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EXPRESSION ANALYSIS OF PROTEINASE INHIBITOR TYPE-2 IN RESPONSE TO SOUTHERN ROOT-KNOT NEMATODE AND ITs CHARACTERIZATION IN *Nicotiana tabacum*

Zeinalabedin SHAHADATI-MOGHADDAM[1*] and Nadali BAGHERI[2]

[1]Department of Plant Biotechnology, Tirtash Research and Education Center, Behshahr, Iran.
[2]Department of Plant Breeding and Biotechnology, Sari Agricultural Sciences and Natural Resources University (SANRU), Sari, Mazandaran, Iran

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Proteinase inhibitors (PIs) are polypeptides that occur naturally in a wide range of plants and are considered to be an essential part of the plant's natural defense system. The aims of this study were to determine effect of PI2-*N. tabacum* in nematode (*Meloidogyne incognita*) resistance and its characterize. Resistant (KY9) and susceptible (Ergo) genotypes were inoculated by *M. incognita* race 2 and total RNA were extracted from roots. Exclusive primers were used for *PI2* (Q40561) and *EF1α* (reference gene) in qPCR to determine *PI2* level changes in two, three and four days after inoculation. Results showed significant difference of *PI2* expression in resistant and susceptible genotypes in two-four days after inoculation. Bioinformatics analysis revealed PI2 is an unstable protein and has three proteinase inhibitory domains with a trance membrane region. Proteinase inhibitory domains are trypsin and chymotrypsin inhibitor. Alignment and phylogenetic relationship of inhibitor precursor-repeats in *Nicotiana* sp. revealed seven groups that were variable in sequence. However, they had conserved residues including eight cysteines, a single proline and three Glycine.
*Keywords***:** Alignment, Dendrogram, Gene expression, *Meloidogyne incognita*, QPCR

Corresponding author: Zeinalabedin Shahadati-Moghaddam, Department of Plant Biotechnology, Tirtash Research and Education Center, Behshahr, Iran, E-mail: Shahadati@yahoo.com, Telephone: +98 914 3539974

INTRODUCTION

Plant proteinase inhibitors (PIs) are proteins which form complexes with proteinases thereby inhibit their proteolytic activity. The constitutive expression of PIs in plants has been correlated with their role in planta whereas their wound-inducibility links them to a defense related role against pests. Both PIs and proteases are ubiquitously found in plants and are involved in regulation of the cellular and metabolic functions (GOMES *et al.,* 2011). The co-occurrence of PIs and proteases in plants indicates their role in mutual control and fine tuning of each other's activities. Several families of PIs have been reported depending on specificity towards target proteases their molecular mass and structure. One of the serine PI families is Wound-inducible (Pin-II) (RYAN, 1990; RAWLINGS *et al*., 2004). Also, PIs are essential for regulating the activity of their corresponding proteases and play key regulatory roles in many biological processes (HABIB and FAZILI, 2007). One of the important defense strategies that are found in plants to combat predators involves PIs which are in particular effective against pathogens. The defensive capabilities of PIs rely on inhibition of proteases secreted by pathogens, causing a reduction in the availability of amino acids necessary for their growth and development (LAWRENCE and KOUNDAL, 2002; HABIB and FAZILI, 2007).

Nematodes changes cellular function of feeding sites by secreting of glands proteins in host cells. Glands proteins of many nematode species include proteinases (DE SOUZA *et al*., 2014; ZINOVIEVA, 2014). It has been shown that proteinases of *M. incognita* participate in different processes during the whole life of the nematode, such as feeding, reproduction and embryogenesis (UDALOVA *et al*., 2014). The potential of PIs as anti-nematode effectors was first explored using the serine PI (*CpTI*) in transgenic potato against *Globodera pallida* (HEPHER and ATKINSON, 1992). It has been reported that sporamin (Kunitz-type trypsin inhibitor) activity in transgenic sugar beet caused cyst nematode resistance and prevention growth and development of female nematodes (CAI *et al*., 2003). Also, a modified rice cystatin, Oc-I delta D86, expressed as a transgene in Arabidopsis thaliana, had a negative effect on the size and fecundity of females for both *Heterodera schachtii* and *Meloidogyne incognita* (URWIN *et al*., 1997). In *Solanaceae*, wounding and insect attack lead to the release of a polypeptide hormone 'systemin' from its precursor (PEARCE *et al*. 1991). In most cases, systemin induces jasmonic acid metabolites through the octadecanoid pathway. Systemin travels rapidly to the distal parts of the plant where it induces the JA pathway and regulates the expression of defense molecules including PIs (RYAN and PEARCE, 2003; SCHMIDT and BALDWIN, 2006). PIs often interact with their target proteases by contact with the active site of the protease, resulting in the formation of a stable protease inhibitor complex that is incapable of enzymatic activity (NORTON, 1991).

Proteinase inhibitor II (PI2) is a serine proteinase inhibitor with trypsin and chymotrypsin inhibitory domains (ATKINSON *et al*., 1993). These 5-6 kDa domains are produced by proteolytic processing of precursor proteins containing multiple PI domains (MILLER *et al*., 2000). PI2 occurs in many *Solanaceae* plants, including tomato (PEARCE *et al*., 1991), potato (BEEKWILDER *et al*., 2000), and tobacco (PEARCE *et al*., 1993). Both tomato and tobacco contain a gene encoding a three-domain inhibitor (TAYLOR *et al*., 1993; BALANDIN *et al*., 1995). Moreover, a gene encoding a six-domain inhibitor has been isolated from the reproductive tissues of the *Nicotiana alata* (ATKINSON *et al*., 1993). A feature of these PI2s is the presence of tandem repeats of a 50-52 amino acid polypeptide called inhibitor precursor (IP)-repeats (ANTCHEVA *et al*., 2001). IP-repeats can vary from 1 to 8 with interconnecting linker peptides. Each IP-repeat contains eight conserved cysteines that form four disulfide bonds (SCHIRRA *et al*., 2010) along

with a reactive site for targeting a protease by binding to enzyme active site (MISHRA *et al*., 2012).

The objectives of this study were to determine effect of PI2-*N. tabacum* in resistance and susceptibility of genotypes inoculated by *Meloidogyne incognita* (Southern Root-Knot Nematode; SRKN) and its characterization.

MATERIALS AND METHODS

Plant materials

One SRKN resistant tobacco genotype (*Nicotiana tabacum;* KY9) and a susceptible genotype (Ergo) were planted in Plastic pots (1.5 L) with sterilized sandy loam soil and maintained in the greenhouse at the Tirtash Research & Education Center, Behshahr, Iran (SHAHADATI-MOGHADDAM *et al*., 2017).

SRKN Inoculation and sample preparation

A population of *M. incognita* race 2 was isolated from fields of Mazandaran province, Iran and were proliferated on susceptible tomato plants (*Solanum lycopersicum* cv. Rutgers) in the greenhouse. Inoculum was collected from tomato roots according to ATAMIAN *et al.* (2012). One week after transplanting of tobacco plants, a hole near each plant in the pot was created. One mL of water suspension containing 2500 J2 nematodes/mL was pipetted in each hole and the holes were covered with soil. Some pots did not inoculate as control. The plants were irrigated daily by hand and the greenhouse temperature was controlled at 27 ± 1°C. Two, three and four days after inoculation (dai) the roots were harvested by gently rinsing off the soil with running water. Three samples of treatments and controls from KY9 and Ergo prepared as biological replicates.

Total RNA extraction and first strand cDNA synthesis

Total RNA was extracted from approximately 100 mg of freshly sampled root tissue using TRIzol reagent (Invitrogen, 15596-027), according to the manufacturer's instructions. Any genomic contamination was removed before cDNA synthesis using RNase-free DNase I (Fermentas, #EN0521), according to the manufacturer's protocols. Nucleic acid quality was estimated by visual analysis on 1.2% agarose gel electrophoresis. RNA concentrations were measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Inc) and only RNA samples with an A_{260}/A_{280} ratio in the range 1.8–2.0 were used. All RNA samples were stored at -80ºC. The first strand of cDNA was synthesized from 1.5µg total RNA with the RevertAid First Strand cDNA Synthesis kit and Oligo $(dT)_{18}$ primer (Thermo Scientific, #K1621) according to user instructions.

Primer design for PInII and reference gene EF1α

Primers were designed using the Primer3 online software (http://primer3.ut.ee) for Proteinase inhibitor type-2 (Q40561) and reference gene (*EF1α*) (SCHMIDT and DELANEY, 2010) (Table 1). Primers were tested by Primer BLAST on *Nicotiana tabacum* Refseq mRNA database to ensure the amplicon specificity. OligoAnalyzer 3.1 was used to determine of primer dimer probability and ΔG. All primers were custom-ordered from a commercial supplier (Generay Biotech, China).

Table 1. Optimized primer sequences for target gene (PI2) and reference gene (EF1α)

Gene symbol	Gene name	Accession number[a]	Primer sequences (5'-3')	Amplicon lenght	Tm (ºC)
PI2	Proteinase inhibitor type-2	Q40561	F-ACTACTTCGGTCAGGATGGC R-GAGTTCGCAACCCTTGTCTG	173 bp	60
EF1α	Elongation factor 1-alpha	P43643	F-CTGTTGGTCGTGTGGAAACC R-CAAACCCACGCTTGAGATCC	186 bp	60

[a] uniprot database

Quantitative PCR

qPCR was performed in 96-well plates using the CFX96 real-time PCR detection system (Bio-Rad, C1000TM Thermal Cycler). Three different biological replicates in each time-course (2, 3 and 4 dai) were used for inoculated and control plants. Two technical replicates for all cDNA samples were loaded in PCR plate. PCR reactions were 14µL in volume and contained 7µL SYBR Green PCR Master Mix (Applied Biosystems, 4309155), 1µL cDNA solution, 1µL (0.5µM) of each forward and reverse primers and 4 µL nuclease-free water. The thermal profile of the reaction was 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Finally, a melt curve was generated by increasing temperature from 55 to 95°C, in order to verify primer specificity. According to LIVAK and SCHMITTGEN (2001) $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression of each gene in inoculated plants to the control plants by Microsoft Excel software.

Characterization of PI2 by bioinformatics tools

Expasy's prot-param server was used to calculation of physical and chemical parameters (http://www.expasy.org/tool/protparam/). The secondary structure of protein was predicted by PSIPRED server (http://bioinf.cs.ucl.ac.uk/psipred/).

Table 2. Families of proteinase inhibitor in Nicotiana sp. from Uniprot database and their IP-repeats (A-F)

Protein name	Accession number	Species	IP-repeats	Reference
Proteinase inhibitor type-2	Q40561	*N. tabacum*	N. tab3-A, N. tab3-B N. tab3-C	Balandin et al. (1995)
proteinase inhibitor type-2	XP_009598130	*N. tomentosiformis*	N. tom3-A, N. tom3-B N. tom3-C	Sierro et al. (2013) Auto. Comp. Anal.
Proteinase inhibitor type-2	A0A1U7XB93	*N. sylvestris*	N.syl3-A, N. syl3-B N. syl3-C	Sierro et al. (2013) Auto. Comp. Anal.
proteinase inhibitor type-2	A0A1J6JJ91	*N. attenuata*	N. att3-A, N. att3-B N. att3-C	Database submission
Proteinase inhibitor	Q9SQ77	*N. alata*	N.ala4-A, N. ala4-B N. ala4-C, N. ala4-D	Miller et al. (2000)
insect injury-induced proteinase inhibitor	Q3SBE6	*N. tabacum*	N. tab4-A, N. tab4-B N. tab4-C, N. tab4-D	Database submission

trypsin proteinase inhibitor precursor	Q1WL50	*N. benthamiana*	N.ben4-A, N. ben4-B N. ben4-C, N. ben4-D	Wu et al. (2006)
Proteinase inhibitor type II NGPI-2	Q9SDW7	*N. glutinosa*	N. glu6-A, N. glu6-B N. glu6-C, N. glu6-D N. glu6-E, N. glu6-F	Choi et al. (2000)
Proteinase inhibitor	Q40378	*N. alata*	N.ala6-A, N. ala6-B N. ala6-C, N. ala6-D N. ala6-E. N. ala6-F	Atkinson et al. (1993)
wound-induced proteinase inhibitor 2-like precursor	B3F0C1	*N. tabacum*	N. tab6-A, N. tab6-B N. tab6-C, N. tab6-D N. tab6-E, N. tab6-F	Srinivasan et al. (2009)
Proteinase inhibitor type II NGPI-1	Q9SDW8	*N. glutinosa*	N. glu8-A, N. glu8-B N. glu8-C, N. glu8-D N. glu8-E, N. glu8-F N. glu8-G, N. glu8-H	Choi et al. (2000)

MEMSAT3 & MEMSAT-SVM methods was used to prediction of signal peptide, transmembrane helix, cytoplasmic and extracellular regions (NUGENT and JONES, 2009). Homology modeling was performed on the SWISS-MODEL workspace server using the function of automatic mode (http://swissmodel.expasy.org) (BORDOLI *et al*., 2009). Eleven proteinase inhibitor sequences of *Nicotiana sp*. were selected from UniProt database (http://www.uniprot.org/) (Table 2). These proteins had three-eight potato type II proteinase inhibitor domains (IP-repeats). IP-repeats were determined from A to H starting from the N-terminus. For evaluation the phylogenetic relationship a dendrogram was generated based on amino acid residues of IP-repeats by MEGA6 software using the UPGMA method. To determine the conserved amino acid in domains, a representative from each group were aligned using Clustal omega (DE HOON *et al*., 2004).

RESULTS AND DISCUSSIN

Expression levels of PI2

Relative expression of *PI*2 indicated significant difference between KY9 (5.95-fold) and Ergo (0.44-fold) at 2 dai (Figure 1). High significant difference was observed between KY9 (57.41-fold) and Ergo (0.26-fold) at 3 dai, also with 103-fold for KY9 and 0.10-fold for Ergo at 4 dai. PI2 had downregulation in susceptible genotype than control at all time-courses.

The potential of *PIs* as anti-nematode effector has been reported in some plants, such as cowpea (HEPHER and ATKINSON, 1992), sugar beet (CAI *et al*., 2003), Arabidopsis (URWIN *et al*., 1997) and rice (URWIN *et al*., 1995). On the other hand, it has been revealed that JA pathway had an important role in upregulation of proteinase inhibitor genes (SUN *et al*., 2011; LARRIEU and VERNOUX, 2016).). This effect has been approved in tomato resistance to *M. incognita* by JA treatment of plants (UDALOVA *et al*., 2014). Also, it has been reported that JA signaling pathway is affect in resistance to nematode in rice (NAHAR *et al*., 2011; KUMARI *et al*., 2016) and soybean (IBRAHIM *et al*., 2011). Increasing trend of *PI2* expression in resistant genotype could likely indicate that a stimulating factor enhanced JA signaling pathway. Low PI2 level in susceptible

genotype relative to the control indicated a reducer system in JA signaling pathway and downregulated involved genes by nematode secretions. This system affected on decrease of PI2 in susceptible genotype, while could not affect in presence of *R* gene in resistant genotype. It has been suggested that effectors secreted by nematodes contribute to plant defense suppression during infection (ROSSO *et al*., 2011).

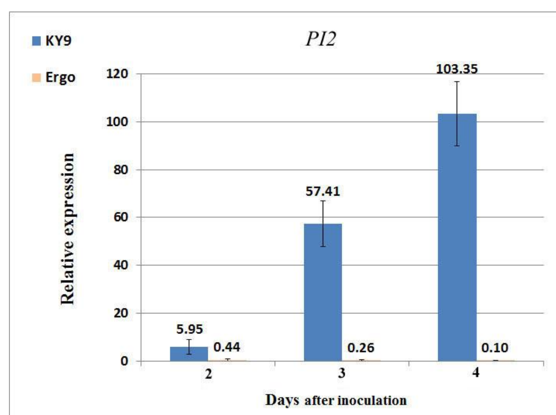

Figure 1. Relative expression of *PI2* in resistant (KY9) and susceptible (Ergo) genotype to *Meloidogyne incognita* race 2 at three time-courses

Three-domains PI2 protein characteristics in N. tabacum

Investigating the biochemical properties of three-domain PI2 protein in *N. tabacum* (Q40561) using Prot-Param server indicated molecular weight of PI2 (197 amino acid) was 20.9 kDa and predicted isoelectric point was 4.49 (Table 3).

Table 3. Biochemical properties of three-domain PI2 protein in N. tabacum and its segments (IP-repeats and domains)

Polypeptide name	Length (aa)	Molecular weight (kDa)	Theoretical pI	Negatively charged residues [a]	Positively charged residues [b]	Instability index [c]	Aliphatic index
PI2	197	20.9	4.49	24	13	47 [us]	56.95
IP-A	50	5.2	6.16	4	4	40.9 [us]	31.4
IP -B	52	5.6	4.2	7	3	41 [us]	35.5
IP -C	51	5.4	4.39	7	4	63.7 [us]	68.8
Domain I	52	5.4	4.44	7	4	67.1 [us]	47.1
Domain II	53	5.7	4.7	5	4	34.6 [s]	33.2
Domain III	51	5.4	4.12	8	3	68.1 [us]	20.9

[a]: (Asp + Glu), [b]: (Arg + Lys), [c]: s= stable, us=unstable

The molecular weight of ~21 kDa for the PI2 has been previously reported (TAMHANE *et al*., 2012). This protein had 24 negative charged residues (Asp + Glu) and 13 positive charged residues (Arg + Lys). Aliphatic index was 56.95, which an index for resistance level of protein to

heat. Furthermore, instability index of PI2 (47) showed this protein is unstable. Instability of PIs was explained by NORTON (1991). However, when PIs affected by the proteinases, create a stable complex with proteinases and inhibit their activity. PI's instability is necessary for a regulatory system. Because of PIs can act only when enzymes are available in the environment.

Cysteine with 12.2% and glycine with 10.2% had the highest contents among the amino acids. Investigating the properties of IP-repeats and domains revealed that they had 5.2-5.7 kDa molecular weight. HEATH *et al*. (1995) have shown that molecular weight of PI domains in *N. alata* is 6 kDa and sequence length is 53 amino acids. Domains had negative charge and only domain II was stable (Table 3).

Secondary structure properties

Conserved domain search in NCBI server approved three IP-repeats in residues 25-74, 82-134, 140-180 on *N. tabacum* PI2 protein (Q40561). MEMSAT method indicated a signal peptide in residue 1-24 and a trance membrane region in residue 7-24, which confirmed by Kyte-Doolittle method in TMHMM server. This region indicates PI2 is attached to the membrane within the cytoplasm. However four-domain PI2s and higher have no trance membrane region. The end of trance membrane region is exactly the beginning of the first IP- repeat (IP-A). The N-terminus of the PI2 was placed outside the cell and domains were located inside the cell (Figure 2). It has been reported that proteinase inhibitors are localized in various compartments of plant cells such as cytosol, vacuoles, cell wall, cytoplasm and nuclei (HABIB and FAZILI, 2007; KIDRIC *et al*., 2014). Secondary structure prediction of PI2 protein in the PSIPRED server revealed an α-helix in residue 4-21 and 10 β-strands (Figure 3). A large part of the helix was placed inside the membrane. There were three β-strands in IP-A and IP-C also four β-strands in IP-B. This additional sheet has been created by insertion of two isoleucines in linker region.

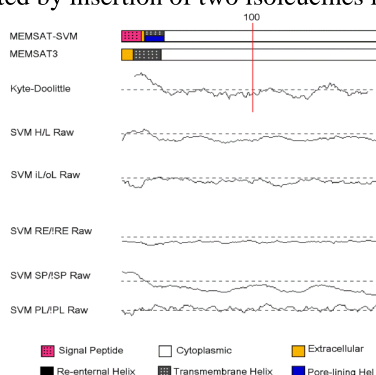

Figure 2. Diagram of the MEMSAT3 and MEMSAT-SVM predictions for the PI2 topologies. The N-terminus of the PI2 is extracellular and has signal peptide residue. A transmembrane region is located in residue 7-24 and cytoplasmic region include PI domains

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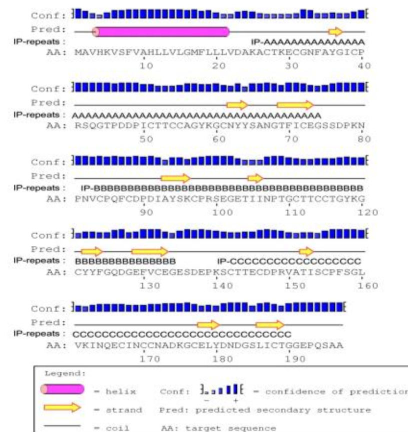

Figure 3. Secondary structure prediction of PI2 in *N. tabacum* using PSIPRED server. PI2 protein contains an α-helix in residue 4-21 and 10 β-strands. Three IP-repeats are indicated by IP-A, IP-B and IP-C

Tertiary structure properties

Each IP-repeat contains eight cysteines, which have a main role in 3D structure by disulfide bonds. Homology modeling in SWISS-MODEL server to prediction of 3D structure revealed a 56% Similarity polypeptide called 1pju.4 (Wound-induced proteinase inhibitor II; *Solanum lycopersicum*), which was identified by X-ray crystallographic method (BARRETTE-NG *et al*., 2003).

Alignment of this polypeptide with PI2 indicated that 1pju.4 covers residue 25-141 and includes two IP-repeats (Figure 4a). Structure of 1pju.4 had eight β-strands and contain two potato type II proteinase inhibitor domains in total (Figure 4b). Four β-strands together produce a domain and there was a linker region between domains. The prediction of cysteine bonds was done based on 1pju.4 disulfide bridges (RAWLINGS *et al*., 2004). Accordingly, Domain-I contained two segments, first segment was in N-terminus and second one was in C-terminus. These two segments are connected together and create Domain-I (Figure 4c).

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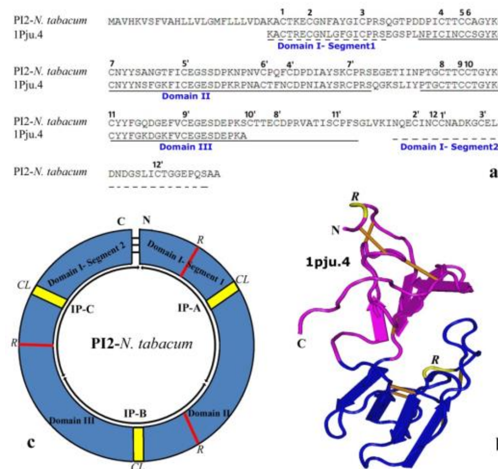

Figure 4. Alignment of PI2-*N. tabacum* (Q40561) and 1pju.4 (*Solanum lycopersicum*). Cysteines are indicated by numbers. The similar numbers make disulfide bridges to each other (a). 3D structure 1pju.4 whit two proteinase inhibitor domains. Reactive sites are marked by R (ATKINSON *et al*. 1993) (b). Diagrammatic representation of the domain organisation of PI2-*N. tabacum* (Q40561) and IP-repeats positions. The precursor protein shown as a linear product, forms a circular structure that is clasped by three disulphide bonds between the N- and C-terminal repeats. Domain I include segment1 from N-terminus and segment 2 from C-terminus. Each domain contains a proteinase reactive site (R). Cleavage in each linker region (CL) releases active domains which inhibits trypsin or chymotrypsin proteinase (c).

Proteolytic cleavage of the precursors (IPs) occurs inside the linker regions by proteinase and release trypsin and chymotrypsin inhibitory domains., that resulting inactivation of target proteinase (BARRETTE-NG *et al*., 2003). Cys-1 and Cys-2 (Domain I), Cys-6' and Cys-4' (Domain II), Cys-10' and Cys-8' (Domain III) help to hold the reactive site loop in a relatively rigid conformation that likely helps to prevent proteolytic cleavage of the inhibitor upon interaction with proteinases (Figure 4a).

IP-repeats properties

The phylogenetic tree for 50 IP-repeats from eleven proteinase inhibitor sequences in *Nicotiana* sp. identified seven cluster. The first cluster comprised IP-Cs of three-domain proteins from *N. tabacum*, *N. tomentosiformis, N. sylvestris* and *N. attenuate*. There were similar conditions for cluster two (IP-Bs) and cluster three (IP-As) (Figure 5). This fact that *N.*

tomentosiformis and *N. sylvestris* are ancestors of *N. tabacum* confirmed by this grouping. Almost all IP-repeats of *N. glutinosa* (six and eight-domain proteins) were in the group seven, except N.glu6-F and N.glu8-H which were in group five. IP-repeats four and six-domain proteins were mostly in the group six. Group four include only N.ben4-D. Almost all IP-repeats of *N. glutinosa* (six and eight-domain proteins) were in the cluster seven, except N.glu6-F and N.glu8-H which were in cluster five. IP-repeats four and six-domain proteins were mostly in the cluster six. Cluster four include only N.ben4-D. PI2 genes in *Nicotiana* sp are important due to their evolution into multiple inhibitory repeat types (2 to 8 IP-repeats) from the ancestral single repeat PI2 precursor (BARTA *et al*., 2002).

Four, six and eight-domains IPs had same linker region EEKNN, but linker region in three-domains IPs was different. Also three-domains IPs were located in separate groups at phylogenetic tree (Figure 5) and had more genetic distance with other groups. Single repeat PIs are thought to be the ancestral members that have given rise to the other forms by series of domain duplication events (BARTA *et al*., 2002). The primary domains were probably trypsin inhibitor. Group 1 has been separated from others by sequence mutation and transformation to chymotrypsin. In the next step, group 2 has similarly branched. But group 3 has retained its identity. Other groups have been later formed in the same manner by change the sequences and nature and duplication of the domain.

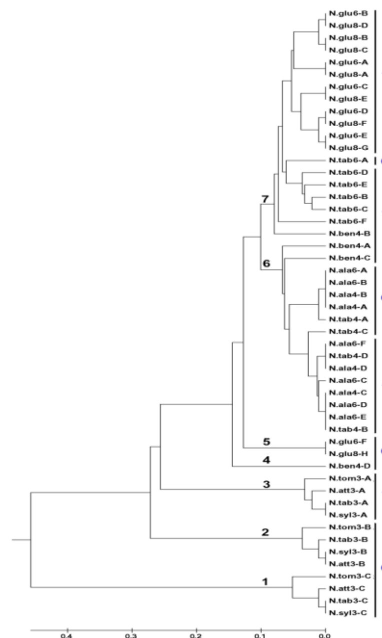

Figure 5. Phylogenetic relationship of IP-repeats in *Nicotiana* sp. using UPGMA approach with MEGA 6 revealed seven groups. Dendrogram indicated three-domain proteins had most deferent from other multi domain PI2s. Trypsin (T) and chymotrypsin (C) inhibitor indicated according ATKINSON *et al.* (1993)

Alignment of seven IP-repeats (one representative from each group) indicated that the sequence of IP-repeats is variable. However, presence of eight cysteines, a single proline residue

and three Glycine residue is conserved throughout IP-repeats. (Figure 6a). The important functional role of the two disulfide bonds anchoring the reactive site loop is reflected by their conserved presence among all known Pin-II inhibitors (TAMHANE *et al*., 2012). Other conserved residues such as proline and Glycines are structurally important, as they belong to the three-turns in 3D structure (KONG and RANGANATHAN, 2008). The amino acid after proline indicates the type of proteinase (trypsin and chymotrypsin) that cleavage the linker region. Enzyme affected sites on IP-repeats of PI2-*N. tabacum* showed IP-A and IP-B are influenced by trypsin and IP-C is influenced by chymotrypsin in post-proline amino acid (Figure 6b).

According ATKINSON *et al*. (1993) reactive site, which interacts by proteinase, determine type of inhibitor (Figure 6a). Amino acids Arg, Lys indicate trypsin inhibitor and Leu, Phe, Thr indicate chymotrypsin inhibitor. However, the structural domains need to be separated for activity. Therefore, the conserved residue (CPX) can indicate the type of PI. Because this residue was located just before linker region and affected by proteases to separate the domains. Accordingly, IP-A and IP-B could be trypsin inhibitor and IP-C could be chymotrypsin inhibitor (Figure 6b).

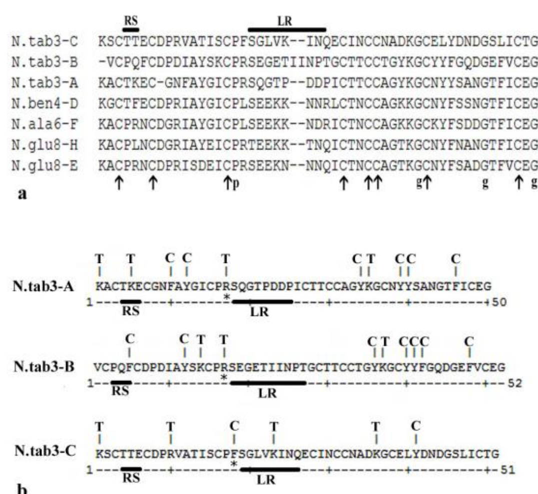

Figure 6. Alignment of seven IP-repeats (one representative from each phylogeny group in *Nicotiana* sp.) by Clustal omega. Conserved residues indicated by arrows (eight cysteines), p (single proline) and g (three Glycine) (a). According Expasy's peptide cutter server trypsin (T) and chymotrypsin (C) can separate the amino acid chain in IP-repeats of PI2-*N. tabacum* at the specified points. However, in the tertiary structure only linker region is available to enzyme, that indicated by asterisk (b). RS: Reactive site, LR: linker region.

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ANALIZA EKSPRESIJE INHIBITORA PROTEINAZE TIP-2 U ODGOVORU NA NEMATODE KORENA I NJIHOVA KARAKTERIZACIJA KOD DUVANA (*Nicotiana tabacum* L.)

Zeinalabedin SHAHADATI-MOGHADDAM[1*] i Nadali BAGHERI[2]

[1]Departman za biljnu biotehnologiju, Tirtaš istraživački i obrazovni centar, Behshahr, Iran
[2]Departman za oplemenjivanje biljaka i biotehnologiju, Sari Univerzitet za poljoprivredne nauke i prirodne resurse, (SANRU), Sari, Mazandaran, Iran

Izvod

Inhibitori proteinaze (PI) su polipeptidi koji se prirodno javljaju kod velikog broja biljaka i smatraju se bitnim delom biljnog prirodnog odbrambenog sistema. Ciljevi ovog rada bili su karakterizacija PI2 i utvrđivanje njegovog uticaja na rezistenciju nematoda (*Meloidogine incognita*) kod duvana (*Nicotiana tabacum*). Otporni (KI9) i osetljivi (Ergo) genotipovi inokulirani su sa *M. incognita* rasa 2, a ukupna RNA je ekstrahovana iz korena. qPCR su izvršeni ekskluzivnim prajmerima za PI2 (Q40561) i EF1 *α* (referentni gen) i odredili promene PI2 nabora tokom dva, tri i četiri dana nakon inokulacije.Rezultati su pokazali značajnu razliku u ekspresiji PI2 u rezistentnim i osetljivim genotipima i tri vremenska kursa. Bioinformatička analiza otkrila je da je PI2 nestabilan protein i ima tri domena inhibicije proteinaze sa regionom trans-membrane. Domeni inhibitor proteinaze su inhibitor tripsina i himotripsina. Dva konzervirana ostatka su u PI2 proteinu, što izgleda vrlo značajno. Filogenetski odnos prekursora inhibitora u *Nicotiana sp*. izvojio je sedam grupa.

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