Designing and experimental evaluation of gene-specific primers of pea (*Pisum sativum*) defense proteins

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Abstract

Real-time gene expression analysis by semi-quantitative and quantitative RT-PCR requires a set of gene-specific primers which should have the ability to amplify the gene of interest specifically. In the present study, we have standardized certain parameters for primer design using the freely available Primer3 software. We have designed the primers for defense genes such as ICS (isochorismate synthase), CCoAOMT (caffeoyl CoA *O*-methyltransferase), C4H (cinnamate 4-hydroxylase), and G-alpha in pea. We have also discussed, the way of sequence retrieval, when the sequence is not reported in the organism of interest. We have evaluated the designed primers using cDNA prepared from mRNA isolated from the pea leaves. By analyzing the results, we have found that primers are perfectly binding with the target and giving single sharp band on a DNA electrophoresis gel. It can be concluded that the parameters used for primer designing by Primer3 play a critical role in the experimental results and parameters defined in the present study resulting in a very good amplification during PCR.

Keywords Primer3 · RT-PCR · Gene expression · Gene-specific primers

Introduction

Quantitative real-time PCR (qRT-PCR) is a type of reverse transcription PCR, for the measurement of transcripts accumulated in a sample. Several methods are used to quantify mRNA, such as Northern blotting and ribonuclease protection assays. However, compared to these methods, qRT-PCR requires very less amount of mRNA. The requirement of less labor and production of a large amount of data make this tool very effective for transcript level analysis. Its user-friendly and sensitivity have made it an important tool for bioinformatics, plant biotechnology, and plant–microbe interaction studies. Like conventional PCR, qRT-PCR uses Taq DNA polymerase, an amplification buffer, dNTP mix, and gene-specific primers for amplification of small amounts of DNA (Ginzinger 2002). Use of a fluorescent signal on

polymerization of DNA and use of computerized thermal cycler make it more efficient, authentic, and sensitive (Die et al. 2016).

The necessity of a good quality primer cannot be ignored for qRT-PCR studies. Several freely available primers designing software products are available (Primer3, Beacon, Gene Fisher, NCBI primer blast, etc.) (Gibson et al. 1996). In general, every primer designing software needs an input sequence for designing the gene-specific primers and the sequences are retrieved from the database available on the World Wide Web, most frequently, the NCBI database. Sometimes, a protein or gene is not reported in that organism or the whole genome of the organism is not sequenced, and then, the problem arises for designing a primer, as the input sequence for the gene is unavailable. In the present study, we tried to remove this problem using gene sequences from closely related organisms with the help of some additional freely available software products like ExPASy translate tool and CLUSTALW.

The laboratory experiment based on PCR includes a general overview of the four major fluorescent chemistries involved in qRT-PCR and addresses the basics involved in primer design. With an understanding of how qRT-PCR uses fluorescent signals to quantify DNA (Eshel et al. 2006; Ginzinger 2002; Huep et al. 2014) coupled with knowledge of

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primer selection criteria and there is need of a step-by-step procedure for designing SYBR1 Green primers using free, online software.

Methodology for primer designing

Retrieval of gene sequence and setting of parameters in software

For the primer designing, first step is to search the sequences which are going to be analyzed by the qPCR system (Gibson et al. 1996). In the present study, we have targeted some defense genes (G-alpha, CCoAOMT, C4H, and ICS) for analysis. G-alpha is the subunit of heterotrimeric G-protein and involved in defense signaling of plants (Patel et al. 2016b). ICS catalyzes in the conversion of the cinnamic acid to salicylic acid. However, genes CCoAOMT and C4H are involved in lignification pathway of the plant. We found that some cds (coding DNA sequences) already reported in pea and retrieved them from the NCBI website. This is well known that the whole genome of pea is not available, and we did not get some proteins in pea plant, so we have taken an alternative path for getting input sequence for those genes. We retrieved the sequences of proteins from closely related pulse crops, whose whole genome is available like *Cicer arietinum*, *Glycine max*, and *Medicago* sp. and gone for multiple sequence alignment by the freely available CLUSTLW software of EMBL. Sequences used in the study taken from leguminous plants (CCoAOMT and C4H from *Medicago* and *Cicer*, G-alpha from pea and ICS from *Glycine max*). The region of DNA sequences which shows maximum similarity were taken as input sequence for Primer3.

Primer length: the standard length of primers is mostly 18–24 base pairs. **Primer TM (melting temperature):** for the present study, we kept minimum (62 °C), optimum (64 °C), and maximum (66 °C). **Product size:** for the present example, we kept the product size between 150 and 200, and increase the sensitivity and appropriate quantification by qRT-PCR. Intense fluorescence produced by SYBR Green detection with larger products than smaller. **Enzyme cofactor Mg^{++} concentration:** default set in the Primer3 software is zero but generally with buffer 25 mM $MgCl_2$ supplied with enzymes by companies and 3–6 mM $MgCl_2$ present in the mix. **Sequence repeats:** repeat of sequence in the primer should be avoided (e.g., ATA TATAT), and the maximum number of repeats should be less than 4. A repeat of single nucleotides (e.g., GAAAAA T) should also be avoided for specific binding of primers. The maximum run should be 3–4. **3' Stability:** the value of delta G (Gibbs free energy) due to the binding of five bases from 3' end, the energy required to break the bonds present at the 3' end. Higher the 3' stability will result in good amplification by primers. **GC Clamp:** means maximum delta G from 5' due

to binding of five bases. Most often called GC clamp, i.e., 5' stability which confers the amount of G and C on 5' end. We have kept 1 or 2; due to this, there is strong binding of primers with the template sequence and gives the primers more power for better specificity and amplification.

Use of Primer3 software

After setting all the parameters, accordingly, we have designed the primer using the Primer3 software. The input sequences accessions were as follows: 1: G-alpha (U97043.1.); 2: CCoAOMT (BT137348.1); 3: C4H (CYP73A9); and 4: ICS (AF078080.1.). By pasting these sequences in FASTA format (for the present Primer3 software, FASTA format is not necessary) and clicking the pick primer option in the software, we can get the number of primers within few seconds. Mostly Primer3 gives more than one primer at a time and at the topmost the best primer was given, but we can select the primers according to parameters.

Selection of best primers designed by Primer3, RNA isolation, and cDNA preparation

A set of primers were designed by Primer3. We have used some other software products for picking best primers. First, we have examined the parameters of primers given by Primer3. We have selected those primers which showed no repeat of sequences, product size between 150 and 200, TM difference zero or less than one, GC content range between 45 and 65, any complementarity less than five, 3' complementarity less than 5, repeats less than 4, and no hairpin loop formation (recently included in Primer3). We have checked the primer dimer formation and hairpin formation by software Oligo calculator, and finally, primers were checked using the primer blast software of NCBI. NCBI primer blast results in the organisms, where the primer is running and provides all the parameters previously provided by Primer3 and showed the degeneracy of primers with the template sequence of specific genes for which it was designed. After seeing these all the parameters, we have selected some primers for synthesis (Table 1). After synthesis, we have gone for semi-quantitative RT-PCR with cDNA prepared using RNA isolated from pea leaves (Patel et al. 2016a) with the help of procedure provided in the paper and semi-quantitative RT-PCR was performed according to Marone et al. (2001) by keeping annealing temperature 60 °C.

Results

The designed primers were synthesized by outsourcing and were checked in a wet lab experiment. Primers were giving very good amplification in semi-quantitative RT-PCR

Table 1 List of primers selected for synthesis and wet lab experiment

S. no.	Gene		Primer sequence 5'–3'	TM (°C)	Product length in base pairs	Best annealing temperature
1	ICS	F	CCCGCAGTTTGTGGGTTTCCA	63.68	164	60 °C
		R	GCACCCAGTTCCTGTTCACCA	64.96		
2	CCoAOMT	F	AGGTCACAGCAAAACACCCATGGAA	65.14	150	
		R	TGGCTAGGGCAGTGGCAAGGA	65.3		
3	C4H	F	CGGCTCCCGAACACGAAACG	64.25	171	
		R	GCGGCTTCCGATTCCCAACC	64.09		
4	G-alpha	F	TGCAGAGGCTGTCTGATGCAAA	62.78	155	
		R	CTGGCCGCCGACATCAAACA	63.68		

with a single band of the size defined by the Primer3 software. By seeing the results, we observed (Fig. 1) that primers were working well in and giving a single sharp band of definite product size on the gel after semi-quantitative RT-PCR. Wet lab results were supporting the primer parameters which we have used during primer designing with freely available online software Primer3. All the designed primers are represented in Table 1 having product sizes between 150 and 200 bp. The best annealing temperature for all the primers was found to be 60 °C during PCR standardization. However, melting temperature of the primers was different such as ICS (F-63.68 °C, R-64.96 °C), CCoAOMT (F-65.14 °C, R-65.93 °C), C4H (F- 64.25 °C, R- 64.09 °C), and Gα (F-62.78 °C, R-63.68 °C). The product sizes were found to be 164 bp, 150 bp, 171 bp, and 155 bp for the genes ICS, CCoAOMT, C4H, and Gα, respectively.

Discussion

Primer designing and selection of primers is the initial step for all the study related to gene expression. Length of the primer is one of the important aspects of selection. Much longer primers will take more time to hybridize, more time to extend, and more to remove, so produces very fewer amplicons. The present study suggested the primer length 18–24 bp. In general, for qRT-PCR, most of the researchers used the product length 80–150 bp (Patel et al. 2016b).

However, the present study suggested 150–200. After being familiar with the online designing software Primer3, we can design a good quality primer for gene expression analysis by semi-quantitative or quantitative RT-PCR. Sometimes, the Primer3 software does not give any output after setting interested parameters. To resolve this problem, we must see the statistics of the Primer3 software. After seeing the statistic, we can change some of the parameters without getting an increase in 5' complementarity and self-complementarity means without creating any primer dimers. This is the fact that no primer designing software is flawless and it is difficult to design the primers for sequences less than 400 base pairs. A less number of base pairs in the input sequences always create a problem in setting appropriate GC content and primer TM. TM is the temperature at which 50% of the primers and its complements are hybridized. Most of the research papers, where qPCR was used shown that annealing temperature of PCR should be 60 °C, so during primer designing, we must take care of while putting the range of primer TM (Ginzinger 2002).

Very few studies were available discussing the primer designing and who those discussed primer designing using the freely available software products, and have not validated their primers in wet lab experiment (Thornton and Basu 2011). In contrast with the present study, we have discussed the primer designing parameters as well as validated our primers in wet lab experiments. Schematic representation is given in Fig. 2. We have also discussed how to select a good

Fig. 1 Semi-quantitative RT-PCR results of primers designed by the Primer3 software, where ICS (*isochorismate synthase*), CCoAOMT (*caffeoyl CoA O-methyltransferase*), C4H (*cinnamate 4-hydroxylase*), and G-alpha (one subunit of heterotrimeric G-protein)

Fig. 2 Diagrammatical representation of steps involved in primer designing and method for selection of input sequence

input sequence of the gene for which no reports available in the NCBI database, not discussed in the previous studies. The present work will help researchers to design better quality primers using the freely available software Primer3.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest related to this study to disclose.

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