

BİOLOGİYA VƏ TİBB ELMLƏRİ

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DESIGNING SPECIFIC PRIMERS FOR QUANTITATIVE REAL-TIME PCR ANALYSIS OF DREB GENES IN WHEAT

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The DREB/CBF is an important class of transcription factors that binds to the drought-responsive cis-acting elements. Quantitative RT-PCR is a popular tool for transcriptomics and optimization of conditions is important for the development of a robust assay. To design primers for the expression analysis of the DREB gene, we determined conservative regions by MAFFT alignment of the nucleotide sequences of this gene obtained from NCBI and the sequencing results of the partial DREB gene isolated from local wheat genotypes Barakatli 95 and Azamatli 95. Primers were designed on the selected conserved sequences using Primer3Plus software.

Keywords: DREB genes, wheat, RT-qPCR, Primer3Plus, primer efficiency

Introduction

DREB, the Dehydration Responsive Element (DRE)-binding proteins family is one of the largest families of transcription factors (TFs) that plays a significant role in the signaling network which modulates many plant processes [1]. They belong to the AP2/ERF superfamily of transcription factors. The DREB TFs have been divided into two categories, DREB1 and DREB2. The common feature of all DREB genes is that they have three conservative regions, the EREBP/AP2 DNA binding domain, the N-terminal nuclear localization signal, and the Ser/Thr-rich region [2].

DREBs are one of the important genes for crop improvement in wheat either through engineering stress tolerance or through crop breeding strategies since it is the major TF that binds to the cis-acting elements of most of the osmotic stress-inducible genes responsible for providing osmotolerance to the plants under stress conditions [3]. According to microarray analyses, most of these target stress-inducible genes contained the conserved DRE or DRE-related core motifs in their promoter regions. Both cDNA and Gene Chip microarrays have revealed more than 40 target genes of DREB1/CBF including TFs, phospholi-

pase C, LEA proteins, KIN (cold-inducible) proteins, sugar transport proteins, desaturase, carbohydrate metabolism-related proteins, osmoprotectant biosynthesis proteins, and protease inhibitors known to function in stress response and are thought to be responsible for the observed stress tolerance of the transgenic plants [4].

Therefore, the study of the level of expression of such genes by RT-qPCR is of great interest. Primer design is a critical step when setting up qPCR or reverse transcription-qPCR assay (RT-qPCR). qPCR primers that anneal poorly or to more than one sequence during amplification can significantly impact the quality and reliability of results. Also, if there are performing a one-step RT-qPCR, the reverse transcriptase will use the reverse primer to prime the transcription reaction. In this scenario, a poor primer would result in both inefficient reverse transcription and inefficient amplification. The aim of this study was to develop effective primers for the examination of the transcript level of DREB genes in wheat.

Materials and methods**Plant materials and growth conditions**

Experiments were undertaken on two durum wheat (*Triticum durum* Desf.) genotypes,

Barakatli 95 (drought-tolerant), Garagylchyg 2 (drought-sensitive), and two bread wheat (*Triticum aestivum* L.) cultivars, Azamatli 95 (tolerant), and Giymatli 2/17 (sensitive). Seeds of plants were provided by the Research Institute of Crop Husbandry of Azerbaijan.

Seeds were pregerminated in filter paper and stratified at 4°C for 24 h. Germinated seedlings were transplanted to 2 (for control and drought treatments) multi-cell seed trays containing the soil-sand mixture and transferred in a growth chamber at 16:8 h light/dark period 24°C and 19°C respectively, relative humidity 50%. Plants were watered 2 times a day with tap water. The 3-leaf stage plants were subjected to water deficiency by withholding irrigation. At 7 days after stress, three uniform leaf samples were harvested for qPCR analysis. Samples were immediately frozen in liquid nitrogen and kept at -80°C until required.

RNA extraction and cDNA synthesis

To extract total RNA from the leaf material, the Monarch Total RNA Miniprep Kit (New England Biolabs, Inc.) was used following the supplier's instructions, and genomic DNA contamination was eliminated using RNase-free DNase I. The quality and quantity of extracted RNA samples were determined using agarose gel electrophoresis. The concentration of total RNA was estimated spectrophotometrically. The single-stranded cDNA synthesis was carried out from total RNA in a final volume of 20 µl using LunaScript RT SuperMix Kit (New England Biolabs, Inc.) by the manufacturer's instructions.

Quantitative real-time PCR

Real-time PCR was carried out using a 7500 fast real-time PCR system in a volume of 20 µl containing 10 µl Luna Universal qPCR Mix (New England Biolabs, Inc.). The PCR program was as follows: Initial denaturation at 94°C for 60 s, followed by 45 cycles of 95°C for 15 s and 60°C for 30 s. No-template controls (NTCs) were included for each primer pair. The

reactions were repeated three times for each of three biological replicates. Elongation factor 1 alpha was used as an internal control gene. Primers were designed on a complete coding DNA sequence of wheat DREB genes from NCBI using Primer3Plus software. Melt curve analysis was performed and primers showing a single amplicon were chosen for further experiments. The primer sets efficiencies of each primer pair were determined by the standard curve method using serial dilutions of cDNA, using the formula: Efficiency (%) = $10^{(-1/\text{slope})} - 1 \times 100$. Dissociation curves for each amplicon were analyzed to verify the specificity of each amplification reaction. The fold change in expression (stressed compared to control) was determined according to the $2^{-\Delta\Delta C_t}$ method (5).

Statistical analysis

The statistical analysis was carried out using SAS software ver9.2.

Results and Discussion

To design primers, first of all, nucleotide sequences of DREB genes with access numbers of AF303376.1, AB193608.1, KM520370.1, DQ195068.1 were obtained from NCBI (*The National Center for Biotechnology Information*). To establish conservative sequences specific for the DREB gene, these sequences along with DREB gene nucleotide sequences isolated by us from local Barakatli 95 and Azamatli 95 varieties were aligned using CLUSTAL format alignment by MAFFT v7.427 (<https://mafft.cbrc.jp/alignment/server/>) software (Fig.1).

The primer should be designed in junction exon-exon of genic sequence to avoid amplification of contaminant genomic DNA, amplifying specifically the target cDNA sequence. But this was not possible in our case, because the areas that are conservative for the DREB gene are located only on the large, main exon, which is the first one.

Fig.1. CLUSTAL format alignment by MAFFT (v7.427). Regions chosen for the primer design are highlighted

PCR product/amplicon size for efficient amplification should be between 70 and 200 bp long. Considering this, conservative areas that are ideally suited for primer design were chosen (Fig.1). The number of primers to return depends on how many options you want to choose from. It will not take long for the program to design several primer pairs, and this should give a reasonable chance of finding a suitable pair. In our research, we decided first to design 5 primer pairs. Melting temperature should be for a minimum of 60°C and a maximum of 63°C; the ideal melting temperature is 60°C (with a maximum difference of 3°C in the melting tempera-

tures, T_m , of the two primers). It is possible to use a T_m calculator to determine these temperatures. Besides, when creating a highly effective primer it is important to make sure the 3' end of the primer contains a C or G residue because T and A residues bind more easily to DNA in a non-specific way. Aim for a GC content of around 40-60% to ensure maximum product stability.

The following primers have been designed using Primer3Plus software (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) and considering all conditions:

[illegible]

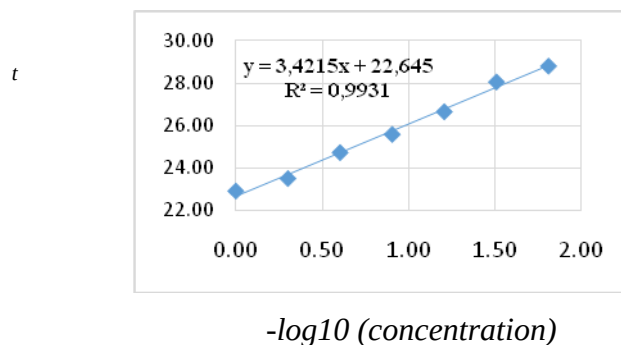


Fig.2. A standard curve constructed from the data of qPCR with Primer pair 3

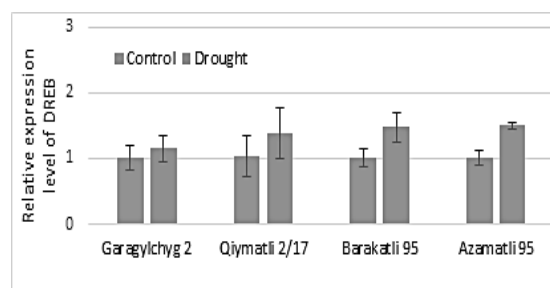


Fig.3. Expression levels of DREB genes in wheat plants under control condition and drought stress

The primer sets efficiencies of each primer pair were determined by the standard curve method using serial dilutions of cDNA: 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625. From the presented primer pairs, Primer pair 3 better met the requirements. The efficiency of the qPCR reaction for this primer pair was 0.96 (Fig.2). Using these primers, the expression level of the DREB transcription factor gene was examined in wheat genotypes under normal and drought conditions.

As seen in Figure 3, the expression of this gene was induced differently under stress in various genotypes. Considering this, it is recommended to use F CTGTGGCTTGGTTCATTCCC / R ACTCCGATTCATCCTTCCCA primer implementing amplification of 208 bp fragment for the expression analysis of the DREB gene in wheat genotypes.

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BUĞDADA DREB GENLƏRİN REAL ZAMANDA GEDƏN qPZR ANALİZİ ÜÇÜN SPESİFİK PRAYMERLƏRİN DİZAYN EDİLMƏSİ

S.M.Rüstəmovə

DREB/CBF - transkripsiya faktorların vacib sinfi olaraq quraqlığa cavabdeh sis-təsir elementləri ilə birləşir. RT-qPZR transkriptomika üçün əsas alətdir və etibarlı analizin işlənilib hazırlanması üçün şəraitin optimizasiyası aparılmalıdır. Bu tədqiqat işində buğdada DREB geninin ekspressiya analizi üçün praymerlərin dizayn edilməsi məqsədilə NCBI-dən götürülmüş bu genə aid nukleotid ardıcılıqları və bizim tərəfimizdən yerli buğda genotipləri olan Bərəkətli- 95 və Əzəmətli- 95 sortlarından ayrılaraq sekvens edilmiş hissəvi DREB geninin nukleotid ardıcılıqları MAFFT düzlənmə proqramı ilə yoxlanılaraq konservativ sahələri müəyyən olunmuşdur. Praymerlər bu ardıcılıqlar üzərində Primer3Plus proqramının köməyiylə dizayn edilmişdir.

Açar sözlər: DREB genləri, buğda, RT-qPZR, Primer3Plus, praymerin effektivliyi

ДИЗАЙН СПЕЦИФИЧЕСКИХ ПРАЙМЕРОВ ДЛЯ КОЛИЧЕСТВЕННОГО ПЦР-АНАЛИЗА В РЕАЛЬНОМ ВРЕМЕНИ ГЕНОВ DREB В ПШЕНИЦЕ

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DREB/CBF является важным классом транскрипционных факторов, который связывается с цис-активными элементами, реагирующими на засуху. Количественный метод ОТ-ПЦР является популярным инструментом для транскриптомики, и для разработки надежного анализа необходима оптимизация условий. С целью проектирования праймеров для анализа экспрессии гена DREB в пшенице, с помощью программы MAFFT, выравнивались нуклеотидные последовательности, взятые из NCBI и результаты секвенирования местных генотипов Баракатли 95 и Азаматли 95. На основе выбранных консервативных последовательностей были разработаны праймеры с использованием программного обеспечения Primer3Plus.

Ключевые слова: гены DREB, пшеница, кПЦР-РВ, Primer3Plus, эффективность праймера