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Research Article

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Screening, Isolation and Purification of New Defensin Like Gene/S (DEFL) From Selected Local Plants of Solanaceae Family

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ABSTRACT

The spread of multi-drug resistant microbial strains and reduced range of drugs available led to an enquiry for therapeutic alternatives. Medicinal plants and peptides isolated from them are being used for their antimicrobial properties. Plant defensins are short peptides that show resistance against a variety of human and plant pathogens with a number of other bioactivities and characteristics. Antimicrobial peptides have also been isolated from Solanaceae family. We therefore focused our research towards isolation of defensin-like genes from two selected plants of Solanaceae family which were Solanum tuberosum and Withania somnifera. Plants genomic DNA were isolated by using CTAB extraction method. The presence of DEFL genes were confirmed by PCR with specifically designed primers in both plants and results were established by using agarose gel electrophoresis. The purified desired PCR product bands of 1100 bp from Withania somnifera & 1000 bp Solanum tuberosum were sent for sequencing for further studies. This research work can further help to comprehend the genetic & functional diversity of the defensin like peptide, their distinctiveness, regulation, and better research based to understand the topic.

Keywords: Bioactive peptides; *Solanum tuberosum*; *Withania somnifera*; Defensin-like peptides; Solanaceae family.

INTRODUCTION

Bioactive peptides are best for to exhibit antifungal, antiviral, antiparasitic, antibacterial, anti-cancerous, antioxidant and anti-inflammatory activities. These bioactive peptides are rich in cysteine and glycine content and disulphide bridges formed between these cysteine residues increases their overall stability [1-3]. Antimicrobial Peptides (AMPs) are essential components of immune system and referred as a Host Defense Peptides (HDPs) [4]. Organisms protect themselves against pathogens by producing antimicrobial peptides [5].

These peptides are ubiquitous in nature and show action against a wide variety of both gram positive and gram-negative bacteria, fungi, viruses, both exo and endo parasites and ultimately to cancerous cells [6]. They have been isolated from different organisms, as they are conserved peptides and found in vertebrates and invertebrates as well as in every plant [7]. These bioactive peptides were called as defensins and were first isolated from wheat grains and barley [8,9].

Plant defensins are cationic peptides that are universal inside of the plant kingdom and have a place with a large superfamily of antimicrobial peptides found in a few living beings on the whole called defensins. The essential structure of these peptides incorporates 45 to 54 amino acids deposits with extensive arrangement variety. Defensins contain cysteine and disulphide bridges among these cysteines provide them stability [8]. α-defensin and β-defensin are two kinds of defensins. Defensins are considered to be best known antimicrobial peptides from plants. Four disulphide bridges are present in all known members of this class and are folded into a globular pattern that have one K-helix and three L-strands [10].

Defensins are mainly found in plant seeds and protect seeds from soil pathogenic fungi and greatly stimulate the survival of plant seedling. Some organs express more than one defensin gene but at least one defensin gene is expressed in each organ. Defensin genes are inducible by pathogen attack which supports their role in defense. In addition, induction of defensin genes also takes place by environmental stress such as cold and salt, drought and the signaling molecules like ethylene, methyl jasmonate and salicylic acid [11]. Plants have a substantial collection of genes encoding defensin-like (DEFL) polypeptides that have a monitored example of cysteine buildups, however, are generally variable in the developed protein [12]. Plant defensin like peptides additionally contain an N-terminal signal sequence, comparable intron size and position and conserved cysteine residues [13,14].

These DEFL specifically interact with membrane receptor kinase to fulfil their antimicrobial mode of activity [15]. Other than defense response against pathogenic organisms defensins and DEFL also work in plant growth and maturity. These activities make them best tools of choice for application in medicinal and agricultural organizations. A variety of DEFL peptides have been discovered but still a lot must be exposed about the isolations and activities of DEFL in the perspective of their diversity [16,17].

The above discussion encourages further studies on defensin like genes as a therapeutic potential agent. The present project was therefore focus towards the isolation of defensin-like gene from selected plant of Solanaceae family.

MATERIALS AND METHODS

Defensin like genes were isolated from selected plants of Solanaceae family by using genomic DNA as A template and subjected to sequence analysis. The whole work regarding this research was carried out in Molecular Biochemistry Laboratory, Department of Biochemistry, University of Agriculture, Faisalabad.

Selection of plants

The seeds of selected plants were procured from AARI (Ayub Agriculture Research Institute), Faisalabad, Pakistan and from local market of Faisalabad. Plants were grown in pots by giving favorable conditions for proper growth in university Botanical garden, UAF.

DNA Sisolation

The fresh leaves of the *Solanum tuberosum* and *Withania somnifera* were used for Genomic DNA isolation. The isolation of the nucleic acid (avoiding RNA by use of RNases) was according to the protocols defined by Doyle and Doyle [18]. The DNA isolation was confirmed on 1% agarose gel (w/v in 1X TAE) and stained with ethidium bromide. Further quantification of DNA was done by spectrophotometric analysis. The purity of the DNA was checked by finding A260/280 ratio. Quantity was determined in ng/mL.

Polymerase chain reaction

Defensin like genes (DEFL) were amplified by PCR in a 50 µL reaction mixture from the genomic DNA of *Solanum tuberosum* and *Withania somnifera* using specifically designed primer according to the available reported cDNA sequence by using online bioinformatic softwares like (www.JustBio.com and http://simgene.com/Primer3) [19]. The conditions to be optimized for the PCR reaction mixture were 20-100 ng of Genomic DNA, 10X PCR buffer (ThermoSCIENTIFCTM), 2 mM dNTPs (Mixture), Forward and reverse primers 0.1-1 µM each, 1-6 mM $MgCl_2$ and 1.25 U/50 µL reaction of Taq DNA Polymerase using Gene Amp PCR System 2400 as thermocycler (Perkin Elmer, USA).

The amplification was done using 3 minute hold for initial denaturation at 94°C followed by 34 cycles of repeated denaturation (94°C for 40 sec), variable optimizeable annealing temperatures (from 45-55°C for 1-2 min) and polymerization (72°C for approximately 1 min) followed by a hold of polymerization of 5 minutes at 72°C. For *Solanum tuberosum* and *Withania somnifera* the designed primers were used ST982F: 5/-CTGCCCTTTCAAAAGTAGTTGC-3/ and ST982R: 5/-CCACTGCTAAATCGCTCTGTACT-3/. The amplicons were confirmed on 1.2% agarose gel electrophoresis.

PCR product purification

FavorPrepTM, Gel Purification Mini KitCat. No.: FAGPK 001 (50 Preps) FAGPK 001-1 (200 Preps) was used for the purification of PCR product. This kit was provided with binding, washing and elution buffer. This kit method was used to purify an amplified PCR product or a fragment of DNA by digesting agarose gel which was also confirmed electrophoretically. Then this purified product was sent for further sequencing and affirmation of isolated genes from *Solanum tuberosum* & *Withania somnifera*.

Sequencing

Sequencing of required and isolated genes were done by sending samples to CAMB, Lahore by using Sanger's method.

RESULTS AND DISCUSSIONS

Selection of plant and DNA isolation

Therapeutic plants speak to a rich source of antimicrobial operators. Plants are utilized therapeutically as a part of distinctive nations and are important source of numerous intense and effective medications for the majority of the world's population [20]. Different plants were studied for the presence of defensins and defensin like genes. Plants of the Solanaceae family were largely unexplored for defensin like genes hence the plants belonging to Solanaceae family were selected to check their possession for defensin like genes. So, the plants of Solanaceae family have been studied most extensively for defensin like genes. A starchy tuber of the plant *Solanum tuberosum* & *Withania*

somnifera (winter cherry) are perennial in the family of Solanaceae. These plants are present both in tropical and subtropical areas. The cellular mass of these local vegetable & fruity plants was therefore used for DNA isolation following the method of Doyle and Doyle [18]. As Tamari et al., [21] isolated DNA from petunia hybrida tissues belonging to Solanaceae family by using CTAB and Edwards DNA extraction method. The isolated DNA was confirmed by gel electrophoresis as shown in Figure 1.

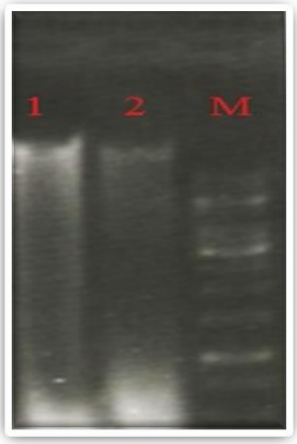

Figure 1. Isolation of genomic DNA from *Solanum tuberosum* and Withania somnifera.

Lane 1,2 shows genomic DNA from *Solanum tuberosum* and *Withania somnifera*, respectively extracted by the method of Doyle and Doyle [18] and treated with RNases to be used for PCR and lane 3 shows the Marker (M) 1 kb DNA Ladder (Fermentas).

Polymerase chain reaction (PCR)

To isolate the defensin like genes of *Withania somnifera* & *Solanum tuberosum* from the genomic DNA. So, a set of oligodeoxyribonucleotide primers were designed from the gene sequence available from GeneBank NCBI based on deduced nucleotide sequence of DEFL gene from Solanaceae family using the CDS region encoding *Solanum tuberosum* gene from cDNA and conserve sequence chose from different sequence for this purpose [22].

The primers were designed using online primer designing tools available on (www.JustBio.com or primer 3.0) for the PCR amplification from the genomic DNA that may possess sequence encoding DEFL gene that was not previously practiced although some non-coding regions that are usually present in the genome.

Therefore much effort was done in optimizing the conditions for the amplification of the gene sequence on genomic DNA regarding the optimization of $MgCl_2$, Primers and DNA concentrations to be used for amplification along with PCR thermal cycling conditions specially annealing temperatures were optimized in our work for the amplification of DEFL gene.

The optimum concentrations for the amplification of PLCPs approximately 1100 bp expected gene size were $MgCl_2$ 2.5 mM, Primer 10 µM each (diluted with sterile H_2O) and DNA 50 ng. The thermal cycling conditions were optimized where initial; denaturation at 94°C for 3minutes and 34 cycles of denaturation at 94°C for 40 sec, annealing at 48°C for 1 minute, extension at 72°C for 1 minutes. Followed by extended polymerization of 5 min at 72°C to add poly A tail and amplify completely. PCR was performed on both the DNA samples S1 and S2 isolated

from leaves of the *Withania somnifera* and *Solanum tuberosum* plants after several tries the product achieved was electrophoretically viewed on 1% agarose gel at 80 volts for 1 hours as shown in Figure 2.

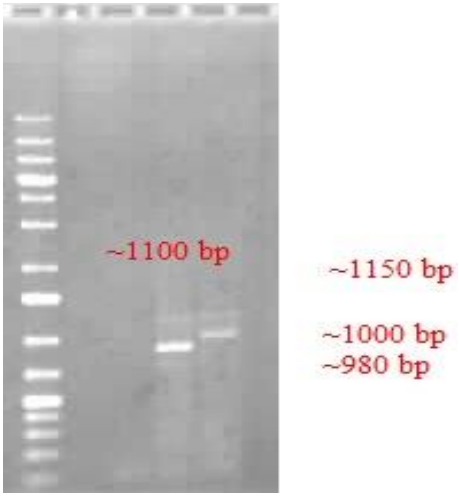

Figure 2. Electrophoresed gel of PCR showing amplification of defensin like gene from *Withania somnifera* and *Solanum tuberosum* by using ST980 F/R primer.

On left side lane M shows 1 kb DNA ladder (Fermentas) of 0.5 µg/µL Lane C shows PCR amplification of template DNA of Withania somnifera and D shows *Solanum Tuberosum*.

Genomic DNA fragments of approximately 1100 bp and 1000 bp from *Withania somnifera* and *Solanum tuberosum* were obtained at the 2.5 mM and 3 mM $MgCl_2$ concentration but clear band was obtained at 2.5 mM concentration of $MgCl_2$ because $MgCl_2$ act as a cofactor for Taq polymerase [20]. Specific DEFL genes close to primer product size were amplified in *Withania somnifera* and *Solanum tuberosum*.

DEFL genes were also reported in *Solanum tuberosum* and *Solnum lycopersicum* plants of Solanaceae family but *Withania somnifera* belonging to Solanaceae family was remained unexplored for defensin like genes. No DEFL gene was previously reported in this plant.

New studies prompted the disclosure of a few extra cysteine-rich peptide families from different plants for example 93 DEFL in *Oryza sativa* and no less than 300 DEFL in *A. thaliana* [16,17]. To verify the *Arabidopsis thaliana* defensin like microarray data (AtDEFL) Tesfaye et al., [23] employed quantitative reverse-transcription polymerasechain reaction (qRT-PCR). Defensin like genes were verified in selected Solanaceae plants by using conventional PCR, PCR with different reaction Recipe and touchdown PCR by using ST980 F/R primer.

PCR product purification by using kit method

The next step was the purification of PCR product from gel. The preferable purification was the gel extraction because only required band separated from the gel and next, we preceded it by Favoprep gel purification mini kit (FAVORGEN Biotech Core cat: FAGPK001).

Amplified and purified DEFL like gene sequence was loaded on 1% agarose gel with 40 µl sample and 1 Kb DNA ladder (fermentas) was run for 1 hour by applying a voltage of 70 V. Apparatus was set as two wells of the comb are combined so as to increase the capacity for sample. The electrophoresed gel is shown in Figure 3.

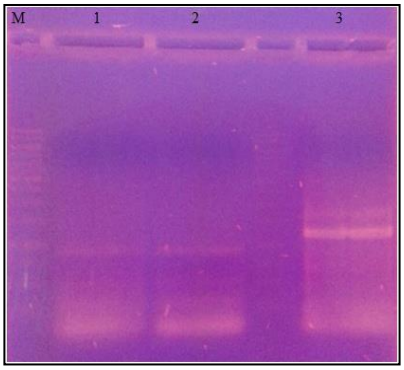

Figure 3. Loading whole sample volumes for gel extraction and purifcation. Lane M represents marker.

Lane 1 & 2 shows amplification of DEFL in *Solanum tuberosum* & while lane 3 represents amplification of DEFL *Withania somnifera*.

The expected purified band of approximate 1000 bp and 1100 bp size of DEFL gene were sent for sequencing to 1st base DNA sequencing Panicon singapore and the sequence obtained was then used for genetic analysis or comparison with reported sequences by using different bioinformatics tools like BLAST and CLUSTUL W. Natarajan et al., [24] used QIAEX II Gel extraction system (Qiagen, Valencia, CA) for purification of desired gene while Favoprep gel purification mini kit was used for the purification of DEFL gene.

Sequencing

DNA sequencing is the Determination of nucleotide sequence in a DNA sample. A modified PCR reaction is used for conducting DNA sequencing. Genomic DNA was isolated from selected Solanaceae plants and targeted genes were amplified by using polymerase chain reaction. Amplification of the desired genes were confirmed by visualizing the electrophoresed gel in gel documentation system. Multiple trials were done to attain clear visualization. After confirmation, the desired genes were purified by using FavorprepTM Gel Purification mini Kit of 50-200 preps size (FAVORGEN, Biotech Core, and Catalogue No. FAGPK001-1).

Primers were diluted according to the requirement of 1st base DNA sequencing service. For this 100 µl primers stock solution was prepared by using TE buffer for each forward and reverse primer and then a working solution of 1:10 ratio was prepared. The forward and reverse gene sequencing of *Solanum tuberosum* and *Withania somnifera* was done by Sanger's first-generation sequencing method. But due to change in climate and habitat of local plant, our sequence analysis showed least similarity with the original DEFL gene sequence.

CONCLUSION

Bioactive proteins involved in defense mechanism by their ability to bind with cellular membranes of pathogens in non-receptor type of binding and act as antioxidants. The present project has main focus on solution of problems concerned with human health and food. Purpose of the study was isolation, screening, and analysis of defensin like genes from unexplored medicinal plants of our region. Sequence analysis of *Solanum tuberosum* and *Withania somnifera* can be use by online bioinformatics tools for comparison of genetic variations, phylogenetic studies and for gene characterization.

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