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Primer Designing for Dreb1A, A Cold Induced Gene

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Abstract

Primer designing for cold induced gene, DREB1A is done using Primer3 software. Seventeen sequences related to DREB1A gene were retrieved from UniGene, GenBank and RefSeq databases using Entrez. On multiple sequence alignment, a 286 bp conserved region was identified. Potential primers for the conserved region and for seventeen nucleotide sequences were determined using Primer3. The properties of these potential primers were analyzed using Premier Biosoft's NetPrimer tool. One forward (5' end) and one backward (3' end) primer having 50 to 60% GC content, 52 to 58°C Tm and absence of secondary structures were finalized. Specificity of the primers was validated by carrying out local alignment against the NCBI's nr database through BLAST. All the alignments showed significant alignment to DREB1A gene validating the specificity of the primers.

Keywords: DREB1A gene; Cold induced gene; Primer design; Primer3; NetPrimer; BLAST

Introduction

In the last 15 years, the computer has become an essential companion for cell and molecular biologists. Bioinformatics refers to the creation and advancement of algorithms, computational and statistical techniques, and useful to solve formal and practical problems arising from the management and analysis of biological data. Bioinformatics is considered as amalgam of biological sciences especially biotechnology with computer science and information technology. Bioinformatics is the application of computer technology to the management of biological information. It is used to gather, store, analyze and integrate biological and genetic information which can then be applied to gene- based drug discovery and development. It also includes the collection, organization, storage and retrieval of biological information from 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/ steps in successful DNA sequencing. Optimal primer sequence and appropriate primer concentration are essential for maximal specificity and efficiency of Polymerase Chain Reaction (PCR) amplification. A poorly designed primer can result in little or no product due to non- specific amplification and/or primer-dimer formation that may become competitive enough to suppress product formation. There are several online tools available that are devoted to serve molecular biologist for effective PCR primer design.

Plants and other living organisms frequently encounter biotic and abiotic stresses causing potential harm to them. As against biotic stresses that are pathogenic (Ramonell and Somerville, 2002), plants, being sessile, are often exposed to abiotic stresses (Smirnoff, 1998; Bohnert and Sheveleva, 1998). Abiotic stress refers to any harmful effect that is caused by nonliving environmental factor(s) and can be an object, substance or process such as drought or dehydration, extreme heat or cold, high light, acute pressure, non-physiological pH, oxidative reactions, high salt levels, mineral deficiencies or excess, high amount of acidity or alkalinity, toxicity, radiations, high wind, mechanical stress and even wounding. Many of the stress factors eventually lead to cellular dehydration or oxidative stress (Smirnoff, 1998; Vinocur and Altman, 2005).

Acquired plant tolerance to abiotic stresses has been achieved by conventional plant breeding, although with limited success. Combined with the use of DNA molecular markers and quantitative trait loci (QTLs), it offers enormous scope to improve abiotic stress tolerance. Alternatively, using genetic engineering, the gene(s) of interests can be transferred to other stress-sensitive

plant variety to engineer stress tolerance (Holmberg and Bulow, 1998). In case of wheat, the DREB (Dehydration Responsive Element Binding) transcription factor has been used to generate transgenic lines that showed increased drought tolerance (Langridge et al., 2006; Pellegrineschi and Hoisington, 2007). Of course, the success of such stress engineering hinge upon field trials. For a successful stress-engineering, the associated high energy cost can not be underestimated. In case of transgenic Arabidopsis plants, the overexpression of DREB1A target genes under unstressed conditions caused dwarfed phenotypes in the transgenic plants (Liu et al., 1998).

The Arabidopsis RD29A/COR78/LTI78 gene is induced by drought, cold, and ABA, and can be activated by either ABA-dependent or ABA-independent responses. In the promoter of this gene, a 9-bp conserved sequence, TACCGACAT, called Dehydration Response Element (DRE), is an essential cis-element for RD29A gene regulation. Similar cis-acting elements, containing core DRE sequence i.e., an A/GCCGAC motif called C-repeat (CRT) and low-temperature-responsive element (LTRE), have been observed to regulate promoters of cold-inducible cor15a. Two DRE/CRT-binding proteins, DREB1/CBF (DRE Binding protein-1/C-repeat binding Factor), and DREB2 (DRE Binding protein-2) have been isolated (Liu et al., 1998). The former is specifically cold-induced, while the latter is induced only by dehydration and high-salinity. DREBs belong to ERF (Ethylene Responsive element binding Factor)/AP2 family of transcription factors (Riechmann et al., 2000; Agarwal et al., 2006). In Arabidopsis, three DREB1/ CBF namely, DREB1B/CBF1, DREB1A/CBF3, and DREB1C/CBF2 have been isolated.

The DRE related motifs have been reported in the promoter regions of many drought-and cold-inducible genes such as kin1, cor6.6, rd17 (Liu et al., 1998). In recent microarry experiments, 16 genes containing DRE motif have been identified (Seki et al., 2001; 2002). These also activated transcription factors such as C2H2 zinc-finger-type and AP2/ERF-type, further downstream and thereby stress-induced secondary gene expressions. Future research would reveal additional cis- and trans-acting elements that function in ABA-dependent and ABA-independent manner **(**Shinozaki et al., 2003; Yamaguchi-Shinozaki and Shinozaki, 2005**).**

Many orthologous genes of DREB1/CBF have been found in several plant species that are involved in biotic and abiotic stress tolerance, suggesting that by gene transfer, it can be effectively used to improve the stress-tolerance of important crop plants against drought, high-salinity and freezing (Agarwal et al., 2006). Overexpression of the Arabidopsis DREB1/CBF genes in transgenic Brassica napus or tobacco plants induced expression of orthologs of Arabidopsis DREB1/CBF-targeted genes and increased the freezing and drought tolerance of transgenic plants. Constitutive overexpression of DREB1B/CBF1 in transgenic tomato increased drought, chilling, and oxidative stress tolerance (Yamaguchi-Shinozaki and Shinozaki, 2006; references cited therein).

Earlier, we have designed forward and backward primers for DREB2A gene (Garg and Kumar, 2006). In the present study, primers for cold induced gene, DREB1A are designed using cDNA sequences of the gene taken from Gene Bank. Validation of the designed primers has been done by local alignment against the NCBI's nr database through BLAST.

Material and Methods

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. There are number of criteria viz., primer length, Tm, GC contents, 3'-end sequence, dimer formation, false priming, specificity, degenerate primers, hairpin loop that need to be established in the design of primers (Dieffenbach et al., 1995; Singh and Kumar, 2001).

Primer length: a hard core factor

Specificity, temperature and time of annealing are at least partly dependent on primer length. The rule-of-thumb is to use a primer with a minimal length that ensures a denaturation temperature of 55-56^{0}C. Primers of 18-24 nucleotides in length are accepted as best in being sequence specific if the annealing temperature of the PCR reactions is set within 5^{0}C of the dissociation temperature of primer-template duplex. Longer primers (25-35 nucleotides) are required only to discriminate homologous genes within different species or when a perfect complementary sequence to the entire template is not expected.

Melting temperature (Tm)

The optimal melting temperature for primers generally lies in the range of 52-58^{0}C. Both the forward and backward oligonucleotide primers should be designed such that they have similar melting temperatures. A good working approximation of this value can be calculated using the formula of Wallace et al. (1979),

$Tm = 2(A+T) + 4(G+C)$.

GC content

GC% is an important characteristic of DNA and provides information about the strength of annealing. Primers should have a GC contents between 50 and 60 percent. GC contents, melting temperature and annealing temperature are strictly dependent on one another.

Dimers and false priming cause misleading results

Annealing between the 3' end of one primer molecule and the 5' end of another primer molecule and subsequent extension results in a sharp background product known as primer dimer. If the primer binds anywhere else than the target site, specificity of the amplification specifically is reduced significantly. This leads to weak output or a smear. When some bases at 3' end of the primer bind to the target sequence and achieve favorable chances of extension, it also leads to weak output or a smear.

Specificity

Primer specificity is at least partly dependent on primer length. It is found that there are many more unique 24 base oligos than 15 base pair oligos. Primers must be chosen so that they have a unique sequence within the template DNA that is to be amplified. A primer designed with a highly repetitive sequence will result in a smear when amplifying genomic DNA.

Terminal Nucleotides Make a Difference

Both the terminals of the primer are of vital importance for a successful amplification. The 3'- end position in the primer affects mis-priming. Runs (3 or more) of C's or G's should be avoided as G+C rich sequence leads to mispriming. The primer should have a stable 5' end and an unstable 3' end. Stretches of A and T are also to be avoided as these will open up stretches of the primer-template complex. A "G" or "C" is desirable at the 3' end. This GC clamp reduces spurious secondary bands.

Software in primer design: The use of software in biological applications has given a new dimension to the field of bioinformatics. Many different programs for the design of primers are now available. Free ware softwares are available on the internet and many Universities have established servers where a user can log on and perform free analyses of proteins and nucleic

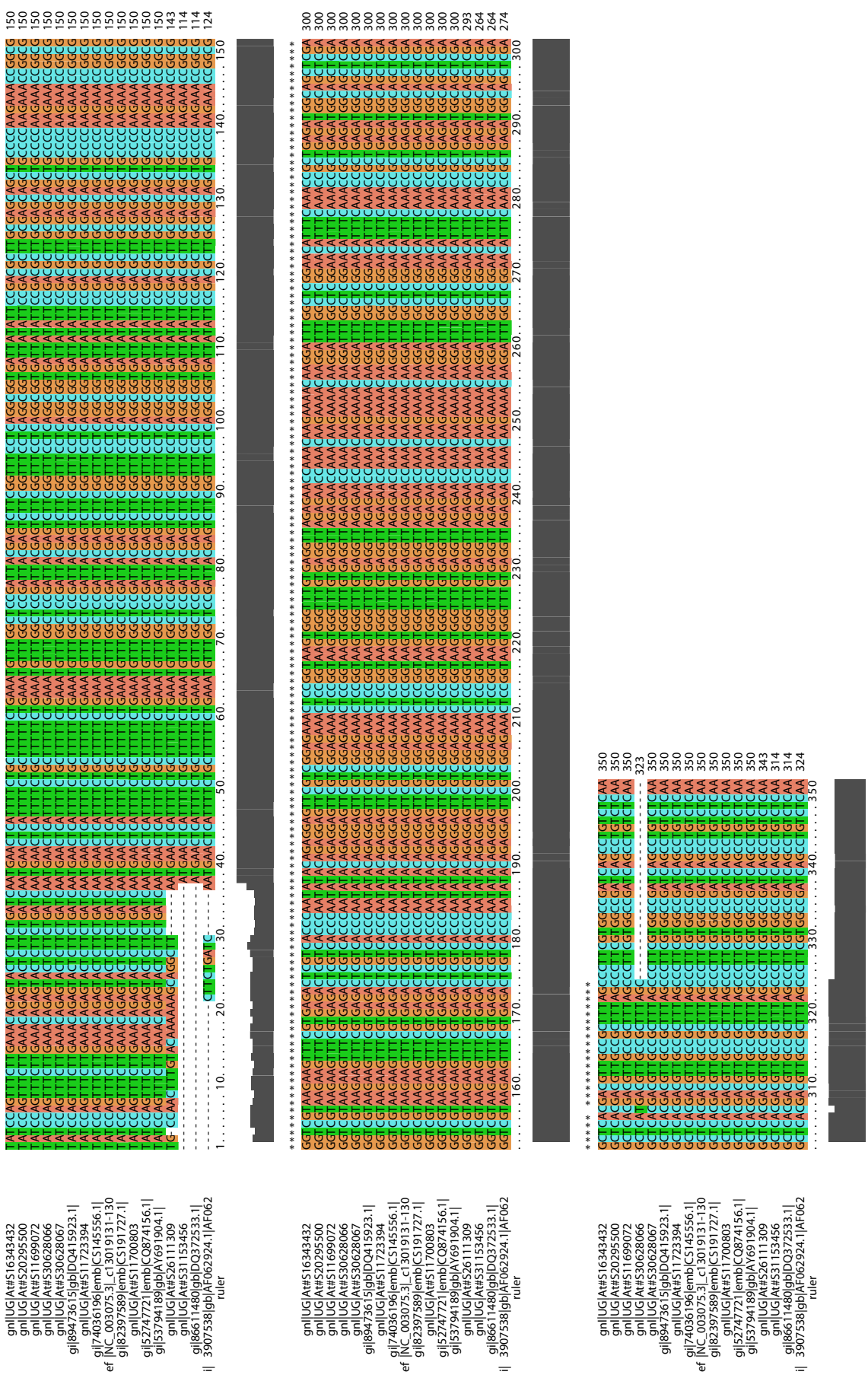

Figure 1: Conserved domain in DREB1A. A highly conserved region in cold induced gene, DREB1A. Asterix represents the postions having 100% identity in the alignment. Also shown is the nucleotide coordinates for the conserved region.

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acid sequences. There are number of stand –alone programs as well as complex integrated networked versions of the commercial software available. These software packages may be for complete DNA and protein analysis, secondary structure predictions, primer design, molecular modeling, development of cloning strategies, plasmid drawing or restriction endonuclease analyses. Many companies all over the world are engaged in biosoftware development. Some scientists have also developed algorithms and computer programs for various purposes of primer design

Primer Design: Twenty-four sequences of DREB1A were taken from GenBank database. After analyzing, very long and very short sequences were neglected. Finally, seventeen sequences were considered. Multiple sequence alignment of those

Name of the primer	Sequence (5' – 3')	Hairpin	Dimer	Tm	GC%
F1	GCTCCGATTACGAGTCTTC	0	0	51.97	52.63
F2	GAGTCTTCGGTTTCCTCAG	0	1	51.51	52.63
R1	CCTCACAAACCCACTTACC	0	0	52.24	52.63
R2	ACCTCACAAACCCACTTACC	0	0	53.74	50
R3	CCACTTACCGGAGTTTCTC	0	1	51.79	52.63

Table1: Various properties of two forward and three backward primers analyzed using NetPrimer.F1 primer was observed most suitable as a potential forward primer for the amplification of DREB1A gene.

Primer3 Output

```
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any   3'  seq
LEFT PRIMER        153   19   55.00   52.63  3.00  2.00 GCTCCGATTACGAGTCTTC
RIGHT PRIMER       290   18   56.26   55.56  5.00  4.00 TTCTCCGACGAACTCCTC
SEQUENCE SIZE: 908
INCLUDED REGION SIZE: 908

PRODUCT SIZE: 138, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 3.00

    1 CCTGAACTAGAACAGAAAGAGAGAGAAACTATTATTTCAGCAAACCATACCAACAAAAAA


   61 GACAGAGATCTTTTAGTTACCTTATCCAGTTTCTTGAAACAGAGTACTCTTCTGATCAAT


  121 GAACTCATTTTCTGCTTTTTCTGAAATGTTTGGCTCCGATTACGAGTCTTCGGTTTCCTC
                                     >>>>>>>>>>>>>>>>>>>


  181 AGGCGGTGATTATATTCCGACGCTTGCGAGCAGCTGCCCCAAGAAACCGGCGGGTCGTAA


  241 GAAGTTTCGTGAGACTCGTCACCCAATATACAGAGGAGTTCGTCGGAGAAACTCCGGTAA
                                     <<<<<<<<<<<<<<<<<<<


  301 GTGGGTTTGTGAGGTTAGAGAACCAAACAAGAAAACAAGGATTTGGCTCGGAACATTTCA


  361 AACCGCTGAGATGGCAGCTCGAGCTCACGACGTTGCCGCTTTAGCCCTTCGTGGCCGATC
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Figure 2: Primer3 output for the designed primers. Primer3 results validating the sensitivity of the designed primers for cold induced gene. *DREB1A*.

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seventeen sequences was done using Bioinformatics tool ClustalX.

ClustalX software: ClustalX is a new windows interface for the ClustalW multiple sequence alignment program (Thompson et al., 1997). It provides an integrated environment for performing multiple sequence and profile alignments for DNA and proteins and analyzing the results. The sequence alignment is displayed in a 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.

NetPrimer: NetPrimer is a web based program that analyzes individual or pairs of primers. It is available free of charge. The program combines the latest primer design algorithms with an intuitive interface allowing the user to analyze primers over the Internet (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html). All primers are analyzed for melting temperature using the nearest neighbor thermodynamic theory to ensure accurate Tm prediction. Primers are analyzed for all secondary structures including hairpins, self-dimers, and cross-dimers in primer pairs. This ensures the availability of the primer for the reaction as well as minimizing the formation of primer dimer. The

Name of the primer	Sequence (5' – 3')	Hairpin	Dimer	Tm	GC%
R1	TCTCCGACGAACTCCTCTG	0	0	54.41	58
R2	TTCTCCGACGAACTCCTCTG	0	0	54.97	55
R3	TTCTCCGACGAACTCCTC	0	0	52.09	56
R4	AGTCTCCAAGCCGAGTCAGC	0	0	57.97	60
R5	AGTCTCCAAGCCGAGTCAG	0	0	54.96	58
R6	ATCCGTCGTCGCATCACAC	0	0	56.85	58
R7	AACATCGCCTCATCGTGC	0	0	54.66	56

Table 2. Various properties of seven backward primers analyzed using NetPrimer. R3 primer was observed most suitable as a potential backward primer for the amplification of DREB1A gene

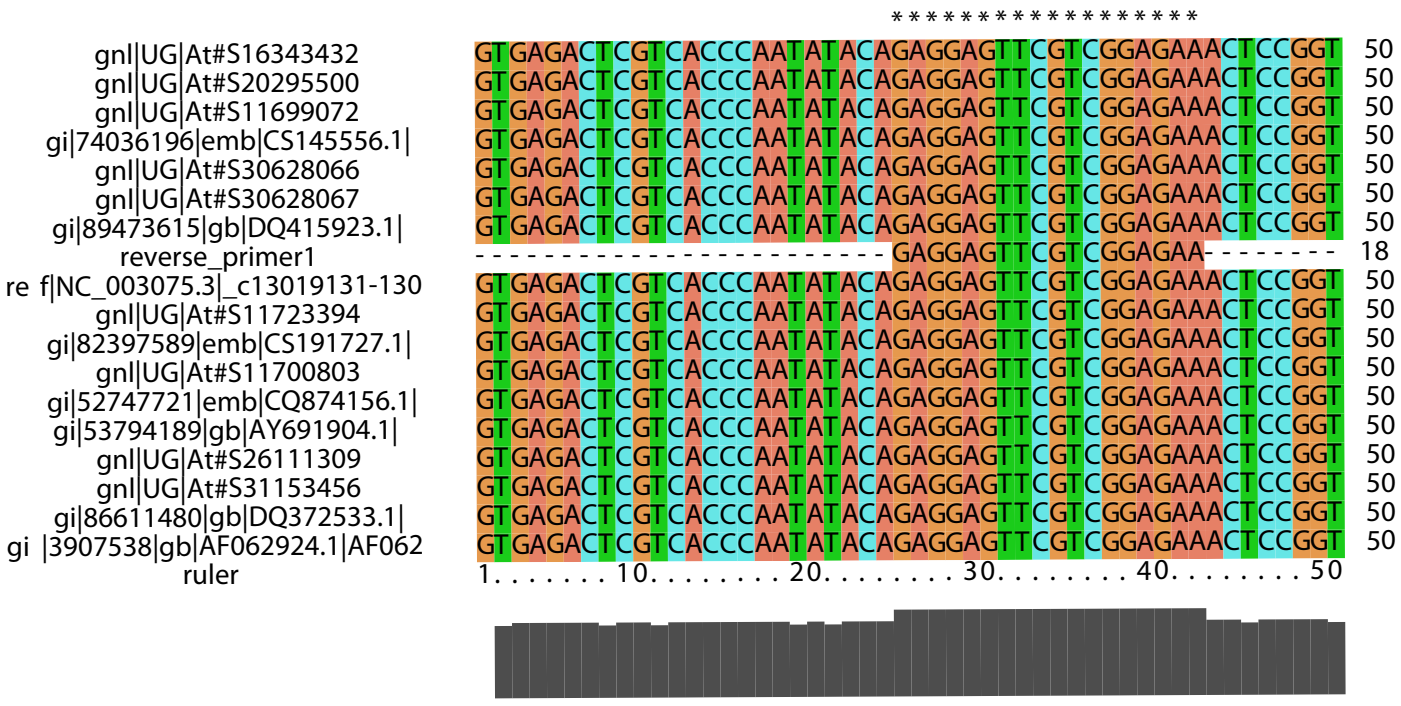

Figure 3: Multiple sequence alignment of the finalized backward primer (reverse complement) sequence with the seventeen nucleotide sequences of cold induced gene, DREB1A. As evident, the primer is highly conserved in DREB1A gene thus is a good candidate for the amplification of DREB1A gene.

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```
>gb|EF156749.1|  Capsella bursa-pastoris DREB1A (DREB1A) gene, complete cds
Length=651

 Score = 38.2 bits (19),  Expect = 0.15
 Identities = 19/19 (100%), Gaps = 0/19 (0%)
 Strand=Plus/Plus

Query  1   GCTCCGATTACGAGTCTTC  19
           ||||||||||||||||||
Sbjct  35  GCTCCGATTACGAGTCTTC  53


>gb|DQ415923.1|  Arabidopsis thaliana cold resistance related AP2 transcription
factor (CRAP2) gene, complete cds
Length=1094

 Score = 38.2 bits (19),  Expect = 0.15
 Identities = 19/19 (100%), Gaps = 0/19 (0%)
 Strand=Plus/Plus

Query  1    GCTCCGATTACGAGTCTTC  19
            ||||||||||||||||||
Sbjct  269  GCTCCGATTACGAGTCTTC  287


>gb|DQ403814.1|  Nicotiana tabacum DREB1A mRNA, complete cds
Length=651

 Score = 38.2 bits (19),  Expect = 0.15
 Identities = 19/19 (100%), Gaps = 0/19 (0%)
 Strand=Plus/Plus

Query  1   GCTCCGATTACGAGTCTTC  19
           ||||||||||||||||||
Sbjct  35  GCTCCGATTACGAGTCTTC  53
```

A

```
>ref|NM_118680.1|  Arabidopsis thaliana DREB1A (DEHYDRATION RESPONSE ELEMENT
DNA binding / transcription factor/ transcriptional activator (DREB1A) mRNA, complete
Length=908

 Score = 36.2 bits (18),  Expect = 0.60
 Identities = 18/18 (100%), Gaps = 0/18 (0%)
 Strand=Plus/Minus

Query  1    TTCTCCGACGAACTCCTC  18
            ||||||||||||||||||
Sbjct  290  TTCTCCGACGAACTCCTC  273


>gb|AY804251.1|  Chorispora bungeana AP2/EREBP transcription factor mRNA, partial
cds
Length=165

 Score = 36.2 bits (18),  Expect = 0.60
 Identities = 18/18 (100%), Gaps = 0/18 (0%)
 Strand=Plus/Minus

Query  1   TTCTCCGACGAACTCCTC  18
           ||||||||||||||||||
Sbjct  22  TTCTCCGACGAACTCCTC  5


>gb|AY691904.1|  Arabidopsis thaliana DREB1A gene, complete cds
Length=805

 Score = 36.2 bits (18),  Expect = 0.60
 Identities = 18/18 (100%), Gaps = 0/18 (0%)
 Strand=Plus/Minus

Query  1    TTCTCCGACGAACTCCTC  18
            ||||||||||||||||||
Sbjct  244  TTCTCCGACGAACTCCTC  227
```

Figure 4: BLAST results for the designed primers. Both forward (A) and backward (B) primers showed significant alignment with *DREB1A* gene.

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program eases quantitation of primers by calculating primer molecular weight and optical activity. To facilitate the selection of an optical 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.

Primer3 software: It is software developed by Rozen and Skaletsky (2000). It is freely available on 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. Primer3 is widely used program for designing PCR (Polymerase Chain Reaction) primers. Primer3 can also design hybridization probes and sequencing primers. It is a tool for automated primer generation according to thermodynamic, primer size, and product size restrictions.

SCPrimer software: It is software developed by Jabado et al. (2006). It is freely available at http://scprimer.cpmc.columbia.edu/SCPrimerApp.cgi. Users are first required to register their user name in order to avail the facility of the software for primer designing. SCPrimer allows design of degenerate primers from multiple sequence alignments in fasta or clustalw format. The algorithm attempts to minimize the number of primers needed to amplify all the sequences in the alignment with a greedy heuristic for the set cover problem.

BLAST: The **Basic Local Alignment Search Tool (BLAST)** finds regions of local similarity between sequences (Altschul et al., 1997). It is an algorithm for comparing primary biological sequence information, either the amino acid sequences of different proteins or the nucleotides of DNA sequences. A BLAST search enables a researcher to compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold. The BLAST program was designed by Eugene Myers, Stephen Altschul et al., 1990 at the NIH and was published.

Results and Discussion

On searching for *DREB*1A in RefSeq database through Entrez, one record corresponding to *DREB*1A was found. The database was accessed from the website, http://www.ncbi.nlm.nih.gov/RefSeq. The Reference Sequence (RefSeq) database aims to provide a comprehensive, integrated, non-redundant set of sequences, including genomic DNA, transcript (RNA), and protein products, for major research organisms (Pruitt et al., 2005). Furthermore, five EST sequences and four mRNA sequences corresponding to *DREB*1A were retrieved from the UniGene database (http://www.ncbi.nlm.nih.gov/UniGene). UniGene database consists of transcript sequences that appear to come from the same transcription locus (Pontius et al., 2003). Besides, four sequences from GenBank database (Benson et al., 2005) and three sequences from EMBL database (Stoesser et al., 1999) were also retrieved for further analysis, resulting in total seventeen nucleotide sequences belonging to *Arabidopsis thaliana* group.

On Multiple Sequence Alignment (MSA) of seventeen nucleotide sequences of *DREB*1A using ClustalX, one onserved region of 286 base pairs was found as shown in Figure 1. The conserved region of the sequence was inserted in the input box of the GUI of Primer3. The parameters were adjusted as follows: Tm min. = 52°C, max. = 58°C; Primer size min. = 18, max.= 24; GC% min.= 50, max.= 60. Afterwards, pick primer button was clicked. The software designed two forward and three backward primers.

The various properties namely Hairpin loop, Primer dimer, Tm and GC% were calculated for all forward and backward primers using Premier Biosoft's NetPrimer tool. The results of NetPrimer for two forward and three backward primers are shown in Table 1 As per results, one forward (F1) primer having 50 to 60 %GC content, 52 to 58°C Tm and absence of secondary structures was finalized. However, of three backward primers, two backward (R1 and R2) primers were found to have runs of C's and A's in them and third backward (R3) primer was found to be forming a dimer. Therefore, backward primer was not finalized. Afterwards, SCPrimer, a web based application for the identification of degenerate primer was used. Sixteen protein sequences for *DREB1*A gene were retrieved from GenBank and EMBL database and were fed as an input to the SCPrimer tool. The parameters for primer designing were set as follows: Tm min. = 52°C, max. = 58°C; Primer size min. = 18, max.= 24; GC% min.= 50, max.= 60. SCPrimer predicted 1513 potential primer (756 forward and 757 backward) for the input sequences. As forward primer was already finalized, only 757 backward primer sequences were retrieved for further primer analysis. Of the 757 backward primers, primers with degeneracy greater than one and having A or T at the 3' end were discarded as presence of G or C at the 3' end of primer prevents breathing of end and increases efficiency of the priming thus resulting in 177 potential primer sequences. All the 177 sequences were then analyzed for the presence of secondary structures like hairpin loops, dimer, palindrome and repeats using NetPrimer software. Primer sequences with none of the secondary structures and having Tm and %GC content with in the favorable range were selected resulting in total seven potential backward primers as shown in Table 2. Primer3 software was further used to examine the sensitivity of all the seven backward primers against the DREB1A gene sequence. As per the results, only one primer (R3) was found suitable as a backward primer as shown in Fig. 2. As it is an utmost requirement for the degenerate primer to be conserved in all the nucleotide sequence for the DREB1A, multiple sequence alignment of the chosen backward primer (R3) was carried out along with seventeen nucleotide sequences for DREB1A using ClustalX. As evident from the Fig. 3, selected primer is having 100% conservation in all the sequences for DREB1A and thus is a good candidate for the amplification of DREB1A gene.

Specificity of the primers was validated by carrying out the local alignment against the NCBI's nr database through BLAST. The BLAST search was carried against the non-redundant (nr) database. All the results showed significant alignment to DREB1A thus validating the specificity of the primers as shown in Fig 4.

Conclusion

In this study, a systematic attempt has been made to design forward and backward primer for the amplification of DREB1A gene. A conserved region ina set of DREB1A gene sequences was identified using ClustalX software and primers (forward and backward) were identified using Primer3 software. Further, due tothe inefficiency of all the backward primers designedusing Primer3 software, SCPrimer software was usedlike presence of secondary structures, Tm or %GC out of range and presence of A or T at the 3' end, one primer sequence was found most suitable as a backward primer. Sensitivity and Specificity measure analysis using Primer3 and BLAST softwares, respectively showed that the designed primers are theoretically appropriate for the amplification of DREB1A gene. The designed forward and backward primers may be used for PCR amplification of DREB1A gene using microbial genomic DNA.

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