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ISOLATION OF NOVEL *CYCLOTIDE* ENCODING GENES FROM SOME *SOLANACEAE* SPECIES AND EVOLUTIONARY LINK TO OTHER FAMILIES

Zirwah Rizwan[1], Nosheen Aslam[1,*], Farheen Zafar[2], Rashida Humma[2] and Amer Jamil[2,*]

[1]Department of Biochemistry, Government College University, Faisalabad, Pakistan. [2]Molecular Biochemistry Lab, Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan
***Corresponding author's e-mail: amerjamil@yahoo.com; afzal_nosheen@yahoo.com**

Antimicrobial resistance is a very serious threat to human health; hence the search for novel antimicrobials is urgently needed. Cyclotides are unique disulfide rich mini-proteins of plant family having nearly 30 amino acids with N and C terminals joined to induce cyclization. The exclusive structural cyclic cystine knot motifs (six conserved cysteine residues) make these stable molecules against resistant antimicrobial strains. The *Solanaceae* family is phylogenetically fourth distinct family of plants expressing conserved cyclotides. We investigated thirteen *Solanaceae* species displaying high antimicrobial activities against different microbial strains for identification and isolation of *cyclotide* genes. The *cyclotide* encoding genes were found in four *Petunia x hybrida* cultivars and in *Solanum lycopersicum.* The homology of the *cyclotide* encoding genes was compared with homology sequence patterns of the already reported *PETUNITIDE* (*Petunia x hybrida*) genes. Phylogenetic analysis of novel cyclotide precursor genes from *P. x hybrida* cultivars revealed that they had a close relationship with their homologues from *Solanaceae* and formed three separate clades while *S. lycopersicum* showed a relationship with *Fabaceae* and formed a single clade. *Solanaceae* precursors are distinct from other previously known plant family precursors. Novel cyclotide precursor discovery within the *Solanaceae* plant species, defined their exclusive structural sequences, array of antimicrobial activities and evolutionary link to their diversity from other families.
Keywords: Cyclotides, *Solanaceae*, Antibacterial, Antifungal, Phylogeny.

INTRODUCTION

The high sequence diversity of cyclotides is due to natural selection in angiosperms (flowering plants), but little is known about the evolutionary background of cyclotide precursor diversity. The prevalence of cyclotides among *Solanaceae* species an agronomically important family remains to be elucidated. Identification of *cyclotide* genes within this plant species will help explore their evolution, distribution, diversity and biological activities. The exploitation of *Solanaceae* precursor genes will permit production of novel, ultra-stable therapeutics, and lead to the improvement of stable crops and the reduction of crop losses to microbial attack.

Cyclotides are a family of plant peptides comprising of 28-37 amino acids with cyclized backbone forming a cyclic cysteine knot (CCK) linking through 6 cysteine residues (Craik *et al.,* 1999; Burman *et al.,* 2014). They were initially discovered in the *Rubiaceae* family from *Oldenlandia affinis*, and then subsequently identified in several plant species from the *Fabaceae*, *Violaceae*, *Solanaceae* and *Cucurbitaceae* plant families (Simonsen *et al.,* 2005; Gruber *et al.,* 2008; Burman *et al.,* 2014). Cyclotides are reputed to have a defense role within plants, according to the published literature, in which they are described as uterotonic (Gran, 1973), antimicrobial activity (Stromsted *et al.,* 2017), insecticidal (Barbeta *et al.,* 2008), anti-HIV (Gustafson *et al.,* 2004), cytotoxic (Lindholm *et al.,* 2002), molluscicidal activities (Plan *et al.,* 2008) and antihelmintic (Colgrave *et al.,* 2008). Cyclotides are effective against a wide range of microbes. They display their activity either by outer membrane lysis of microbes or by membrane piercing and interacting with specific intracellular targets (Svangard *et al.,* 2007; Burman *et al.,* 2011; Henriques *et al.,* 2011).

In recent years, a wide variety of plants have been screened with the purpose of identifying and revealing novel peptides of the cyclotide family (De Veer *et al.,* 2019; Kan and Craik, 2020; Ravipati *et al.,* 2017). Cyclotides have been identified by screening from *Violaceae* and *Rubiaceae* species. Even though the *Violaceae* and *Rubiaceae*are not closely related taxonomically, it has been believed that cyclotides did not independently evolve in these two families, but rather have evolved from a single ancestral gene. The failure of screening to discover cyclotides in a diverse array of plants might be due to the fact that cyclotides may have been lost or inactivated in the majority of plants (Mulvenna *et al.,* 2006).

The key purpose of the current research was to evaluate antimicrobial potency of protein extracts of *Solanaceae* plants family against several fungal and bacterial species *in vitro*, to discover whether genes encoding cyclotides copiously are more widely distributed than has been perceived and to further analyze the evolutionary link to other known

cyclotides bearing families. A bioinformatics approach with searches based on sequence homology patterns and phylogenetic analysis was adopted to identify sequence divergence. In this work we provide insight into the evolution of plant families *cyclotide* genes by studying the *Solanaceae*, particularly the species of *Petunia* and *Solanum lycopersicum* and extensively describe screening of *Solanaceae* plant species for the presence of cyclotides. Cyclotide-like sequences are not widely present in a wide range of *Solanaceae* plants that has been previously reported. Still, the likely gene evolution was examined by observing homology patterns and phylogenetic analysis of the identified *P. x hybrid* cultivars and *S. lycopersicum* precursor genes. These novel sequences, distinct from other family sequences still increase the probability that evolution of *Solanaceae cyclotide* genes is from aprimeval gene common to several plant kingdom members.

MATERIALS AND METHODS

***Materials*:** *Petunia X hybrida* cultivars, *Solanum lycopersicum*, *Solanum nigrum*, *Cestrum nocturnum*, *Brunfelsia pauciflora*, and *Capsicum annuum* were sourced from Evergreen Nursery (Faisalabad, Pakistan) and the botanical gardens of the University of Agriculture Faisalabad respectively, and identified taxonomically by taxonomist Prof. Dr. Mansoor Hameed in the Herbarium Botany Department, University of Agriculture, Faisalabad, Pakistan.

***Crude extracts preparation*:** Leaves of the plant species were rinsed with distilled water and dried at room temperature. The samples were then fine powdered under liquid nitrogen and stored at -80 °C till further analysis.

***Protein extraction*:** The proteins of fresh leaves from selected medicinal plants were extracted in extraction buffer (15 mM NaH_2PO_4, 10 mM Na_2HPO_4, 2 mM EDTA, 100 mM KCl, 1mM PMSF, 2 mM Thiourea and 1.5%PVPP) (Saravanan and Rose, 2004). Briefly, the leaf powder was resuspended in extraction buffer in 1:3 (w/v) with 1 mM PMSF (phenyl methyl sulfonyl fluoride) added. The mix was homogenized on ice at 2 °C using a polytron. The samples were centrifuged at 10,000 rpm for 30 min (Hermle). Proteins were precipitated in four volumes of chilled acetone containing 10% TCA, 2% β-mercaptoethanol, and 1% PVPP at -40 °C overnight. The samples were centrifuged again at 10,000 rpm for 30 min at 4 °C, and pellets were dissolved in DMSO.

***Antimicrobial assay*:** Antimicrobial potential of protein extracts against two bacterial *Bacillus subtilis* and *Escherichia coli* and two fungal *Fusarium solani* and *Trichoderma harzianum* strains was assessed using disc diffusion method. The cultures were inoculated in nutrient agar and potato dextrose agar medium respectively for bacterial and fungal cultures. Filter paper discs were impregnated with 30 µL of the plant protein extract, chloramphenicol and fluconazole were used as positive control for antibacterial and antifungal assay respectively while, DMSO as a negative control. The petri plates were incubated at 37 °C for 24 hours (antibacterial) and 48 hours (antifungal). The extracts having antimicrobial activity were assessed by zone reader (NCCLS, 2002).

***DNA Isolation*:** Genomic DNA of the plant species *Petunia X hybrida* cultivars (*Petunia F-1 hybrid* falcon, *Petunia F-1 Grfl* hulahoop), *Solanum lycopersicum*, *Solanum nigrum*, *Cestrum nocturnum*, *Brunfelsia pauciflora*, and *Capsicum annuum* was isolated from the plant leaves following CTAB method (Doyle, 1990).

***Primer Designing and Amplification*:** The cyclotide genes were amplified from the *Solanaceae* plant species using family specific designed primers against PETUNITIDE1 gene. PCR reaction conditions for gene amplification were optimized according to the primer parameters using components of ThermoSCIENTIFIC (Mushtaq and Jamil, 2012). Following primers were used in the study:

Forward: 5′CACACTCTCTCTCTCCCTCTAT
Reverse: 5′ACTCCGTTCATTACGTCTAACC

The amplification settings were optimized as: Initial denaturation at 95 °C for 3 min, 35 cycles of 30 s at 95 °C, 40 s at 50 °C, and 50 s at 72 °C, with a final extension of 5 min at 72 °C. Amplicons were detected and visualized on 1% agarose gel after ethidium bromide staining. The amplicons of probable size were purified from gel through a Gel Purification Mini Kit (FavorPrep™, Cat. No. FAGPK001).

***Molecular Cloning and Sequencing*:** Purified DNA fragments were ligated into pTZ vector using TA cloning Kit (Fermantas) and sequenced from a commercial sequencing service (Molecular Biology Products Inc.).

***Bioinformatics Analysis*:** Clustal in BioEditv.7.2.5 was used to validate the cyclotide gene homology and phylogenetic tree was constructed using MEGA6.0 software (Hall, 2013; Tamura *et al.*, 2013).

RESULTS

Antimicrobial and molecular studies were conducted on protein extracts from different plant species viz *Petunia x hybrida* cultivars (*Petunia F-1 hybrid* falcon, *Petunia F-1 Grfl* hulahoop), *Solanum lycopersicum*, *Solanum nigrum*, *Cestrum nocturnum*, *Brunfelsia pauciflora* and *Capsicum annuum* by disc diffusion method. Screening of cyclotides genes was performed by PCR of thirteen plant species of the *Solanaceae* family (Table 1) and study of sequence homology patterns.

***Antibacterial activity*:** Antibacterial activities of the protein extracts were evaluated with chloramphenicol (6 mg/mL) as positive control and DMSO as negative control by disc diffusion method (Fig.1).

Clear zones of inhibition suggest that most ethno-directed plant samples displayed moderate effectiveness against both

the bacterial species *E. coli* (gram negative) and *B. subtilis* (gram positive).

Table 1. List of *Solanaceae* family medicinal plants species selected for *cyclotide* gene identification

S.No.	Plant species
1.	*Petunia x hybrida* cv Mid blue
2.	*Petunia x hybrida* cv Pink vein
3.	*Petunia x hybrida* cv Blue
4.	*Petunia x hybrida* cv Lilac
5.	*Petunia x hybrida* cv Picotee rose
6.	*Petunia x hybrida* cv Picotee rose 1
7.	*Petunia x hybrida* cv Picotee blue
8.	*Petunia x hybrida* cv Picotee burgundy
9.	*Solanum lycopersicum*
10.	*Solanum nigrum*
11.	*Cestrum nocturnum*
12.	*Capsicum annuum*
13.	*Brunfelsia pauciflora*

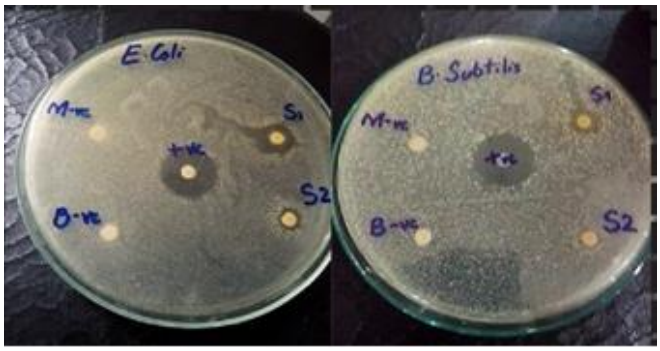

Figure 1. Antibacterial activities of *Solanaceae* plant species protein extracts against *B. subtilis* and *E. coli* with chloramphenicol as positive control (+) and DMSO as negative control (-). S1 and S2 represent plant samples of *Petunia x hybrid* cv Picotee rose and *Brunfelsia pauciflora*, whereas M^- and B^- represent the negative control of samples respectively

Protein extracts of *Petunia hybrid* cv Lilac and *Petunia hybrida* cv Picotee rose were most active against *E. coli* (Figure 1). Overall, it was observed that *P. hybrid* cv Lilac, *P. hybrida* cv Picotee rose, *P. hybrida* cv Picotee blue and *S. lycopersicum* showed broad spectrum activity against both *E. coli* and *B. subtilis* by developing clear inhibition zones. However, the rest of the plant extracts displayed significant inhibition against the selected bacterial strains (Fig. 2).

Antifungal activity: Antifungal activities of the protein extracts were determined with fluconazole (15 mg/250 uL) as a positive control and DMSO as negative control (Fig.3).

Protein extract of *Solanum lycopersicum* had the highest inhibition zone of 17 mm against *F. solani* (Fig. 4). *P. hybrid* cv Lilac and *P. hybrid* cv Picotee rose displayed 16 mm zone each against *F. solani.* Overall, protein extracts from all the plant species exhibited comparable degree of activity against both the fungal strains *F. solani* and *T. harzianum*.

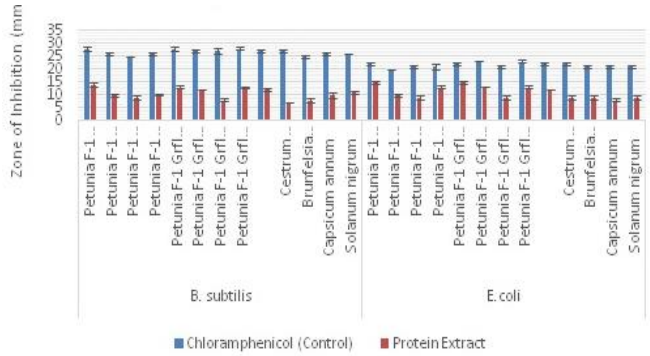

Figure 2. Comparison of inhibition zones of protein extracts from thirteen *Solanaceae* plant species against two bacterial strains *B. subtilis* and *E. coli* with chloramphenicol as control

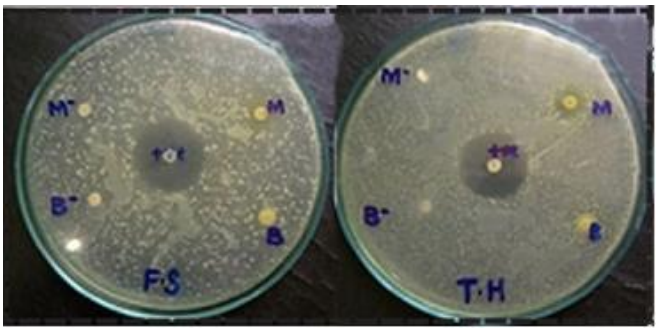

Figure 3. Antifungal activities of protein extracts from *Solanaceae* plant species against *F. solani and T. harzianum* with fluconazole as positive control (+) and DMSO as negative control (-). M and B represent plant samples of *Petunia x hybrid* cv Picotee rose and *Brunfelsia pauciflora*, whereas M^- and B^- represent the negative control of samples respectively

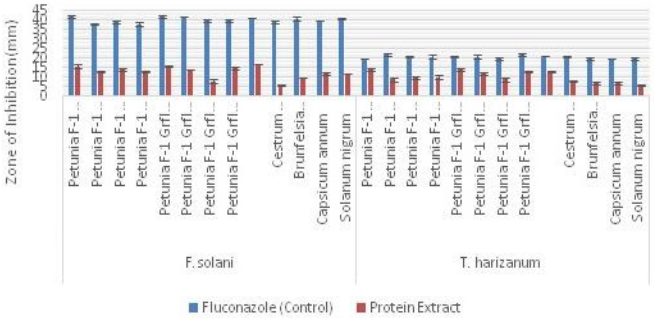

Figure 4. Comparison of inhibition zones of protein extracts from thirteen *Solanaceae* plant species against two fungal strains *F. solani* and *T. harzianum* using fluconazole as control

DNA isolation and screening of Solanaceae plant species for cyclotide encoding genes: Genomic DNA of the selected plant species of *Solanaceae* family was isolated using CTAB methodology (Doyle, 1990).

The cyclotide gene was amplified after optimizing the PCR conditions. Primers were designed from the existing sequences accessible in Genbank (NCBI).Out of the eight different cultivars of *P. hybrida* plants, *Petunia x hybrida* cv Picotee rose and *Petunia x hybrid* cv Lilac showed the presence of two isoforms of an individual gene that might be with or without intronic regions, while from *Petunia x hybrida* cv Picotee rose 1, *Petunia x hybrid* cv Picotee blue and *Solanum lycopersicum* revealed the presence of a single gene (Fig. 5). The resultant amplified bands were excised from the gel and ligated in pTZ57R/T cloning vector.

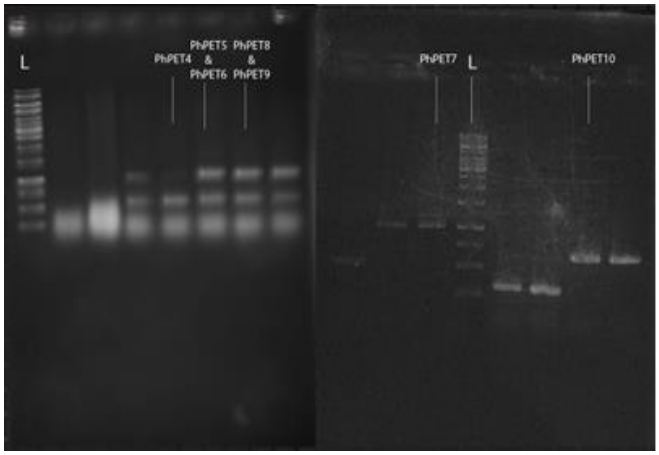

Figure 5. PCR amplification of *cyclotide* genes from *Solanaceae* plants. PCR amplicons of *P. hybrida cultivars* (*PhPET4, PhPET5, PhPET6, PhPET7, PhPET8* and *PhPET9*) and *S. lycopersicum* (*PhPET10*) having isoforms of approximately 500 and 1000bp sizes amplicons whereas, (L) stands for the 1 kb DNA ladder

***Sequencing and insilico homology study*:** After extensive PCR centered screening of the targeted genes, their nucleotide sequences were obtained from a commercial sequencing service (Molecular Biology Products, Inc). Sequences of particular cyclotide genes were then examined and cleaned using various online tools and their contigs were made in BioEdit program. Alignments and BLASTn along with different tools of BioEdit were utilized to get maximum information regarding selected cyclotide gene.

Seven genes were isolated from the four *Petunia* cultivars (*P. hybrid* cv Picotee rose, two from *P. hybrida* cv Lilac, *P. hybrida* cv Picotee blue and two from *P. hybrida* cv Picotee rose 1) and *S. lycopersicum*, using primers designed against the PETUNITIDE precursor gene of *Petunia x hybrida*. These genes were named *PhPET4*, *PhPET5*, *PhPET6*, *PhPET7*, *PhPET8*, *PhPET9* and *SlPET10* respectively, based on their homology (Fig. 6).

The genes from *P. x hybrida* and *S. lycopersicum* were named and numbered *PhPET1-9* and *SlPET10* because a *PETUNITIDE1, 2* and *3*genes have already been isolated from *P. x hybrida*, which should be named according to the new system. Fig. 7 shows an alignment of the base pairs of DNA isolated from four *Petunia* cultivars and *S. lycopersicum* together with previously reported three cyclotide encoding genes from *Petunia x hybrid.* Overall the genes showed 74-90% resemblance except *PhPET9* and *PhPET10* when compared with *PETUTINIDE1 PETUTINIDE2* and *PETUTINIDE3*genes (Table 2).

***Phylogenetic analysis*:** In the current study, 25 (=8 + 17) sequences were analyzed to investigate *Solanaceae* cyclotides evolution. Among them, 8 precursor sequences were discovered from the *Petunia x hybrida* cultivars and *Solanum lycopersicum* (listed in Table 3). These recently discovered eight precursor sequences were pooled with other previously identified seventeen precursor sequences retrieved from Cybase of other plant families (i.e. *Violaceae*, *Rubiaceae*, *Fabaceae*, *Solanaceae* and *Poaceae*).A phylogenetic profile to study the evolutionary relationship among *P. x hybrid* and *S. lycopersicum cyclotide* precursor genes with other known *cyclotide* genes from different plant families was generated using MEGA6.0 software (Fig. 7). The phylogenetic analysis of *cyclotide* precursor genes was evaluated using Maximum likelihood method based on the Tamura and Nei-based distance model.

Table 2. Differences at the DNA level between *PETUNITIDE* homologues

Gene	Species	PETUNITIDE1		PETUNITIDE2		PETUNITIDE3	
		nm[a] (bp)	match[b] (%)	nm (bp)	match (%)	nm (bp)	match (%)
PhPET4	*P. x hybrida* cv Picotee rose	68	78.9	54	83.2	240	25.5
PhPET5	*P. x hybrida* cv Lilac	67	78.8	63	80.2	64	80.0
PhPET6	*P. x hybrida* cv Lilac	36	88.7	54	83.0	69	78.3
PhPET7	*P. x hybrida* cv Picotee blue	35	90.5	67	81.8	73	80.1
PhPET8	*P. x hybrida* cv Picotee rose 1	58	74.3	62	72.6	67	70.4
PhPET9	*P. x hybrida* cv Picotee rose 1	210	48.5	207	49.3	204	50.0
SlPET10	*S. lycopersicum*	295	43.2	288	44.5	278	46.4

[a]: Non-match (nm), No. of different base pairs; [b]:percent of identical base pairs

Table 3. *Cyclotide* gene sequences used in this study.

Cyclotide Gene name	Total Nucleotides (bp)	Accession Number	Source
Kalata B1	840	AY630566	*Viola odorata*
Kalata B1	724	AF393825	*Oldenlandia affinis*
kalata S	608	EU910548	*Viola baoshanensis*
Viba 17	769	EU910552	*Viola baoshanensis*
kalata B2	993	AF393828	*Oldenlandia affinis*
Tricyclon A	867	FJ211183	*Viola tricolor*
cliotide T2	494	JF931989	*Clitoria ternatea L*
cliotide T8	515	JF931994	*Clitoria ternatea L*
Panitide L2	473	KC182531	*Panicum laxum*
Panitide L4	489	KC182529	*Panicum laxum*
caripe 2	343	AGQ04614	*Carapicheai pecacuanha*
chassatide C18	236	JQ309975	*Chassalia chartacea*
Phyb A	613	DC242826	*Petunia x hybrida*
Phyb C	634	JQ886399	*Petunia x hybrida*
Phyb D	453	JI361658	*Petunia integrifolia*
Cter 1	225	KR911962	*Clitoria ternatea L*
Cter 3	231	KR911983	*Clitoria ternatea L*
cT23	185	AML32982	*Clitoria ternatea L*
PhPET4	322	This study	*Petunia x hybrida*
PhPET5	316	This study	*Petunia x hybrida*
PhPET6	318	This study	*Petunia x hybrida*
PhPET7	368	This study	*Petunia x hybrida*
PhPET8	226	This study	*Petunia x hybrida*
PhPET9	407	This study	*Petunia x hybrida*
SlPET10	519	This study	*Solanum lycopersicum*

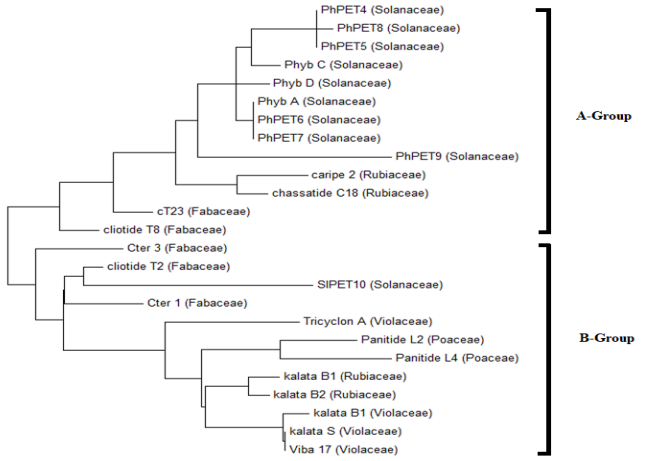

Figure 7. Molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree was constructed in MEGA6.0. Phylogenetic tree showing the relationship among cyclotide precursors found in *Solanaceae*, *Violaceae*, *Rubiaceae*, *Fabaceae* and *Poaceae* plant families. The analysis involved 25 nucleotide sequences with 17 already reported precursor sequences, 8 of *P. x hybrida* cultivars and *S. lycopersicum* novel precursor sequences (Table 3)

Figure 6. DNA alignments of seven genes of *Petunia* cultivars and *S. lycopersicum* compared to *PETUTINIDE* precursor genes. The *PETUTINIDE1*, *2* and *3* genes were used as reference. Deduced seven newly isolated genes encoding *cyclotides* from *P. x hybrid* cultivars (*P. hybrid* cv Picotee rose, two from *P. hybrida* cv Lilac, *P. hybrida* cv Picotee blue and two from *P. hybrida* cv Picotee rose 1) and *S. lycopersicum* are compared for homology to *PETUTINIDE* precursor genes. Dot above indicates homologous base pairs

DISCUSSION

Plants synthesize several antimicrobial compounds that take part in the protection mechanism against pathogenic microbes (Khameneh *et al.,* 2019). In the current study, we scrutinized *Solanaceae* plant family following the antimicrobial assay by targeting *Solanaceae* plant species leaf protein extracts. After screening the target plants, cyclotide encoding gene was isolated, cloned and sequenced for *insilico* studies.

***Susceptibility of bacterial and fungal strains*:** All the extracts displayed comparable level of antimicrobial activities as determined by conventional extraction and assay methods conducted on fresh leaves. So, the *Solanaceae* plant species were classified as having high, moderate and mild antimicrobial activity. Nearly all of the ethno-directed plant samples revealed modest efficacy.

The plant protein extracts showed more inhibition to Gram-negative bacteria as compared to the Gram-positive ones. *E. coli* is the most potent agent for various bacterial diseases such as bacteremia, cholecystitis, diarrhea, cholangitis, urinary tract infection, pneumonia and neonatal meningitis (Bisi-Johnson and Obi, 2012).The literature studies revealed that *Ficus carica*, *Capparis decidua*, *Ziziphus jujube* and *Syzygium cumini*, had powerful antibacterial compounds against *E. coli* (Shad *et al.*, 2014). Similarly, activity of ethno botanical plant extracts showed promising potential against Gram-negative bacterial species as compared to Gram-positive (Tadhani and Subhash, 2007; Gillon *et al.,* 2008). Likewise, *Syzygium cumini* and *Ficus carica* presented activities against both fungal and bacterial strains (Shad *et al.,* 2014). In our research, fungal strains were found to be more sensitive to the plant extracts as compared to the bacterial strains. Further detail of our fungal results indicate that plant protein extracts showed best inhibition zones against *F. solani* as compared to *T. harzianum.* Chandrasekaran and Venkatesalu (2004), also reported that fungal strains were much receptive to the medicinal plant extracts than the bacterial strains. Similarly, Kim *et al*. (2019) demonstrated that tomato leaves exhibited activity against *F. solani* and other fungal pathogens. Overall, different microbial strains have been found sensitive to plant protein extracts of *Solanaceae* family. Probable cause of this activity might be the existence of antimicrobial peptides and phytochemicals in the *Solanaceae* plant species (Chowanski *et al.,* 2016). The antimicrobial peptides provide us scientific basis for the utilization of these plant species for cyclotides screening.

***Solanaceae: Antimicrobially potent family*:** *Petunia* is known for anti-microbial and antioxidant activities (Rahman *et al.,* 2008). Its leaves produce an insecticide, broadly used as natural insecticides. Our findings revealed that four *Petunia* cultivars and *S. lycopersicum* leaf protein extract had moderate inhibition zones both against bacteria and fungi, while rest of the *Solanaceae* plant species displayed negligible zones of inhibition activity. *Petunia* and *S. lycopersicum* have also been shown to possess moderate anti-oxidant activity (Rahman *et al.,* 2008; Kim *et al.,* 2019). *Petunia* plant extracts exhibited substantial antimicrobial activities against different bacterial and fungal species (Thenmozhi and Sivaraj, 2011; Kim *et al.,* 2019) demonstrating pharmacological potential (Borhade, 2012).

This study demonstrates that adequate antimicrobial activity is due the fact that active components are present in fresh leaves of plant. *Solanaceae*, phylogenetically fourth distinct plant family producing structurally conserved cyclopeptides, proposing either transfer of *cyclotide* encoding gene sequences within the plant kingdom or concurrent evolution upon the CCK motif.

***Cyclotide encoding gene from P. x hybrid cultivars and S. lycopersicum*:** The *Solanaceae* hosts two most vital vegetable crops globally cultivated i.e. *Solanum lycopersicum* and *Solanum tuberosum*, yearly production beyond 450 million tons. *Solanaceae* produces structurally conserved cyclopeptides, proposing either transfer of *cyclotide* encoding gene sequences within the plant kingdom or concurrent evolution upon the CCK motif. *Cyclotide* genes were isolated from different cultivars of *Petunia x hybrida* and *Solanum lycopersicum* (of *Solanaceae* family) by specific primers PET1F/ PET1R designed from *Petunia x hybridaPETUNITIDE1* gene reported for 629 bp complete CDS of cyclotide precursor 1 mRNA (accession number JQ886398), having no introns. The estimated cyclotide product size was 500bp whereas; we got 1000 bp amplicon as well besides 500 bp amplicon, which might be explicitly an isoform of similar cyclotide sequence. Rest of the *Solanaceae* plant species showed no amplicon. *Solanaceae* family has not been found to be abundant in cyclotides; *cyclotide* genes have been reported from *Petunia x hybrid* (*Solanaceae*) (Poth *et al.,* 2012; Ravipati *et al.,* 2017). In contrast no cyclotide genes have been reported from *S. lycopersicum* demonstrating report of novel *cyclotide* encoding gene from our study.

We could amplify *cyclotide* encoding genes from four *Petunia* cultivars viz (*Petunia x hybrid* cv Picotee rose *Petunia x hybrid* cv Lilac, *Petunia x hybrida* cv Picotee rose 1 and *Petunia x hybrid* cv Picotee blue) and *Solanum lycopersicum* out of thirteen *Solanaceae* plant species. BLAST search using isolated *cyclotide* encoding genes of *Petunia* cultivars sequences revealed matches to *PETUNITIDE1*, *PETUNITIDE2* and *PETUNITIDE3* from *Petunia x hybrid* previously reported by Poth *et al*. (2012). Additionally, the *S. lycopersicum* BLASTn query displayed novel architecture of *cyclotide* encoding gene having no similarity with any of the above previously reported *PETUNITIDE cyclotide* precursor gene. There is little information regarding distribution of *cyclotide* encoding genes in individual plant species as they had not been identified so far from any other plant species of *Solanaceae* family except for *Petunia*. Our results suggest exclusivity of the precursor gene sequence, among members of the

Solanaceae, especially among the different cultivars of *Petunia x hybrida* that further suggests in spite of having altered precursor structure, *Solanaceae* cyclotides are coded by unique *cyclotide* genes.

Homology comparison and phylogenetic profiling of novel cyclotide encoding genes from Petunia cultivars and S. lycopersicum: Nucleotide sequences from *Petunia* cultivars and *S. lycopersicum* were multiply aligned with reference to the earlier identified*PETUNITIDE1, 2* and *3* precursor genes from *Solanaceae* family. The putative nucleotide sequences of each gene were deduced and compared with the reference *PETUNITIDE* genes using Clustal in BioEdit program (Hall, 2013).

Previously, three *cyclotide* encoding genes have been reported with slight base pair difference: they are homologs of each other (Poth *et al.,* 2012). This finding clearly demonstrates that more than one *cyclotide* encoding gene exist within the petunia species. Whereas, our results showed that two of *Petunia* cultivars i.e. *P. x hybrid* cv Lilac and *P. x hybrid* cv Picotee rose 1 with same primer gave two PCR products each. Further *insilico* analysis of the gene sequences based on alignment suggested that presence of gene duplication and divergence which means that the copies of gene sequences are becoming different from each other even within the same plant species, might be due to accumulation of mutations over time. In contrast, the other two *Petunia* cultivars i.e *P. x hybrid* cv Picotee rose and *P. x hybrid* cv Picotee blue and *S. lycopersicum* gave only a single PCR product during isolation that showed they have single gene locus (Rasul, 2012). During multiple alignments with three of the already reported *PETUTINIDE1, 2* and *3* precursor genes displayed that all of our *Petunia* cultivars genes *PhPET4*, *PhPET5*, *PhPET6*, *PhPET7* and *PhPET8* were homologs of *PETUTINIDE* genes. Whereas one of the cultivar genes i.e. *PhPET9* and *S. lycopersicum* gene i.e. *SlPET10* showed only 40% resemblance to all three *PETUTINIDE* gene precursors that suggest they are non-homologs and have their own new identity. All the gene sequences displayed some degree of homology but have unique novel sequences as well. This study also confirmed that all cultivars of *Petunia* and the rest of the *Solanaceae* plant species did not possess *cyclotide* encoding genes; this conclusion suggests that cyclotides are not ubiquitous within the *Solanaceae* family (Ravipati *et al.,* 2017). Direct confirmation of this is still not been known; the likely role of the genes will be discovered by observing the expression patterns of the revealed *cyclotides* encoding genes of *Solanaceae* family.

It is predicted from the outcome of our results that the variances among the precursor gene sequences of interrelated molecular species were small however in rest they are quite large. In this perspective, big difference is well-defined as an indel. With exception of these indel regions, precursor sequences are homologous. This suggests that regardless of the sequence variances, these molecular species are related. This conclusion can be related to punctuated theory of equilibrium (Eldredge and Gould, 1997), which refers to evolutionary tendencies at organism level.

Phylogenetic profiling of *Petunia* cultivars and *S. lycopersicum cyclotide* genes was done through MEGA6.0 software (Tamura *et al.,* 2013). As cyclotides have conserved sequence, we assume that signature sequences are highly conserved over the course of evolution. This conserved signature sequence helped us to generate phylogenetic relationships between plant families (Park *et al.,* 2017). The phylogenetic tree showed those *Petunia* cultivars and *S. lycopersicum cyclotide* genes were separated into four distinct clades, depending upon the similarity-based nucleotide sequences. The assessment of phylogenetic relationship indicates that there are two types of *cyclotide* precursor genes i.e. the A-group and the B-group (Fig.7). *Cyclotide* precursor genes in A-group were distributed in eight clades. Seven precursor genes of *Solanaceae* family isolated from *Petunia* cultivars in our current study belonged to A-group and were classified in three clades. *PhPET4, PhPET8* and *PhPET5* were in one clade with *phyb C* (*Solanaceae*). *PhPET6* and *PhPET7* were in another clade with *phyb A* (*Solanaceae*). PhPET9 formed a clade distinct from other *cyclotide* precursor genes. However, *SIPET10*gene isolated from *S. lycopersicum* form a clade with *cliotide T2* (*Fabaceae*) of B-group. The phylogenetic tree shows that our novel *Petunia* cultivars *cyclotide* genes cluster with their homologues from the *Solanaceae* (*Phyb A, Phyb C* and *Phyb D*) family whereas *S. lycopersicum cyclotide* genes cluster *Fabaceae* (*cliotide T2*) family.

Results indicated that the *Solanaceae* species evolved prior to the most common ancestor. Also, there are some molecular species that could have occurred by genetic changes between the parental lineages. These *cyclotide* gene precursors are quite distinct from those in other previously known families. Phylogenetically distinct *Violaceae, Rubiaceae, Fabaceae* and *Poaceae* precursors might explain the low yield of cyclotides among *Solanaceae* plants. Therefore, discovery of novel *cyclotide* encoding precursor genes within the *Solanaceae* family might facilitate their application as an alternative route for circular cyclotide production to augment crop protection among *Solanaceae* plant species significant to the human diet, for instance tomato, capsicum and potato via genetic integration of customized *cyclotide* encoding domains.

***Conclusion*:** In the *Solanaceae,* an agronomically important plant family, neither its antimicrobial activity nor cyclotide diversity has been recognized. The cyclotide discovery among this family is thrilling with significant development. Even though the amino acid arrangement and architecture of the mature peptides enclosed by these freshly studied sequences have not so far been elucidated, genes isolated in the current study are noteworthy for their homology to the

PETUNITIDE cyclotide encoding genes. The matches consist of many chief residues that are significant for the compact structural arrangement of cyclotides. Here we conclude that *cyclotide* encoding genes are not abundant within the *Solanaceae* plant family.

Similar to *PETUNITIDE* genes the *Petunia* cultivars genes are more compact besides formerly known *cyclotide* gene precursors. Therefore, the finding of novel *cyclotide*-encoding genes within the *Solanaceae* plant family has excessive potential to enhance the worth of *Petunia* cultivars and *S. lycopersicum* as research tools for studying cyclotide processing and also facilitate their utilization as an alternative route for circular peptide construction contrasted to *cyclotide* genes. This research confirms the exclusivity of *Solanaceae* precursor sequences and suggests *cyclotide* gene evolution occurred independently within *Solanaceae* species. Subtle *cyclotide* gene sequence difference and phylogenetically distinct *Rubiaceae*, *Fabaceae*, *Violaceae* and *Poaceae* precursors explains their low cyclic products yield. Thus, this discovery might make it possible to connect the evolution of new *cyclotide* precursor genes with its pharmaceutical function by expression of both designed and natural cyclotide precursor genes to enhance crop protection within *Solanaceae* plant species.

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