobacco seedlings at indicated time period by analyzing the histochemical *GUS* assay in response to wounding.

Overall results showed uniform *GUS* expression in transgenic plants following wounding treatment at all indicated time period (Figure 34). Upon wounding, *GUS* activity was found to increase with an increase in time period reaching to a maximum level after 36 hours. Moreover, the present results revealed that wounding induces the expression of *GUS* gene not only in wounded leaves, but also in the leaves distant from wounded site suggesting the systemic wound-induced induction of *Os*RGLP2 promoter. The similar systemic wound induced response was reported in transgenic rice plants by introducing the Pin2-GUS construct, however very low expression was observed in that case (Xu et al., 1993). It has been established that many defensive traits are expressed in plants distal to the site of injury as a result of wounding (Green and Ryan, 1972). The combined effect of local and systemic defense responses provide broad-spectrum resistance against biotic attacks, and constitute a form of induced immunity (Jones and Dangl, 2006; Howe and Jander, 2008; Fu and Dong, 2013).

In an earlier study, Keil et al. (1989) also demonstrated higher *GUS* expression in leaves, floral bud and tubers upon wounding with a fusion of Pin2 promoter and *GUS* reporter gene. Likely, the two *PI-II* genes of *Solanum american* designated as *SaPIN2*a and *SaPIN2b* were also shown to be up-regulated in response to wounding (Xu et al., 2001). Furthermore, the expression of *Sa*PIN2b promoter fused to a *GUS* gene was investigated in both transgenic nightshade plants and tobacco plants and significant increased *GUS* activity was observed by fluorometric assay in the leaves of transgenic plants in response to wounding treatment after 24 hours (Liu et al., 2006). In the same study, histochemical staining assay indicated that *SaPIN2b:GUS* expression was widespread in wounded leaves and prominent in the area surrounding the incision wounding site, while in unwounded leaves *GUS* activity was only present in the trichome

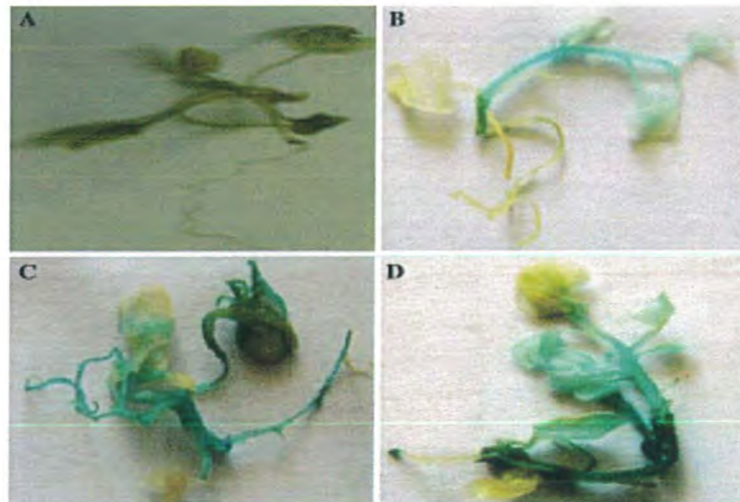


Figure 34: *GUS* expression of transgenic seedlings in response to wounding. (A) Untreated WT seedling as control, (B) *GUS* expression after 12 hours (C) *GUS* expression after 24 hours (D) *GUS* expression after 36 hours.

(Liu et al., 2006). Similar effect of wounding was detected for the expression of *CanPIs* of pepper plants in which both local and systemic leaves were found to have increased *CanPI* activity upon wounding (Tamhane et al., 2009; Mishra et al., 2012). In the light of above mentioned reports, it can be revealed that *Os*RGLP2 promoter has an active role in plant defense against pathogens or insect attacks, and therefore can be used to enhance the tolerance against biotic stresses.

Microscopic study revealed strong *GUS* expression in vascular bundles of leaves, stems and roots which may suggest that wounding signals are transported through vascular bundles (Keil et al., 1989; Xu et al., 1993). In leaves, *GUS* activity was detected in all parts such as leaf epidermis, guard cells, mesophyll cells, and midrib, which was comparable to an earlier study by Xu et al. (1993) where transgenic rice plants expressing the *GUS* gene driven by Pin2 promoter exhibited strong *GUS* expression in leaf epidermal guard cells, vascular tissues, leaf sheath and trichome upon wounding. After 12 hours of injury, diffused *GUS* expression was observed in vascular tissues and mesophyll cells of leaves which become intense with the passage of time (Figure 35). In stem, high level of *GUS* activity was detected in vascular bundles and relatively low *GUS* expression was observed in outer cells after 36 hours of injury (Figure 36). Similarly, in roots, microscopic analysis revealed that *GUS* activity was mainly associated with vascular bundles, root epidermal layer, and root hairs (Figure 37). Root showed prominent *GUS* expression in vascular region than cortex after 24 hours. However, *GUS* activity increases after 36 hours and become more uniform in cortex as well as in vascular bundles, while low expression was also detected in outer root epidermal layer (Figure 37). Such histochemical observations were also reported in both wounded leaves and roots of transgenic dicot plants transformed with pin2/Act1 intron/GUS construct (Keil et al., 1989) and in the leaves of transgenic nightshade plants expressing *SaPIN2b:GUS* construct (Liu et al., 2006) after 24 hours of wounding. In future, the wound inducible property of *Os*RGLP2 promoter can be of particular importance in driving tissue specific expression and producing insect resistant crops.

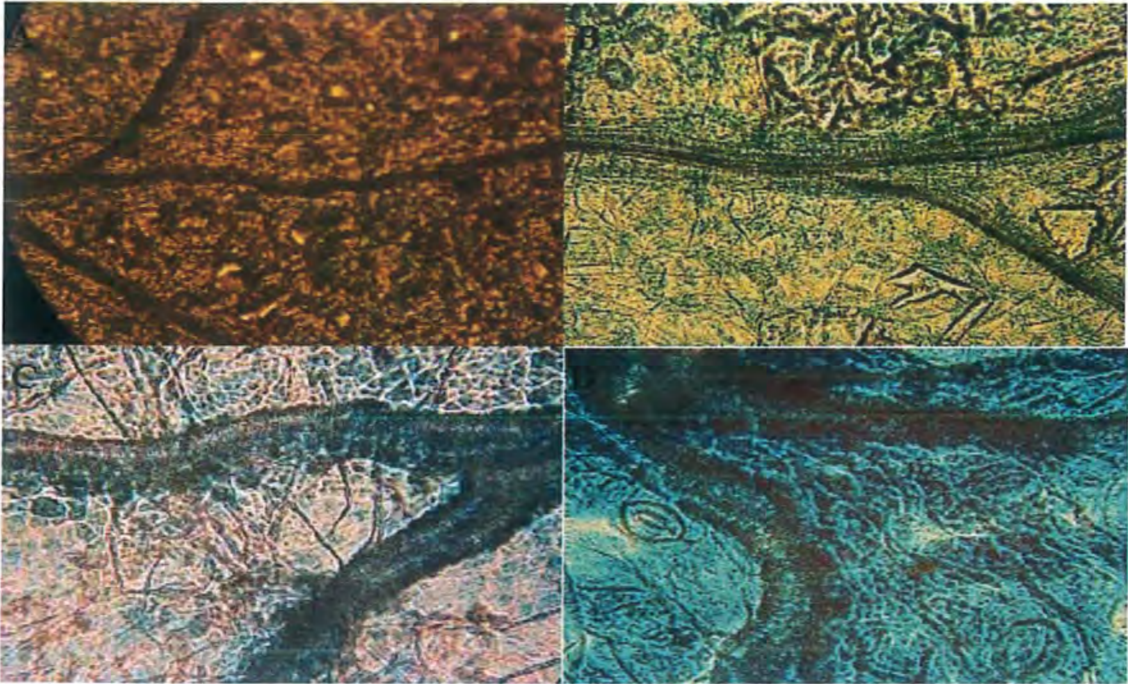


Figure 35: *GUS* expression in tobacco leaf tissues after wounding. A) No *GUS* expression in control, B) Low diffused *GUS* expression in midrib and surrounding tissues after 12 hours, C and D) High *GUS* activity in vascular bundles and stomatal guard cells after 24 and 36 hours.

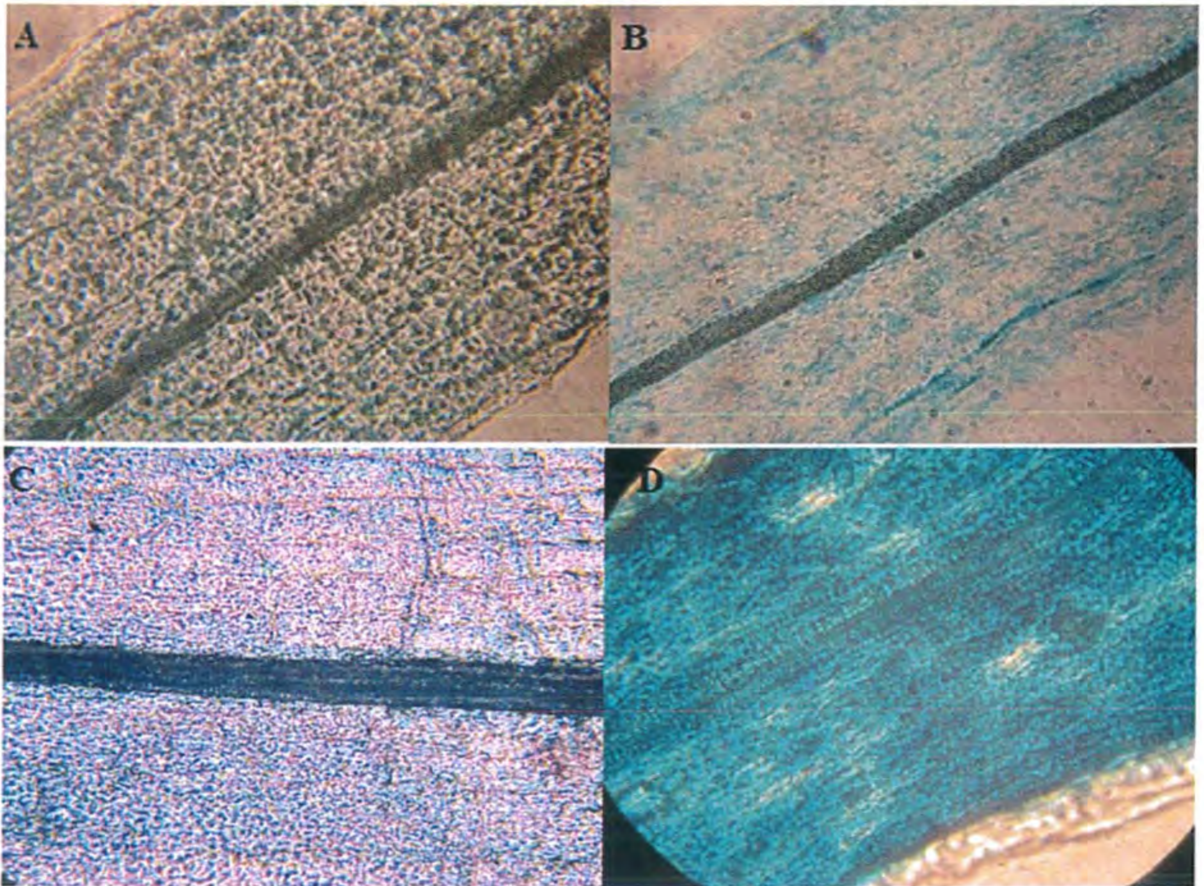


Figure 36: *GUS* expression in tobacco stem in response to wounding. A) No *GUS* expression in control, B) Diffused *GUS* expression in stem after 12 hours, C and D) *GUS* activity in stem after 24 and 36 hours.

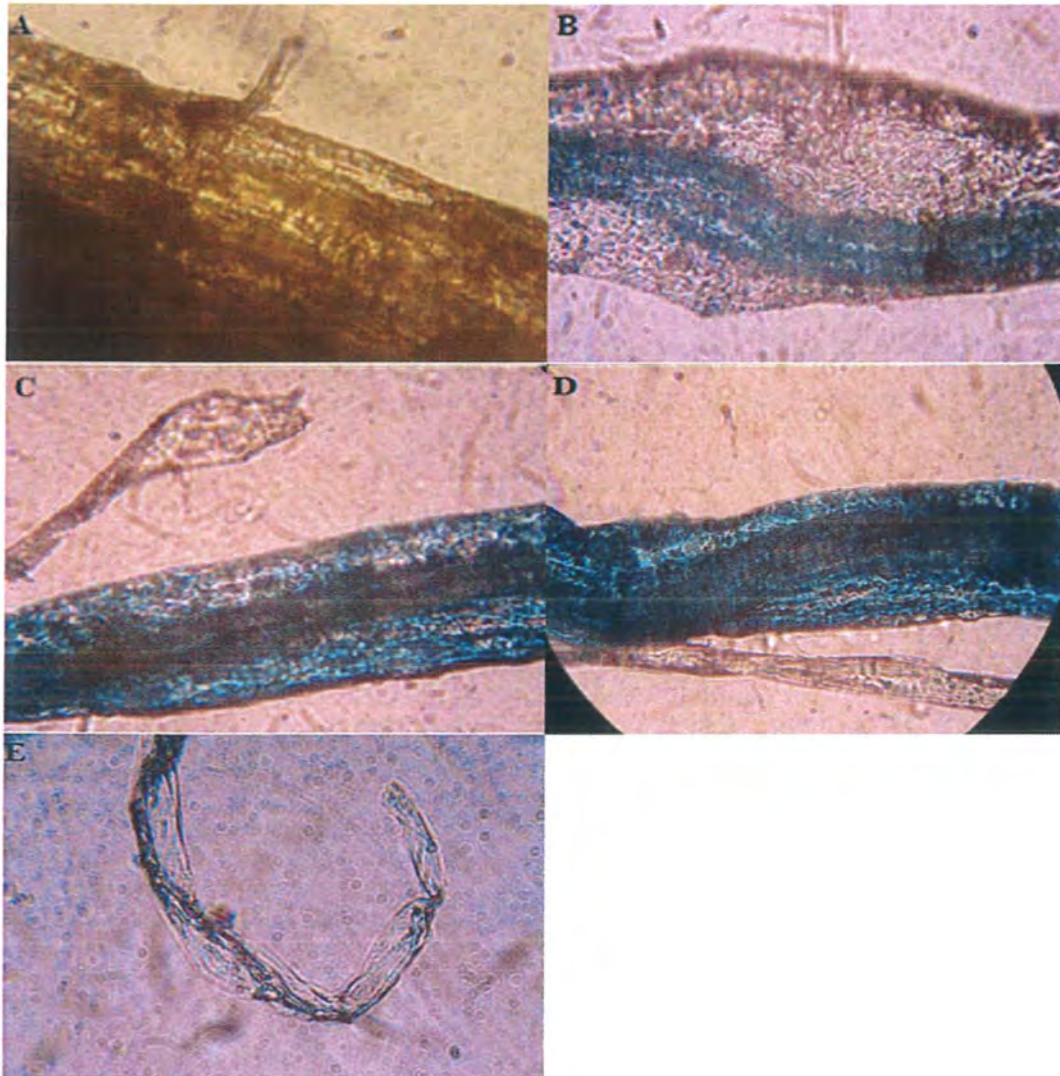


Figure 37: *GUS* expression in tobacco root in response to wounding. A) No *GUS* expression in control, B) *GUS* activity in root showing prominent expression in vascular tissues after 12 hours, C and D) *GUS* activity in stem after 24 and 36 hours, E) Slight *GUS* expression in outer epidermal layer and root hairs.

3.3 Conclusion

The results of *in silico* analysis confirmed the presence of highly conserved domains in all *PI-II* sequences. Based on phylogenetic studies, it was found that multi domain PI-II proteins in Solanaceae are most likely to be originated by duplication from ancestral single PI-II domain proteins. From expression analysis of tomato *PI-II* gene in transgenic plants, it was concluded that this gene under the regulation of *Os*RGLP2 promoter was responsive to all applied stresses such as ABA, MeJA and NaCl applications. Quantitative real time PCR analysis revealed considerable level of *PI-II* induction in response to both ABA and MeJA treatments. ABA triggered *Os*RGLP2 driven *PI-II* gene expression to a maximum level at higher concentration (100 μM) signifying its major role in plants during abiotic stress conditions. While in response to MeJA treatment, *PI-II* activity was stimulated up to a certain concentration (50 μM) identifying it as an effective elicitors to regulate many aspects of plants including plant defensive responses, plant growth and developmental processes. Moreover, under salt stress there was significant up-regulation of *PI-II* induction at low concentration (100 mM NaCl). However, too much salt concentration has reduced the expression of *PI-II* gene in transgenic plants. Hence it can be proposed that *PI-II* gene can be used as a potential candidate for generating transgenic crops resistant to both biotic and abiotic stresses. Furthermore, the relative *GUS* expression analysis in response to wounding, ABA, MeJA and NaCl gives clear indication towards the active role of *Os*RGLP2 promoter in up-regulating the downstream genes under stress conditions.

3.4 Future Perspectives

Following are the future perspectives regarding the use of presently achieved results;

- To characterize the functional role of *Os*RGLP2 driven *PI-II* gene for fungal and insect resistance in transgenic plants.
- To work out on the biotechnological relevance of the *PI-II* gene under *Os*RGLP2 promoter for other abiotic stresses in valuable crops.

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APPENDICES

Appendix-1

LB medium

Trypton	10g/L
Yeast extract	5g/L
NaCl	10g/L

pH was adjusted to 7.0

Appendix-2

Plasmid isolation from bacterial culture

1. A single colony of bacteria was inoculated into 3 ml of LB media containing the appropriate antibiotic in a test tube. The medium was incubated overnight at 37 °C for 16 hours in case of *E.coli* and for 36-48 hours at 28 °C in case of *A. tumefaciens* with vigorous shaking.
2. The culture was poured into an eppendorf tube and was pellet down by centrifugation at 12000 rpm for 60 seconds at 4 °C in a microfuge. The supernatant was poured out.
3. Bacterial pellet was re-suspended in 100 μl of ice-cold solution I by vigorous vortexing.

   Solution I

   50 mM Glucose

   25 mM Tris HCL (pH 8.0)

   10 M EDTA (pH 8.0)

   Solution I was prepared in batches of approximately 50 ml, autoclaved and then stored at 4°C.

4. Then 200 μl of solution II was added. The cap of the eppendorf was tightly closed and the contents were mixed by inverting the tube rapidly for five minutes. Then eppendorf was stored on ice.

   Solution II

   0.2 N NaOH (freshly diluted from a 10 N stock)

   1 % SDS

5. Then 150 μl of solution III was added. The eppendorf was again tightly closed and was vortexed in an inverted position for 10 seconds to disperse solution III through the viscous bacterial lysate. The eppendorf was again stored on ice for 3-5 minutes.

   Solution III

Potassium Acetate 5M	60 ml
Glacial Acetic Acid	11.5 ml
H2O	28.5 ml

6. The contents were centrifuged at 14000 rpm for 5 minutes at 4 °C in a microfuge. The supernatant was transferred to a new eppendorf.
7. An equal volume of phenol:chloroform was added in to the supernatant and contents were vortexed. Then eppendorf was centrifuged at 14000 rpm for 2 minutes at 4 °C in a microfuge. The supernatant was again transferred to new eppendorf.
8. The double stranded DNA was precipitated by adding an equal volume of isopropanol. The eppendorf was kept at room temperatures for 10 minutes and then centrifuged at 14000 rpm for 5 minutes at 4 °C.
9. The supernatant was removed by carefully inverting the eppendorf and the DNA in pellet form was washed with 70 % ethanol.
10. The pellet was air dried and was suspended in nanopure water followed by treatment with RNase. The dissolved pellet of DNA was stored at 20°C till further use.

Thesis

ORIGINALITY REPORT

%15	%7	%12	%0
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	J. Liu. "The Nightshade Proteinase Inhibitor IIb Gene is Constitutively Expressed in Glandular Trichomes", Plant and Cell Physiology, 07/20/2006 Publication	%1
2	scholar.sun.ac.za Internet Source	%1
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