In Silico Design of PCR Primers to Amplify the Salt Tolerance Gene in Soybean

by Florentina Kusmiyati

In Silico Design of PCR Primers to Amplify the Salt Tolerance Gene in Soybean

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ABSTRACT— *Soybean yield is decreased because of many stresses such as salt stress. Ionic and osmotic stresses are the effects of salt stress. An effective way of maintaining sustainable production in salt-affected soil is through breeding high salt tolerance soybean, which can be detected by PCR. The optimal PCR plays an important role in gene expression analysis. The success of a PCR-based method largely depends on the optimal primer sequence analysis in silico prior to a wet-bench experiment. Here we described designing of primer using web-based tools. Many types of online primer design software are available, which can be used free of charge to design desirable primers. The objective was to design of PCR primers to amplify the salt tolerance gene in soybean. A highly conserved region of 411 bases was detected by Clustal Omega. Primers were predicted using Primer3 based on conserved region, considering ideal conditions for primer length, hairpin, dimer, Tm, and GC%. The predicted forward and reverse primers were validated using NetPrimer. Both forward and reverse primers have shown significant similarity with salt tolerance gene and recommended to be used to amplify the salt tolerance gene in soybean.*

Keywords— *FASTA, Glycine max, NCBI, NetPrimer, Primer3*

1. INTRODUCTION

Soil salinity is one of the major environmental factors contributing to the loss of crop productivity and quality [1]. Worldwide, more than 45 million hectares of irrigated agricultural land are affected by salinity, and then 50 % of agricultural lands will be lost by the middle of the 21st century [2]. It also become a severe threat to global food security and agricultural profits [3]. Soybean is classified as a salt-sensitive glycophyte, and salinity stress adversely inhibits all developmental stages [4], since it is the most important food crop because its content presents 40-42 % high quality protein, and 18-22 % oil comprising up of 85 % unsaturated fatty acid [5]. However, salinity at higher levels generates both osmotic and ionic stess on soybean: an osmotic stress caused by reduced water availability in soil and an ionic stress due to imbalance of solutes in the cytosol [6]. Salt stress significantly affects on almost all development stages during the soybean life-cycle, including seed germination and seedling establishment [7]. Salt stress also significantly reduced plant height and total biomass [8], decrease the number of internodes and pods [9], decreases protein content and seed quality [10], and causes chlorosis and necrosis symptoms, in addition to general loss in productivity [11]. Meanwhile, the soybean yield decrease of up to 14 – 35.3 % under salt stress [12].

Development of salt tolerant cultivars will help reduce loss of yield in soybean production under saline conditions [13]. A better understanding of the tolerance mechanisms and identifying the genes involved will enable breeders to develop new strategies to enhance salt tolerance of soybeans [6]. Genotypic variation in salt tolerance of soybean indicates there is potential for identifying genes that increase soybean production, and that the tolerant genotypes exhibit greater nodulation and nitrogen uptake [14]. The causal genes underlying the salt-tolerance locus have been identified by map-based cloning, and found that the dominant gene associated with salt tolerance (e.g. GmCHX1, GmSALT3, and GmST1) [15 – 20]. To speed up the process of creating a new generation with promising agronomic traits (salt tolerance elite soybean lines), salt related genes should be used in research via comparative genomics for plant breeding, transcriptome analysis for plant breeding, genotyping and marker-assisted breeding, and recombinant DNA technology [21]. Molecular markers in breeding programs have played an important role to be efficient in plant breeding with the aid of polymerase chain reaction (PCR) [22]. The use of this technique is becoming a common tool for detecting and quantifying expression profiles of selected genes [23].

PCR is an inexpensive technology for copying a specific sequence of DNA [24]. However the outcome of PCR can be

influenced by many other conditions, such as the template DNA preparation and reaction conditions, designing a good pair of primer is a critical factor [25]. It is sensitive to mismatches between primer and template, and mismatches can lead to a distortion of the true relative abundance of targeted regions [26]. The primer which are unique for the target sequence to be amplified, so primer length, GC%, melting and annealing temperature, 3' end specifity and 5' end stability are important features playing important role in the efficiency of a primer [27]. Primer design is essential to the successful outcome of a PCR experiment, and if the sets of primers are poorly designed, resulting in no production of copies or the occurrence of mismatches [28]. There are numerous web-based primer design software are available. Though most of the software are freely available, but use of these online software requires practice, as online manuals may not be available to assist users. Designing primers for salt tolerance genes through in silico studies can aid in the amplification of the genes that is a prerequisite for further studies. The objective was to design of PCR primers to amplify the salt tolerance gene in soybean.

2. MATERIALS AND METHODS

Nucleotide sequences were searched at the National Center for Biotechnology Information (NCBI, http:// ncbi.nlm.nih.gov/) through Entrez for GmCHX1, GmSALT3, and GmST1. The information about those sequences were reported in Table 1, and those sequences were subjected to Multiple Sequence Alignment (MSA) using Clustal Omega (EMBL-EBI, https://www.ebi.ac.uk/Tools/msa/clustalo/). Therefore the consensus sequence was further used as input for designing primer using Primer3 [29] (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). The parameters were adjusted as follows: Pick left primer: check; Pick right primer: check; Sequence Id: GmST; Targets: skip this option; Excluded regions: blank; Product size ranges: 80-150 100-200; Number to return: 10; Max 3' Stability: 9; Max repeat mispriming: 12; Pair max repeat mispriming: 24; Max template mispriming: 12; Pair max template mispriming: 24; Primer size: min: 18, opt: 21, max: 24; Primer T_m: min: 52, opt: 54, max: 58; Maximum T_m difference: 2; Table of thermodynamic parameters: SantaLucia1998; Product T_m: opt:50; Primer GC: min 35, opt: 65, max = 80; Max self complimentary: 4; Max 3' self-complimentary: 3; Max #N: 0; Max poly-x: 3; Inside target penalty and outside target penalty: default; First base index: 1; GC clamp: 0; Conc. of monovalent cations: 50; Salt correction formula: SantaLucia1998; Conc. of divalent cations: 3.5; Conc. of dNTPs: 0.20; Annealing oligo concentration: 50; Objective fuction penalty weights for primers: T_m Lt: 1 Gt:1, Size Lt:1 Gt:1, Self complementarity: 3, 3' self complementarity: 3; #N's: 2; All other values: 0; Objective function penalty weight for primer pairs: Product T_m Lt: 1 Gt:1, T_m difference: 2, Any complementarity: 3, 3' complementarity: 3, Primer penalty weight: 1, All other values: 0.

Table 1: Information regarding four nucleotide sequences belonging to *Glycine max* gene associated with salt tolerance

Accession name	Accession number	Sequence type	Sequence length
GmCHX1	KF879912	Complete cds	1131
GmSALT3	NM_001353420	mRNA	3259
GmST1	KU871394	Partial cds	411

The primers obtained may or may not be optimal for PCR. It is important to analyze the primer melting temperature (T_m), primer properties, primer secondary structures, primer rating, comprehensive reports, primer pairs, and comprehensive help using an additional software like NetPrimer (PREMIER Biosoft, https://www.premierbiosoft.com/netprimer/). Once the primers have been checked for these various features, it is important to also verify that the amplicon does not form secondary structures. This can be done using UNAFold (IDT, http://sg.idtdna.com/UNAFold). The parameters were adjusted as follows: Nucleotide type: DNA; Temperature: 60 ^{0}C, Na concentration: 50 mM; Mg concentration: 3 mM; Suboptimality: 50 %, Sequence type: linear; Max foldings: 20; Start position: blank; Stop position: blank. Again those nucleotide sequences were subjected for designing forward and reverse primer using GeneFisher2 (BiBiServ, http://bibiserv.cebitec.uni-bielefeld.de/genefisher2) followed by selection of suitable primer using NetPrimer. The parameters for primer prediction were adjusted as per previous case mentioned above. Furthermore, most gene expression assays contain cDNA made from total RNA collected from tissue, it is necessary to verify that the primers do not hybridize with another gene. So, the specifity of both forward and reverse primers was checked using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3. RESULTS AND DISCUSSION

GmCHX1, GmSALT3, and GmST1 nucleotide sequences were taken for designing primer for salt tolerance gene in soybean. A highly conserved region (100 % identical) having 411 bases shown in Figure 1. The Primer3 predicted five forward and seven reverse primer based on consensus sequence (Table 2). Forward primers (F1-F5) having length 18 -20 bp, 40 - 50 % GC content, 52- 54 ^{0}C Tm and absence of secondary structure were finalized. F3 was the first candidate forward primer which should be chosen because it has 50 % GC content (recommendation 50 – 60 %), 53.80 ^{0}C Tm (recommendation 52 – 58 ^{0}C), zero for hairpin and dimer. Besides, F1 was the second candidate forward primer because it has 50 % GC content recommendation 50 – 60 %), 53.73 ^{0}C Tm (recommendation 52 – 58 ^{0}C), and zero for hairpin. In this case, there were 24492 primer pairs considered, 19501 were unacceptable product size, and 2132 had too large Tm difference. This information is useful in determining which parameters have been set too stiff. However there was no reverse primers (R1-R7) which have 50 % GC content, so we supposed the primer obtained may not be optimal for PCR.

In short, the key to developing good primers is to find a primers pair that are very specific, do not produce primer dimers, produce short amplicons, and are efficient enough to produce results that are consistent and reproducible [30]. According to [31], the predicted primers should be subjected to check various properties such as cross dimers are formed between forward and reverse primers, self dimers form between two primers of the same tipe, and hairpins form when primers fold back onto each other, to get the best primer pairs for PCR.
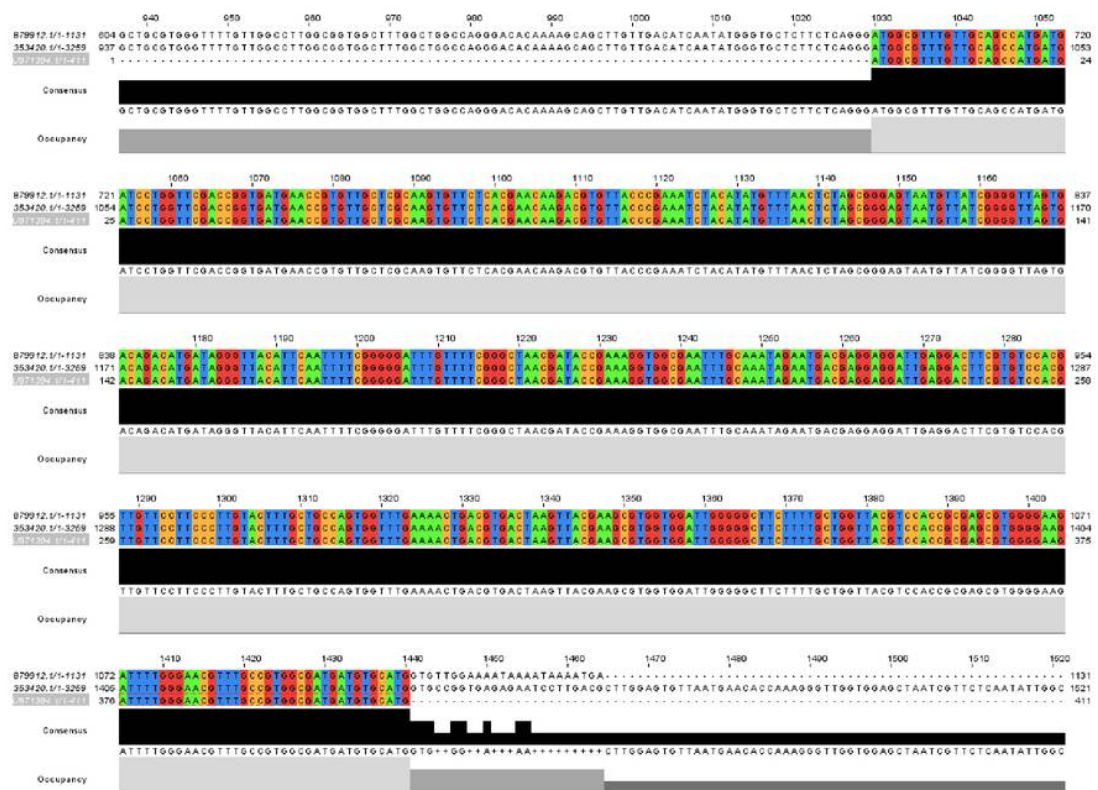

Figure 1: Jalview of ClustalW showing consensus sequence of highly conserved domain having 411 bases

Table 2: Various properties of forward and reverse primers

Primer	Sequence (5'-3')	Length	Hairpin	Dimer	Tm	% GC
F1	GTGACAGACATGATAGGG	18	0	1	53.73	50.00
F2	TAACGATACCGAAAGGTG	18	0	2	53.71	44.44
F3	GGGAGTAATGTTATCGGG	18	0	0	53.80	50.00
F4	TAGTGACAGACATGATAGG	19	0	1	52.76	42.11
F5	TTAGTGACAGACATGATAGG	20	0	0	53.86	40.00
R1	TACAAGGGAAGGAACAAC	18	0	1	53.63	44.44
R2	CAAAGTACAAGGGAAGGAA	19	0	0	54.43	42.11
R3	AGCAAAGTACAAGGGAAG	18	0	0	54.06	44.44
R4	CCTCCTCGTCATTCTATTT	19	0	0	53.78	42.11
R5	GTACAAGGGAAGGAACAA	18	0	0	53.63	44.44
R6	AGTACAAGGGAAGGAACA	18	0	0	54.49	44.44
R7	CAGCAAAGTACAAGGGAA	18	0	0	54.41	44.44

Note: Primer analized by Primer3. F: forward primer; R: reverse primer; Start: the position of the 5'base of the primer, for the forward primers it is the position of the leftmost base and for reverse primers it is the right most base; Length: length of the primer; Hairpin: form when primers fold back onto each other; Dimer: form between two primers of the same tipe; Tm: melting temperature of the primer; % GC: the percent of G and C bases in the primer; ANY: self-complementary score of the primer; SELF: 3'self-complementary of the primer.

**Table 3:** Various properties of F3 and F1 with reverse primers

Forward	Sequence (5'-3')	Reverse	Sequence (5'-3')	Cross Dimer	Self Dimer	Hairpin
F3	GGGAGTAATGTTATCGGG	R1	TACAAGGGAAGGAACAAC	-6.24	0	0
		R2	CAAAGTACAAGGGAAGGAA	0	-3.65	0
		R3	AGCAAAGTACAAGGGAAG	0	-3.65	0
		R4	CCTCCTCGTCATTCTATTT	-6.24	0	0
		R5	GTACAAGGGAAGGAACAA	-6.24	-3.65	0
		R6	AGTACAAGGGAAGGAACA	-6.24	-3.65	0
		R7	CAGCAAAGTACAAGGGAA	0	-3.65	0
F1	GTGACAGACATGATAGGG	R1	TACAAGGGAAGGAACAAC	0	0	0
		R2	CAAAGTACAAGGGAAGGAA	0	-3.65	0
		R3	AGCAAAGTACAAGGGAAG	0	-3.65	0
		R4	CCTCCTCGTCATTCTATTT	-5.67	0	0
		R5	GTACAAGGGAAGGAACAA	0	-3.65	0
		R6	AGTACAAGGGAAGGAACA	0	-3.65	0
		R7	CAGCAAAGTACAAGGGAA	0	-3.65	0

Note: Primer analized by NetPrimer. F: forward primer; R: reverse primer; Start: the position of the 5'base of the primer, for the forward primers it is the position of the leftmost base and for reverse primers it is the rightmost base; Cross Dimer: form between forward and refers primers; Hairpin: form when primers fold back onto each other; Dimer: form between two primers of the same tipe; Tm: melting temperature of the primer

Table 4: Secondary structure analysis

Primer	Sequence (5'-3')	ΔG	T_m	ΔH	ΔS
F1	GTGACAGACATGATAGGG	-0.52	33	-19.6	-64.01
R1	TACAAGGGAAGGAACAAC	-	-	-	-

Note: Primer analized by UNAFold. F: forward primer; R: reverse primer; Start: the position of the 5'base of the primer, for the forward primers it is the position of the leftmost base and for reverse primers it is the rightmost base
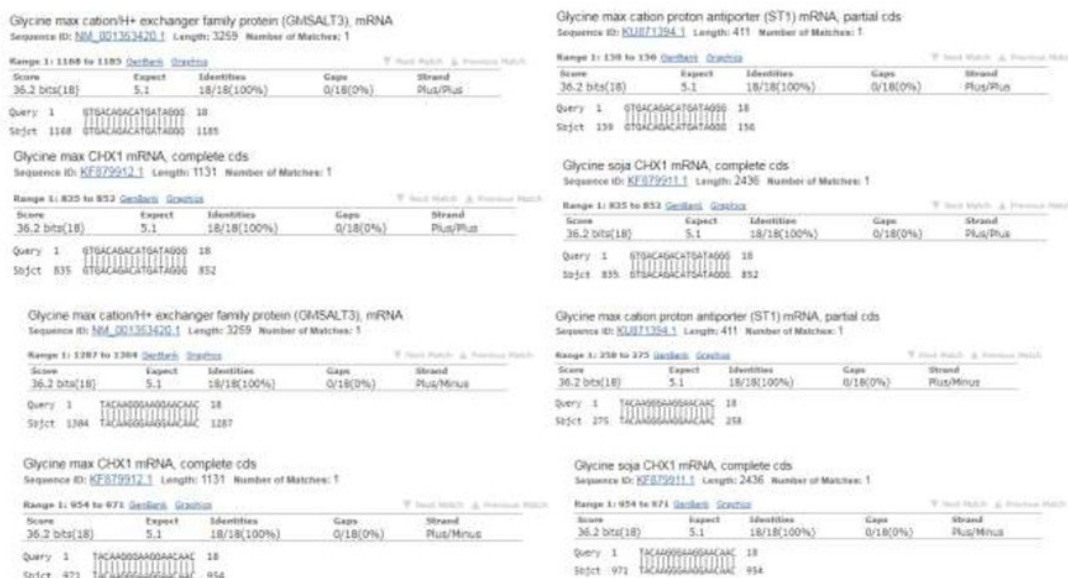
Figure 2: BLAST result for the designed forward and reverse primers

The primer properties were checked between F3 and F1 with reverse primers by NetPrimer (Table 3). F3 and reverse primer pairs showed cross dimer and self dimer together were found in R5 and R6, so this pairs should be avoided because it showed false positives. It is, therefore, critical to design primers that reduce the chance of dimerization and non-specific amplification. However, F1 and R1 primer pair showed zero for cross dimer, self dimer, and hairpin. Table 4 explain that

R1 was not formed a structure and F1 only have a structure. The primer specifity was checked for F1 and R1 primers (Figure 2) which has shown significant alignment with salt tolerance gene. Pridicted primers are important steps before primer synthesis to be used for PCR. In the current study a systematic approach has been made to predict a theoretical primers. The predicted forward and reverse primers have shown significant similarity with for salt tolerance gene in soybean. Hence it may be considered that the designed primers would be used for amplification from heterologous sources leading to discovery of new genes and allele mining.

4. CONCLUSIONS

A highly conserved region of 411 bases was detected by Clustal Omega. Primers were predicted using Primer3 based on conserved region, considering ideal conditions for primer length, hairpin, dimer, Tm, and GC%. The predicted forward and reverse primers were validated using NetPrimer. Both forward (GTGACAGACATGATAGGG) and reverse (TACAAGGGAAGGAACAAC) primers have shown significant similarity with salt tolerance gene and recommended to be used to amplify the salt tolerance gene in soybean.

5. ACKNOWLEDGEMENT

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