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Research Article

PRIMER DESIGNING AND VALIDATION OF STARCH PHOSPHORYLASE GENE FROM *VIGNA SINENSIS* VARIETY PUSA KOMAL

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Abstract- Primer designing for Starch Phosphorylase Gene is done using Primer 3 software, with wet lab validation work has been carried out by PCR operation with agarose gel electrophoresis. Similar sequences for Starch Phosphorylase (*Vigna sinensis*) were retrieved from NCBI`s RefSeq database through Entrez, on multiple sequence alignment, conserved clusters were identified. The potential primers were 18-24 nucleotides selected in this region by all combination. The potential primer was analyzed using Primer Biosoft's' Net primer tool. The forward primer having 5` end and backward primer having 3` end and 50 to 60% GC content , and 54 to 58^{0C} Tm and without secondary structure were finalized. Specificity of the Primer was validated by carrying out the local alignment against the NCBI nr database through BLAST. All the alignment showed significant alignment to starch validating the specificity of the Primer.

Keywords- Starch phosphorylase, Primer designing, *Vigna Sinensis*.

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Introduction

Computer technology helps in the management of biological information; it offers computational, molecular biologists and another research scientist an open and extensible environment in which to explore ideas, prototype, and new algorithms. Bioinformatics Toolbox software provides access to genomics and proteomic data formats, analysis technique and specialized visualization for genomic, proteomic sequence and microarray analysis. It also includes collection, organization, storage and retrieval of biological information from the databases selection of oligonucleotide primers for polymerase chain reaction (PCR) oligo-hybridization and DNA sequencing. Proper primer design is actually one of the most important factors in successful DNA sequencing. For maximal specificity and efficiency of polymerase chain reaction appropriate primer concentration and optimal primer sequence is necessary. A poorly designed primer having little or no product due to nonspecific amplification and primer dimer formation having competitive enough to suppress product formation. There are several online tools available that are devoted to serving molecular biologist for effective PCR primer design [1].

Starch phosphorylase (EC. 2.4.1.1); alpha-1, 4-glucan: orthophosphate, alpha-glucosyl transferase) catalyzes' the reversible conversion of starch into glucose-1-phosphate in the present of orthophosphate. Normally starch consists of two components amylose and amylopectin; both are the polymer of glucose molecules. Amylose is a linear alpha-1, 4 D-glucan polymers and amylopectin is branched alpha-1, 6 D-glucan polymers. Starch phosphorylase is the first enzyme in the degradation of starch in plants. Starch phosphorylase is widely distributed in the plant kingdom [2-4] and has been demonstrated in several plant parts. Multiple forms of the enzyme have also been suggested as a biochemical response to the heterogeneity of starch molecules and starch granule structure [5]. Multiplicity may occur at the post-transcriptional level due to present of different genes or at the post-transcriptional level due to differences in the splicing of mRNA. It can be used for the production of glucose-1-phoshate a cytostatic compound used in cardio-therapy [6-7]. Starch phosphorylase used to estimate he amount of inorganic phosphate in serum under the pathological conditions and amount of inorganic hosphate pollution in the environment detected. After considering the importance of starch phosphorylase enzyme in the present study, we have designed primers for starch phosphorylase genes validation of the designed primer is done through PCR.

Materials and Methods

Seeds of *Vigna sinensis* variety Pusa Komal were collected from a farm. Seeds were germinated in plant growth chamber at 28°c and 60% relative humidity. After 3 days, the germinated seeds were used for further work.

Web-based resources for primer design: There are numerous Web-based resources for PCR primer design. Though most are freely available, they are of variable quality and not well maintained. Therefore, web-based resources often result in missing links and Web sites that have been useful previously may not be functional at a later date. So many criteria such as primer length, GC contents, Tm, dimer formation false priming, hair pin loop should be established in design of pimers [1].

Software in primer design:

The use of software in the biological application has given a new dimension to the field of bioinformatics. Many different programs for the design of primers are now available. Free software is available on the internet and many universities have established servers where a user can log on and perform free analyses of proteins and nucleic acid sequences. There is a number of the stand-alone program as well as complex integrated networked version of the commercial software available These software package may be used for secondary structure predictions, DNA and protein analysis, primer designing, molecular modeling,

plasmid drawing. [8]

Primer designing:
345 Sequence of Starch phosphorylase (EC.2.4.1.1; α-glucosyl transferase) were screened out of which 45 suitable sequences were taken from Gene Bank and multiple sequence alignment was done using bioinformatics tool CLUSTAL W. After alignment, the conserved region was taken and primers were designed using software designed using software primer 3 [9].

Primer 3 software: It is software developed by Rozen and Skaletky [9]. It is freely available on the Internet (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi). This software is provided by the Whitehead Institute "as is " and any express or implied warranties, including, but not limited to, the implied warranties of merchantability and fitness for a particular purpose are disclaimed. The conserved region of the sequence was inserted in the box of the GUI of primer 3. The parameters were adjusted as follows: Tm mini=54°C,max =58°C,primer size min.=18,max.=24, GC%min.=50,max.=60[10].

Gene Runner software: Gene Runner is a simple and convenient program for sequence analysis. It may be downloaded from the website http://www.generunner.com runner now has protein motif database searching abilities. One may visit NCBI website and download Genbank flat files for searching. One can search dbEST or GeneBank for secondary protein structure by protein motifs in all 6 translation frames using Motif Runner [11].

NetPrimer: Netprimer combines the latest primer design algorithms with a web-based interface allowing the user to analyzed primer over the Internet (http://www.premierbiosoft.com/netprimer/netprlaunch.html). All primers are analyzed for melting temperature using the nearest neighbor thermodynamic theory to ensure accurate Tm prediction. The Primers are used for analyzing all secondary structure, including self -dimers, hairpins, cross dimer and minimizing the formation of primer dimer. The program used for quantization of primers by optical activity and calculating primer molecular weight. To facilitate the selection of an optimal primer, each primer is given a rating based on the stability of its secondary structures. A comprehensive analysis report can be printed for individual primers or primer pairs.

ClustalX software: ClustalX software having new windows interfaces for the ClustalW multiple sequence alignment programs [10]. This gives integrated environment for performing multiple sequences, analyzing the research and profile alignments for DNA and proteins. The sequence alignment is displayed in the window on the screen. It calculates the best match for the selected sequences and lines them up so that the identities, similarities, and differences can be seen. Evolutionary relationships can be seen via viewing Cladograms or phylograms.

MACAW: (Multiple Alignment constructions and Analysis Workbench), its use for user to construct multiple alignments by analyzing, locating, editing and combining "blocks "of aligned sequence segments. It is programming for semi-manual local multiple alignments of DNA and protein sequences. The user delimits the sequences and regions in which to search for blocks and decides which blocks to keep. Blocks can also edit or even defined by the user. The significance of each block is given a statistical value.

BLAST: The Basic Local Alignment Search Tool (BLAST) use for finding regions of local similarity between sequences [12]. This program used for the compares nucleotides or proteins sequences to sequence the databases and measure statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families.

Results and Discussion
On searching for families of Starch Phosphorylase around 45 suitable sequences were selected, from which 7 different sequences were selected on bases of our gene closeness, with a high divergence between all sequences. The database accessed from website, http://www.ncbi.nlm.nih.gov/RefSeq. The Reference Sequence (RefSeq) database aims to provide a comprehensive, integrated, non-redundant set of sequence, including genomic DNA, Transcript (RNA) and Proteins products, for major research organism. Sequence Alignment (MSA) of Starch Phosphorylase (one nucleotide sequence alignment was found and each sequence was highly divergent from another sequence as evident by the Phylogenetic Tree is shown in [Fig-1] (A), the Phylogenetic tree is constructed using NJPLOT program in ClustalX Software.

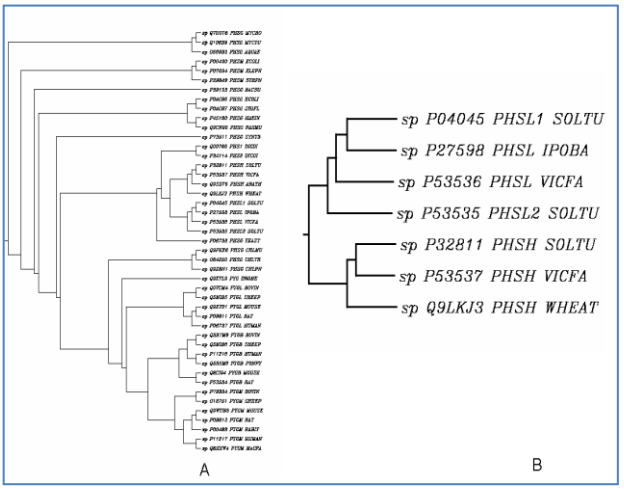

Fig-1 A) Phylogenetic tree for starch phosphorylase families B) Seven sequence of closely related families of genes retrieved from the family tree, For various sources, the high divergence between all sequences were analyzed.

On MSA, four conserved regions were found as shown in [Fig-2]. Nucleotide sequence corresponding to each conserved region in MSA of protein was determined from the corresponding nucleotide sequence all combination of 18-20 nucleotide (ideal length of Primer) long sequences was selected as the potential primer. Nucleotide sequence corresponding to a second conserved region, being highly rich in G+C content was not used for further work.

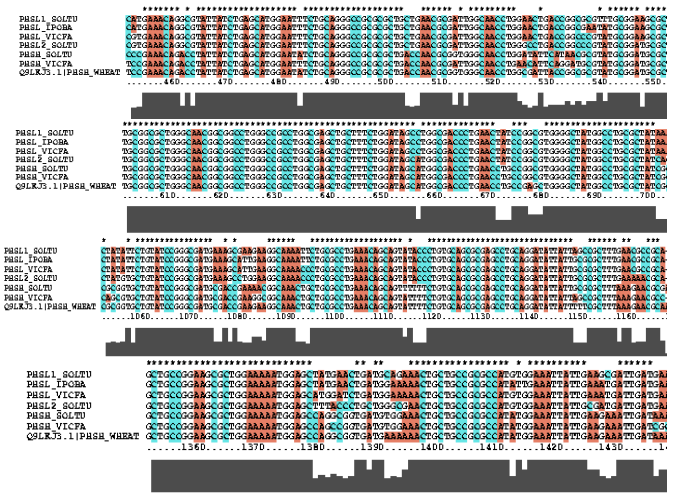

Fig-2 Conserved and consensus regions in starch phosphorylase gene which are obtained for ClustalW, with suitable candidates for our Primers

The various Properties namely Hairpin Loop, Primer dimmer, Tm and GC% were calculated using Primer Biosoft`s Net Primer tool. The result of Net Primer four forward and four backward are shown in [Table-1]. As per result, one forward (F1) and the backward (R2) primer having 50 to 60% GC content, 54 to 58°C Tm and absence of secondary structure were finalized. The primer3 result showed the primers.

Specificity of the primers was validated by carrying out the local alignment against the NCBI`s nr database through BLAST [Fig-3]. The BLAST search was carried against the non-redundant (nr) database. The designed forward and backward primer can be used for PCR amplification of starch Phosphorylase Gene for *Vigna sinensis*.

```
>  emb|X52385.1| Potato mRNA for starch phosphorylase
Length=4942
 Score = 42.1 bits (21), Expect = 0.021
 Identities = 21/21 (100%), Gaps = 0/21 (0%)
 Strand=Plus/Plus
Query  1     CCTGAGGCACTGGAGAAATGG  21
             |||||||||||||||||||||
Sbjct  3190  CCTGAGGCACTGGAGAAATGG  3210

>  dbj|D00520.1|POTGP Solanum tuberosum mRNA for alpha-glucan phosphorylase
precursor,
Complete cds
Length=3101

 Score = 42.1 bits (21), Expect = 0.021
 Identities = 21/21 (100%), Gaps = 0/21 (0%)
 Strand=Plus/Plus
Query  1     CCTGAGGCACTGGAGAAATGG  21
             |||||||||||||||||||||
Sbjct  1331  CCTGAGGCACTGGAGAAATGG  1351

>  gb|L25626.1|IPBSTAPHOA  Ipomea batatas starch phosphorylase gene,
complete cds
Length=6594

 Score = 42.1 bits (21), Expect = 0.021
 Identities = 21/21 (100%), Gaps = 0/21 (0%)
 Strand=Plus/Plus
Query  1     CCTGAGGCACTGGAGAAATGG  21
             |||||||||||||||||||||
Sbjct  3389  CCTGAGGCACTGGAGAAATGG  3409

>  gb|M64362.1|IPBSPP  Sweet potato starch phosphorylase mRNA, complete cds
Length=3292

 Score = 42.1 bits (21), Expect = 0.021
 Identities = 21/21 (100%), Gaps = 0/21 (0%)
 Strand=Plus/Plus
Query  1     CCTGAGGCACTGGAGAAATGG  21
             |||||||||||||||||||||
Sbjct  1352  CCTGAGGCACTGGAGAAATGG  1372
```

Fig-3 BLAST results for designed primers. Primer showed alignment to starch phosphorylase gene.

Table-1 *Various properties of four forward and four backward primers analyzed using the net primer*

Name of the primer	Sequence	Hairpin	Dimer	Tm °C	GC%
F1	CCTGAGGCACTGGAGAAATGG	0	0	61.88	57.14
F2	TGAGGCACTGGAGAAATG	0	0	51.26	50
F3	GAGGCACTGGAGAAATGG	0	0	52.73	55.56
F4	GATGCTGCTCTAGGAAATGG	0	0	61.3	50
R1	ACGACATTTACCTCAACGGC	0	0	57.59	50
R2	CGACATTTACCTCAACGG	0	0	51.47	50
R3	GACATTTACCTCAACGGC	0	0	50.42	50
R4	TACCCTGGAATTGGCACATC	0	0	64.5	50

DNA Isolation and PCR amplification: DNA was extracted by CTAB miniprep methods. 7 µl of DNA sample from stock was diluted with 35 µl of double distilled water, 10 µl of this diluted DNA was loaded on 0.8 percent agarose gel and electrophoresis was done for about 2 hours at the constant current of 50 volts. The gel was observed on UV-transilluminator and picture was recorded on a gel documentation system. 5 µl of DNA from stock was diluted up to 20 times using 95 µl of double distilled water (nanopore).The diluted DNA was subsequently used for PCR amplification. PCR reactions were performed in the total volume of 25 µl reaction volume containing PCR buffer 1X with 1.5 mM $MgCl_2$, 0.25mM dNTPs, 0.100 µM of each primer, 3U Taq polymerase and 50 ηg of genomic DNA as a template. After an initial denaturing step of 2 min at 94°C, 35 cycles were performed for 30 sec at 94°C, 51°C for 1 minute and 72°C for 1 minutes, with a final extension at 72°C for 5 min.

PCR amplification was generated with a set of 4 primers. The primer used for this purpose is listed in [Table-1]. PCR amplified product was (mixed with 6x loading dye and loaded on 3 per cent Agarose) prepared in 1x TBE buffer along with 100bp plus ladder. Electrophoresis was done for 2 hours at 120 volts. Gels were visualized on UV transilluminator and photographed by using SynGene gel documentation system.

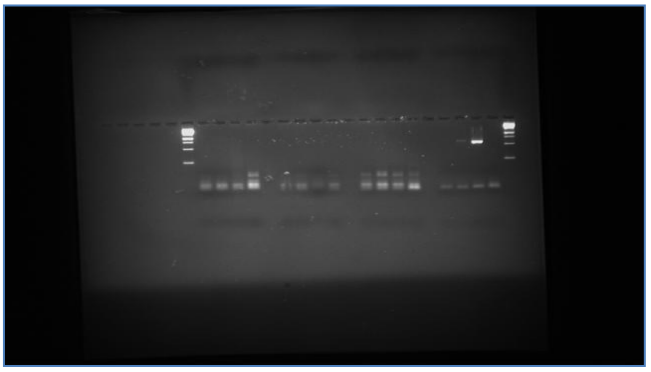

Fig-4 PCR amplification using all four primers sequence Ladder, P1 (4 sample) P2 (4 sample) P3 (4 sample) P4 (4 sample), Ladder

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Conclusion: These research finding may be useful in future for cloning and expression of genes.

Author Contributions: Both author equally contributed

Abbreviations: PCR: Polymerase Chain Reaction, BLAST: Basic Local Alignment Search Tool, NCBI: National Centre for Biological Information

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest: None declared

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