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PCR Primer Design: DREB Genes

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Abstract

The polymerase chain reaction (PCR) is an enzymatic reaction which follows simple, predictable and well understood principles. Selective amplification of nucleic acid molecules, that are initially present in minute quantities, provides a powerful tool for analyzing nucleic acids. In this context, efficiency and sensitivity of the PCR largely depends on the efficiency of the primers that are employed for the amplification of a concerned gene. Environmental adversities like drought resulting in scarcity of water have detrimental effects on crop yields worldwide. Sustainable agricultural and food productivity requires development of stress resistant plant species like drought resistant crops that can with stand and flourish in scanty water level environments. A key to underlying such attempts is the molecular understanding of the discrete stress processes that are interwoven at multiple levels. In this review, we discuss about some of the contemporary developments in the area of stress resistance by plants along with the various approaches for the PCR primer designing of two key genes involved in drought resistance, *DREB1*A and *DREB2*A.

Introduction

Ice, flood and drought have been the scourge of agriculture over the ages resulting in poor harvests and threat of famine. Today, the importance of crop resistance to water stress, extremes of salinity and harsh temperature is likely to increase further as the range of environments in which crops are cultivated expands and the incidence of extreme weather conditions increases with the spectre of global warming (Ramonell and Somerville, 2002). Plants being sessile are more susceptible to abiotic stresses than other living organisms. Abiotic stress refers to any harmful effect that is caused by nonliving environmental factors(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 (Smirnoff, 1998). Being an essential component for the plants, water has always been a crucial factor for the normal growth of plants. Environmental adversities like drought resulting in scarcity of water have detrimental effects on crop yields worldwide. Sustainable agricultural and food productivity requires development of stress resistant plant species like drought resistant crops that can with stand and flourish in scanty water level environments. A key to underlying such attempts is the molecular understanding of the discrete stress processes that are interwoven at multiple levels. Various genetic and biochemical approaches have attempted to study the key genes responsible for drought resistance. Such studies complemented by newer comparative and functional genomics have provided details about drought-induced gene expression leading to the accumulation of specific proteins, conferring drought-resistance. With the recent surge in the number of completely sequenced plant genomes available online, *In Silico* analysis of these genomes is crucial for the functional annotation of genes. In this context, *In Silico* identification

and analyses of genes encoding for proteins that lead to stress resistance in plants is very much important. Besides, aiding in understanding the molecular mechanism of these proteins in stress resistance, such studies will also help in identifying novel proteins that are crucial for imparting tolerance to various stress factors in plants. Designing primer for stress resistant genes through *In Silico* studies can aid in the amplification of stress resistant genes that is a prerequistite for further wet lab studies on a gene. Besides, designed primers can also be crucial in deciding whether a particular variety of a plant has specific gene(s) for tolerating a particular stress condition or not. In this review, we discussed about some of the contemporary developments in this area along with the various bioinformatic approaches for the PCR primer designing of two key genes involved in drought resistance, *DREB1*A and *DREB2*A.

Stress Activated Transcriptional Regulation

Shinozaki et al., (2003) described an approach for bolstering plant resistance against harsh environmental conditions like drought, cold and flood. By overexpressing a single transcript factor, they have succeeded in inducing the expression of several stress- related genes leading to striking improvements in plant tolerance to freezing, salt loading and dehydration (Fig 1.). Till date, various genes and transcription factors crucial for stress tolerance have been studied in various plants species and have been discussed below.

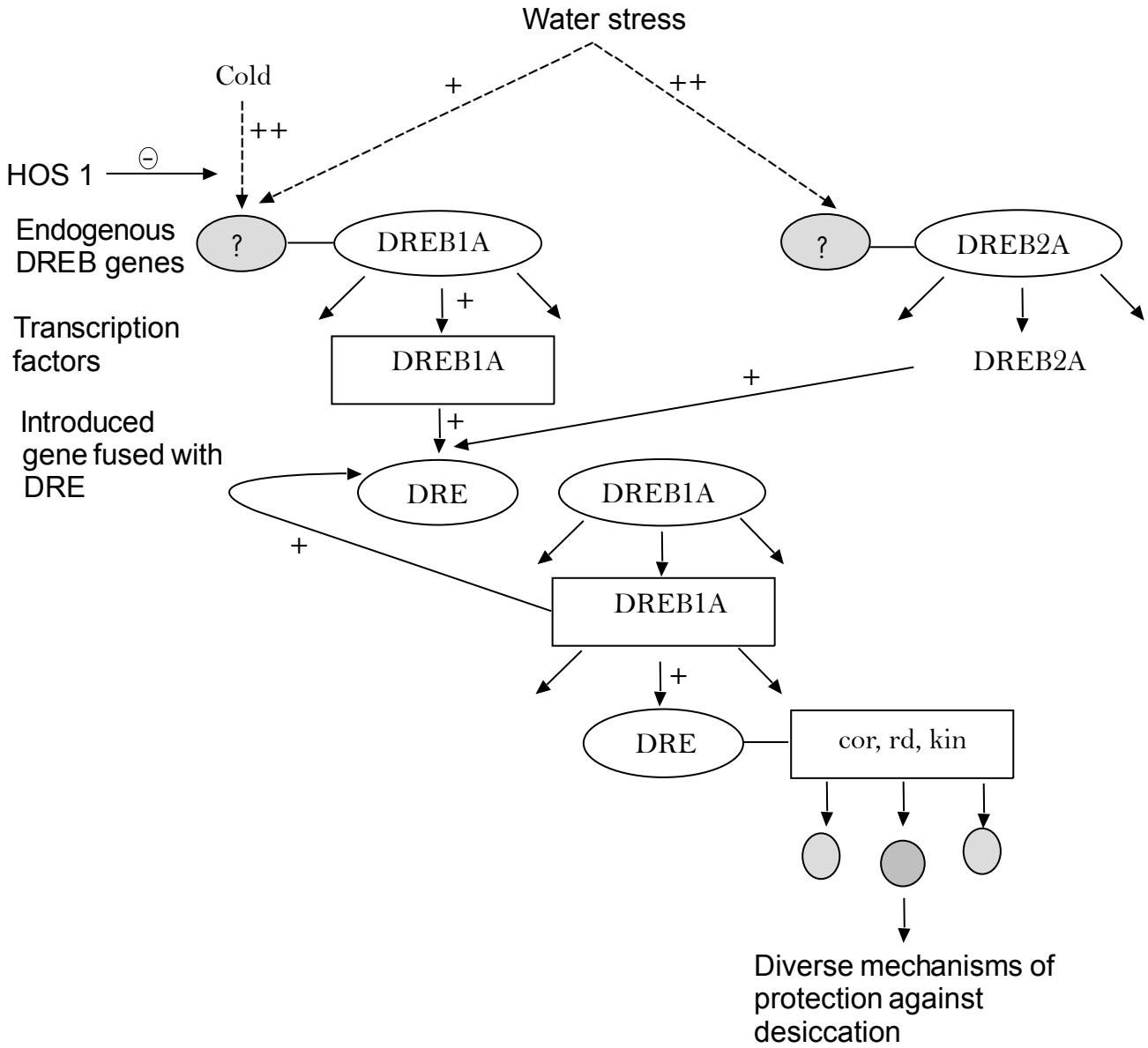

Figure 1: Effect of cold, salt loading and dehydration on expression of stress- related genes leading to striking improvements in plant tolerance.

Stress Tolerance in *Arabidopsis*

Over-expression of a stress – regulatable transcription factor in *Arabidopsis* improves tolerance to dehydration, freezing, and salt stress. The *DREB* genes that are induced by cold and water stress, encodes transcription factors that bind to the DRE promoter element of stress related genes, and turn on their expression. Shinozaki et al., (2003) have amplified this signaling pathway by over-expressing a fusion of a DRE-containing promoter with a DREB gene. The induced *DREB*1A expression in response to stress led to strong induction of DRE-containing target genes (Kasuga et al., 1999). The early steps of this signaling pathway are poorly understood, but genetic analysis of *Arabidopsis* mutants suggested that HOS1 functions as a negative regulator of stress- related genes (Ishitani et al., 1998). It has already been identified a dehydration – responsive transcription factor (*DREB*1A) that mediates transcription of several genes in response to cold and water stress (Liu et al., 1998). Stress related gene expression is induced by the binding of *DREB*1A protein, which is itself induced by cold and water stress, to a *cis*-acting DNA (DRE) element in the promoters of genes such as *rd29A, rd17, cor6.6, cor15a, erd10* and *kin1*. This binding initiates synthesis of gene products implicated in plant acclimation response to low temperature and water stress (Ingram and Bartel, 1996; Gilmour et al., 1998). Over-expression of *DREB*1A gene improved drought and low temperature stress tolerance in tobacco. Shinozaki and Yamaguchi-Shinozaki, (2000) studied molecular responses to dehydration and low temperature. They showed that a major transcription system controls ABA-independent gene expression in response to dehydration and low temperature. The system includes the *DRE/CRT* (dehydration-responsive element/C-repeat) *cis*-acting element and its DNA-binding protein, *DREB/CBF* (*DRE*-binding protein/C-repeat binding factor), which has an AP2 domain. *DREB/CBF* contains two subclasses, *DREB1/CBF* and *DREB2*, which are induced by cold and dehydration, respectively, and control the expression of various genes involved in stress tolerance. They also discussed evidence of differences between dehydration-signaling and cold-stress-signaling cascades, and of cross-talk between them.

Sakuma et al., (2002) studied DNA binding specificity of the *ERF/ AP2* domain of *Arabidopsis DREBs*, transcription factors involved in dehydration and cold inducible gene expression. They showed that DRE/CRT is a *cis*-acting element that is involved in gene expression responsive to drought and low-temperature stress in higher plants. *DREB*1A/*CBF3* and *DREB*2A are transcription factors that specifically bind to *DRE/CRT* in *Arabidopsis*. They precisely analyzed the DNA-binding specificity of *DREBs* and showed that both *DREBs* are specifically bound to six nucleotides (A/GCCGAC) of *DRE*. However, these proteins had different binding specificities to the second or third nucleotides of *DRE*. Gel mobility shift assay using mutant DREB proteins showed that the two amino acids, valine and glutamic acid conserved in the *ERF/AP2* domains, especially valine, have important roles in DNA-binding specificity. In the *Arabidopsis* genome, 145 *DREB/ERF*-related proteins are encoded. These proteins are classified into five groups-*AP-2* subfamily, *RAV* subfamily, *DREB* subfamily, *ERF* subfamily, and others. The *DREB* subfamily included three novel *DREB*1A- and six *DREB*2A-related proteins. They also analyzed expression of novel genes for these proteins and discussed their roles in stress-responsive gene expression. Kasuga et al., (2004) showed that a combination of the *Arabidopsis DREB*1A gene and stress inducible *rd29*A promoter improved drought and low temperature stress tolerance in tobacco by gene transfer. They observed that stress inducible *rd29*A promoter minimized the negative effects on the plant growth in tobacco. They also detected over-expression of stress inducible target genes of *DREB*1A in tobacco. On the basis of the results obtained, they concluded that a combination of the *rd29*A promoter and DREB1A is useful for improvement of various kinds of transgenic plants that are tolerant to environmental stress. Gutterson and Reuber, (2004) reported that AP2 transcription factor family, found only in plants, includes several genes that encode proteins involved in the regulation of disease resistance pathways. These genes are members of the ethylene response factor (ERF) subfamily of AP2 transcription factor genes, which have only a single DNA-binding domain and are distinct from members of the dehydration-responsive element binding (DREB) subfamily. Some ERF subgroups are enriched in such genes, suggesting that they have conserved functions that are required for the regulation of disease resistance pathways. The expression of several ERF genes is regulated by plant hormones, such as jasmonic acid, salicylic acid and ethylene, as well as by pathogen challenge. On the basis of phylogenetic overview of these genes, with a focus on *Arabidopsis*, rice and tomato, they suggested that despite broad conservation of their function in monocots and dicots, some structural elements are specialized within each of these two lineages.

Kasukabe et al., (2004) showed that polyamines play pivotal roles in plant defense to environmental stresses. They cloned spermidine synthase cDNA from *Cucurbita ficifolia* and the gene was introduced to *Arabidopsis thaliana* under the control of the cauliflower mosaic virus 35S promoter. The transgene was stably integrated and actively transcribed in the transgenic plants. As compared with the wild-type plants, the T2 and T3 transgenic plants exhibited

a significant increase in spermidine synthase activity and spermidine content in leaves together with enhanced tolerance to various stresses including chilling, freezing, salinity, hyperosmosis, drought, and paraquat toxicity. During exposure to chilling stress (5°C), the transgenics displayed a remarkable increase in arginine decarboxylase activity and conjugated spermidine contents in leaves compared to the wild type. A cDNA microarray analysis revealed that several genes were more abundantly transcribed in the transgenics than in the wild type under chilling stress. These genes included those for stress-responsive transcription factors such as *DREB* and stress-protective proteins like *rd29A*. On the basis of the results, they suggested an important role for spermidine as a signaling regulator in stress signaling pathways, leading to build-up of stress tolerance mechanisms in plants under stress conditions. Maruyama et al., (2004) showed that transcriptional factor *DREB/CBF* (dehydration-responsive element/C-repeat-binding) specifically interacts with the dehydration-responsive element (DRE)/ C-repeat (CRT) cis-acting element (A/GCCGAC) and controls the expression of many stress-inducible genes in *Arabidopsis*. Transgenic plants over-expressing *DREB*1A showed activated expression of many stress-inducible genes and improved tolerance to not only drought, salinity, and freezing but also growth retardation. They also searched for downstream genes in transgenic plants over-expressing *DREB*1A using the full-length cDNA microarray and Affymetrix GeneChip array. They confirmed candidate genes selected by array analyses using RNA gel blot and identified 38 genes as the *DREB*1A downstream genes, including 20 unreported new downstream genes. Many of the products of these genes were proteins known to function against stress and were probably responsible for the stress tolerance of the transgenic plants. The downstream genes also included genes for protein factors involved in further regulation of signal transduction and gene expression in response to stress. The identified genes were classified into direct downstream genes of *DREB*1A and the others based on their expression patterns in response to cold stress. They also searched for conserved sequences in the promoter regions of the direct downstream genes and found A/GCCGACNT in their promoter regions from -51 to -450 as a consensus DRE. They showed that the recombinant *DREB*1A protein was bound to A/GCCGACNT more efficiently than to A/GCCGACNA/G/C. Kim et al (2004) identified a *cis*-acting element responsive to cold and drought, the C-repeat/dehydration-responsive element (C/DRE) in the *Arabidopsis thaliana* stress-inducible genes *RD29A* and *COR15a* and found in other cold-inducible genes in various plants. They also showed that C/DRE-binding factor/DRE-binding protein (CBF/DREB) is an essential component of the cold-acclimation response, but the signaling pathways and networks are mostly unknown. They used targeted genetic approach to isolate *A. thaliana* mutants with altered cold-responsive gene expression (*acg*) and to identify *ACG1* as a negative regulator of the *CBF/DREB* pathway. They found that *acg1* flowered late and had elevated expression of FLOWERING LOCUS C (FLC), a repressor of flowering encoding a MADS-box protein. They also showed that *acg1* is a null allele of the autonomous pathway gene FVE. The FVE gene encodes a homolog of the mammalian retinoblastoma-associated protein, a component of a histone deacetylase (HDAC) complex involved in transcriptional repression. They also showed that plants sense intermittent cold stress through FVE and delay flowering with increasing expression of FLC. Dual roles of FVE in regulating the flowering time and the cold response may have an evolutionary advantage for plants by increasing their survival rates.

Albrecht et al., (2003) reported that calcium sensor CBL1 integrates plant responses to abiotic stress. Calcium ions represent both an integrative signal and an important convergence point of many disparate signaling pathways. Calcium-binding proteins, like calcineurin B-like (CBL) proteins, have been implicated as important relays in calcium signaling. They reported the *in vivo* study of CBL1 function in *Arabidopsis*. Analyses of loss-of-function as well as CBL1-overexpressing lines indicated a crucial function of this calcium sensor protein in abiotic stress responses. Mutation of CBL1 impairs plant responses to drought and salt stresses and affects gene expression of cold-regulated genes, but does not affect abscisic acid (ABA) responsiveness. Conversely, over-expression of CBL1 reduces transpirational water loss and induces the expression of early stress-responsive transcription factors and stress adaptation genes in non-stressed plants. Their data indicated that the calcium sensor protein CBL1 may constitute an integrative node in plant responses to abiotic stimuli and contributes to the regulation of early stress-related transcription factors of the C-Repeat-Binding Factor/dehydration-responsive element (CBF/DREB) type. Yamaguchi-Shinozaki and Shinozaki, (2007) showed that over-expression of the cDNA encoding *DREB*1A in transgenic *Arabidopsis* plants activated the expression of many stress tolerance genes under normal growing conditions and resulted in improved tolerance to drought, salt loading and freezing. On using strong constitutive 35S cauliflower mosaic virus (CaMV) promoter to drive expression of *DREB*1A resulted in severe growth retardation under normal growing conditions. They also showed that *DREB1A* cDNA and the *rd29A* promoter may be useful for improving the stress tolerance of agriculturally important crops by gene transfer. Schramm et al., (2008)

showed that *DREB2A* is induced by heat stress (hs) and is a regulator of the heat stress response of *Arabidopsis*. They also showed that among the 21 members of the *Arabidopsis* Hsf family, HsfA3 is the only Hsf that is transcriptionally induced during heat stress by *DREB2A*, and *HsfA3* in turn regulates the expression of Hsp encoding genes. They reconstructed this transcription cascade in transient GUS reporter assays in mesophyll protoplasts by showing that *DREB2A* could activate the *HsfA3* promoter, whereas *HsfA3* in turn was shown to be a potent activator on the promoters of Hsp genes. They showed direct binding to the corresponding promoters by electrophoretic mobility shift assays and the involvement of *HsfA3* in the heat stress response *in vivo* directly by observation of reduced thermotolerance in *HsfA3* mutant lines. Altogether, on the basis of these data, they demonstrated that *HsfA3* is transcriptionally controlled by *DREB2A* and important for the establishment of thermo-tolerance.

Stress Tolerance in *Atriplex*

Shen et al., (2003a) characterized a DRE binding transcription factor from a halophyte *Atriplex hortensis*. They showed that environmental stresses, such as salinity, drought and cold, can induce the expression of a large amount of genes. Among these are many transcription factors that regulate the expression of downstream genes by specifically binding to *cis*-elements or forming transcriptional complexes with other proteins. They isolated a DREB-like transcription factor gene named *AhDREB1* from a halophyte *Atriplex hortensis*. *AhDREB1* encoded a protein containing a conserved EREBP/AP2 domain featuring the *DREB* family. In yeast one-hybrid analysis, *AhDREB1* protein was specifically bound to *DRE* elements and activated the expression of the reporter genes of *HIS3* and *LacZ*. The *AhDREB1* gene was expressed in roots, stems and leaves of *A. hortensis*. Salinity induced its expression in roots, but not in other organs. Over-expression of *AhDREB1* in transgenic tobacco led to the accumulation of its putative downstream genes. They also tested the performance of the transgenic lines under stressed conditions and two lines were found to be stress-tolerant. On the basis of the results, they suggested that the AhDREB1 protein functions as a DRE-binding transcription factor and play roles in the stress-tolerant response of *A. Hortensis*.

Stress Tolerance in *Triticum*

The same group (Shen et al., 2003b) isolated one transcription factor of DRE-binding proteins (*TaDREB1*) from a drought-induced cDNA library of wheat (*Triticum aestivum* L.). The *TaDREB1* gene contains one conserved *EREBP/AP2* domain, and shows similarity with *Arabidopsis thaliana DREB* family members in both overall amino-acid sequences and the secondary structure arrangement within the DNA-binding motifs. In yeast one-hybrid system, *TaDREB1* can specially activate the genes fused with the promoter containing three tandemly repeated copies of the wild-type DRE sequence: TACCGACAT. In different wheat cultivars, the *Ta DREB1* gene is induced by low temperature, salinity and drought; and the expression of Wcs120 that contains DRE motifs in its promoter is closely related to the expression of *TaDREB1*. On the basis of the results, they suggested that *TaDREB1* functions as a *DRE*-binding transcription factor in wheat. They also observed the dwarf phenotype in transgenic rice (T0) over-expressing *TaDREB1*. Latini et al., (2007) isolated and characterized a gene in *Triticum durum* namely *TdDRF*1, that belongs to the *DREB* gene family and produces three forms of transcripts through alternative splicing. They also assessed the relationship between the expression profile of the *TdDRF*1 gene and water stress by using real time reverse transcription polymerase chain reaction in a time course experiment up to 7 days. They selected water stress experimental conditions to relate changes in gene expressions during a time frame reflecting as closely as possible those during which water stress starts having a visible effect under field conditions. Among the three isoforms of *TdDRF*1, the truncated form *TdDRF*1.2 was at all times the most expressed. Its expression together with the *TdDRF*1.3 transcript increased sharply after 4 days of dehydration but then decreased at 7 days. The *TdDRF*1.1 transcript was the least expressed overall and varied least with the duration of dehydration. The same group (Latini et al., 2008) showed that the expression profile of *TdDRF*1 upon water stress was genotype dependent. They used quantitative RT-PCR to monitor the expression profile of the three transcripts produced by the *TdDRF*1 gene under stressed (minimally irrigated) and non-stressed (fully irrigated) conditions. They analyzed tolerant and susceptible cultivars and compared the results with those from greenhouse testing.

Stress Tolerance in *Oryza*

Dubouzet et al., (2003) isolated five cDNAs for DREB homologs: *OsDREB*1A, *OsDREB*1B, *OsDREB*1C, *OsDREB*1D, and *OsDREB*2A from rice (*Oryza sativa*). Expression of *OsDREB*1A and *OsDREB*1B was induced by cold, whereas expression of *OsDREB*2A was induced by dehydration and high-salt stresses. The *OsDREB*1A and *OsDREB*2A proteins specifically bound to *DRE* and activated the transcription of the GUS reporter gene driven by *DRE* in rice protoplasts. Over-expression of *OsDREB*1A in transgenic *Arabidopsis* induced over-expression of target

stress-inducible genes of *Arabidopsis DREB*1A resulting in plants with higher tolerance to drought, high-salt, and freezing stresses. This indicated that *OsDREB*1A has functional similarity to *DREB*1A. However, in microarray and RNA blot analyses, some stress-inducible target genes of the *DREB*1A proteins that have only ACCGAC as DRE were not over-expressed in the *OsDREB*1A transgenic *Arabidopsis*. The *OsDREB*1A protein bound to GCCGAC more preferentially than to ACCGAC whereas the *DREB*1A proteins bound to both GCCGAC and ACCGAC efficiently. The structures of *DREB*1-type *ERF/AP2* domains in monocots are closely related to each other as compared with that in the dicots. *OsDREB*1A is potentially useful for producing transgenic monocots that are tolerant to drought, high-salt, and/or cold stresses. Wang et al., (2008a) showed that DREB transcription factors play key roles in plant stress signaling transduction pathway, they can specifically bind to *DRE/CRT* element (G/ACCGAC) and activate the expression of many stress inducible genes. They cloned a novel rice *DREB* transcription factor, *OsDREB1F* and characterized via subtractive suppression hybridization (SSH) from upland rice. Expression analysis revealed that *OsDREB1F* gene was induced by salt, drought, cold stresses, and also ABA application, but not by pathogen, wound, and H_2O_2. Subcellular localization results indicated that *OsDREB1F* localizes in nucleus. Yeast activity assay demonstrated that *OsDREB1F* gene encodes a transcription activator, and can specifically bind to *DRE/CRT* but not to ABRE element. Transgenic plants harboring *OsDREB1F* gene led to enhanced tolerance to salt, drought, and low temperature in both rice and *Arabidopsis*. The further characterization of *OsDREB1F*-overexpressing *Arabidopsis* showed that, besides activating the expression of COR genes which contain *DRE/CRT* element in their upstream promoter regions, the expression of *rd29B* and *RAB18* genes were also activated, suggested that *OsDREB1F* may also participate in ABA-dependent pathway.

Stress Tolerance in *Brassica*

Gao et al., (2002) characterized and studied regulation of four CBF transcription factors from *Brassica napus*. They isolated four orthologues of the *Arabidopsis* CBF/Dreb transcriptional activator genes from the winter *Brassica napus*, cv. Jet neuf. All the four BNCBF clones encoded a putative *DRE/CRT* (LTRE)-binding protein with an AP2 DNA-binding domain, a putative nuclear localization signal and a possible acidic activation domain. Deduced amino acid sequences suggested that BNCBFs 5, 7and 16 are very similar to the *Arabidopsis* CBFI whereas BNCBF17 is different in that it contains two extra regions of 16 and 21 amino acids in the acidic domain. Transcripts hybridizing specifically to BNCBF17 and to one or more of the other BNCBFs accumulated in leaves within 30 min of cold exposure of the *Brassica* seedlings and preceded transcript accumulation of the cold-inducible BN28 gene, a *Brassica* orthologue of the cor6.6 or KIN gene from *Arabidopsis*. Cold-induced accumulation of BNCBF17 mRNA was rapid but was short-lived compared to transcripts hybridizing to BNCBF5/7/16. Transcripts hybridizing to one or more of BNCBF5/7/16 accumulated at low levels after the plants were subjected to prolonged exposure to salt stress. BNCBF17 was not responsive to salt stress. BNCBF transcript accumulation was similar in both spring and winter *Brassica* but the persistence of the transcripts in the cold were generally shorter in the spring than in the winter type. BNCBF5 and 17 proteins bind *in vitro* to the LTRE domains of the cold-inducible *BN115* (*cor15a* orthologue) or *BN28* promoters. Mutation of the core CCGAC sequence of the LTRE indicated that BNCBF17 had a lower sequence binding specificity than BNCBF5. Furthermore, experiments indicated that the LTREs were able to drive BNCBF5 and 17 trans-activation of the Lac-Z reporter gene in yeast. On the basis of results obtained, they concluded that the BNCBFs could function as trans-acting factors in low-temperature responses in *Brassica*, controlling the expression of cold-induced genes through an ABA-independent pathway.

Zhao et al., (2006) reported two groups of *DREB* like genes isolated from *Brassica napus* and named as Group I and Group II. The two groups of genes were both induced by low temperature, but the expression of Group I preceded that of Group II. The Group I *DREBs* could specifically bind with the *DRE cis*-acting element and activate the expression of downstream genes, but Group II factors were trans-inactive although they still had the ability to bind with DRE, which was confirmed by electrophoretic mobility shift assay. Fluorescence quenching assays indicated that the DRE binding ability of the two groups was similar. Co-expression of Group II could depress the trans-activation activity of Group I *DREB* in a concentration-dependent manner. The results strongly suggested that the trans-active Group I *DREBs* were expressed at the early stage of cold stress to open the DRE-mediated signaling pathway in cold stress, whereas the trans-inactive Group II *DREBs* were expressed at the later stage to close the signal pathway in a competitive manner. Dengfeng et al., (2008) reported construction of the T-DNA double dual carrier pCDMARpWDT-Hyg, marker gene hpt (hygromycin phosphotransferase) and anti-retroviral factor gene *DREB* are located in two separate T-DNA. They reported that with *Agrobacterium* mediated transformation of maize embryo callus through the medium resistance to strict screen-

ing, in access to the regeneration of the plant transformation, integration hpt DREB gene and the gene into a total rate of 26.3%.

Cong et al., (2008) isolated a novel DREB gene from *Brassica juncea* and designated it as *BjDREB1B*. They found that the gene contains a conserved EREBP/ AP2 domain and classified it into the A-1 subgroup of the DREB sub family based on the phylogenetic tree analysis. Using RT-PCR, they showed that *BjDREB1B* was induced by abiotic stresses and exogenous phytohormones, such as drought, salt, low temperature, heavy metals, abscisic acid, and salicylic acid. Gel shift assay revealed that *BjDREB1B* specifically bound to the DRE element *in vitro*. Over-expression of *BjDREB1B* in tobacco up-regulated the expression of *NtERD10B*, and *BjDREB1B* transgenic plants accumulated higher levels of proline than control plants under normal and saline conditions, together showing that *BjDREB1B* plays important roles in improving plant tolerance to drought and salinity.

Stress Tolerance in *Aloe*

Wang and He, (2007) isolated a new cold induced dehydration- responsive element binding (*DREB*) gene encoding an AP2/ ethylene response element binding protein transcription factor from *Aloe vera* L. by rapid amplification of complementary DNA ends (RACE). The deduced protein contained a putative acidic activation domain and an AP2 DNA binding domain of 64 amino acids. They also showed that the transcripts accumulated rapidly under cold stress and peaked at 12 hours, then decreased to the original level. They showed localization of *DREB*1 gene in the nucleus, however, it lacks typical nuclear localization signal. On the basis of the results analyzed, they showed that Aloe *DREB*1 might function as a transcription activator involved in the regulation of cold responsive genes via signal transduction pathway dependent or independent of the DREB transcription factor.

Stress Tolerance in *Festuca*

Tang et al., (2005) isolated a new DRE binding protein gene *FaDREB*1 encoded for an AP2/ERFBP type transcription factor by RACE-PCR from *Festuca arundinacea* Schreb seedlings. They also sequenced its cDNA and found it having 988 bp. From the nucleotide sequence, protein was also deduced with 216 amino acid residues and a predicted molecular weight of 23479 daltons and a pI of 4.70. Using Protein Blast Data, they revealed that this protein can be classified as a typical member of the AP2/ERFBP family of DNA binding proteins. Using tissue organ specific expression pattern of the *FaDREB*1 gene, they showed that its transcripts were abundant in leaves and leaf sheaths and scarce in roots. Its mRNA accumulation profiles made clear that its expression was strongly induced by cold treatment, weakly induced by drought and salt stress but did not respond to abscisic acid treatment. Based on the results, they concluded that the protein *FaDREB*1 may be involved in the process of plant response to cold stress through an abscisic acid independent pathway.

Stress Tolerance in *Chrysanthemum*

Yang et al., (2007) isolated two *DREB* like genes designated as *DmDREBa* and *DmDREBb* from *Chrysanthemum* by RACE approach. These two genes code for two proteins of 191 and 185 amino acid residues with the predicted molecular weight of 21.66 and 20.99 KDa, respectively. Both *DmDREB*s proteins comprised a typical *EREBP*/ AP2 domain, a *DREB*1 type nuclear localization signal before the *EREBP*/ AP2 domain and a *DSAWR* sequence after the domain. Phylogenetic analysis suggested both the genes to belong to *DREB*1 sub group. With the help of quantitative real time PCR analysis, they also showed that both genes were accumulated more in leaves and stems than in roots and flowers. The *DmDREBb* reacted earlier and accumulated with higher levels than *DmDREBa* under cold treatment. They also showed that expression of both *DmDREBa* and *DmDREBb* decreased dramatically within 30 minutes of exposure to 100 micromolar abscisic acid (ABA). However, *DmDREBb* was recovered to pre- ABA levels at 2, 4 and 12 hours, whereas, *DmDREBa* expression remained low during the 24 hours exposure. Furthermore, both genes expression was totally inhibited at 40°C. Besides, they also observed that these two *DmDREB* proteins also have transcriptional activity and had the DRE-binding capacity as shown using yeast one hybrid system and were found to be localized in the nuclei of the cells. These authors claimed that they for the first time reported isolation of *DREB* like genes in *Chrysanthemum*.

Stress Tolerance in Soyabean

Li et al., (2005) isolated three DREB homologue genes, *GmDREBa*, *GmDREBb*, and *GmDREBc* from soybean and showed that each of the deduced proteins contains an AP2 domain of 64 amino acids. Yeast one-hybrid assay revealed that all of the three dehydration-responsive, element-binding proteins specifically bound to the dehydration-responsive element. Analysis of transcriptional activation abilities of these proteins in yeast indicated that *GmDREBa* and *GmDREBb* could activate the expression of a reporter gene,

whereas *GmDREBc* could not. The transcriptions of *GmDREBa* and *GmDREBb* were induced by salt, drought, and cold stresses in leaves of soybean seedlings. The expression of *GmDREBc* was not significantly affected in leaves but apparently induced in roots by salt, drought, and abscisic acid treatments. On the basis of results, they suggested that these three genes function specifically in response to abiotic stresses in soybean.

Chen et al., (2007) isolated a novel *DREB* homologous gene, *GmDREB2* from soybean. Based on its similarity with AP2 domains, they classified *GmDREB2* into A-5 subgroup in *DREB* subfamily in *AP2/EREBP* family. Expression of *GmDREB2* gene was induced by drought, high salt, and low temperature stresses and abscisic acid treatment. The *GmDREB2* bound specifically to DRE element *in vitro*. Furthermore, the over-expression of *GmDREB2* activated expression of downstream genes in transgenic *Arabidopsis*, resulting in enhanced tolerance to drought and high-salt stresses and did not cause growth retardation. Analysis of free proline contents in transgenic tobacco indicated that the over-expression of *GmDREB2* accumulated higher level of free proline compared to the wild type plants under drought condition. Based on the results, they indicated that this novel soybean *GmDREB2* gene functions as an important transcriptional activator and may be useful in improving of plant tolerance to abiotic stresses in plants. Wang et al., (2008) identified a gene belonging to AP2/ ERF transcription family from developing soybean seeds. They identified an EST expressed specifically in the developing soybean seeds and full length of the gene was obtained through RACE analysis and named as *GmSGR*. Its AP2 domain has the highest similarity with that of the A-3 member AtAB14 of *DREB* subgroup in the *AP2/ ERF* family in *Arabidopsis*. However, GmSGR could not exhibit transcriptional activation activity in the yeast assay system. On the other hand, it over-expressed in *Arabidopsis* and the germination rates of the transgenic seeds were significantly higher than that of the wild type seeds under higher concentration of abscisic acid and glucose, respectively. However, the germination rates of the transgenic seeds were lower than that of control under salt stress. The expression of *AtEm6* and *AtRD29B* was higher in the seedlings of the transgenic plants than that in the wild-type seedlings. On the basis of these results, they suggested that GmSGR may confer reduced ABA sensitivity and enhanced salt sensitivity to the transgenic seeds through regulating the expression of *AtEm6* and *AtRD29B* genes.

Stress Tolerance in *Pennisetum*

Agarwal et al., (2007) showed that stress inducible *DREB2A* transcription factor from *Pennisetum glaucum* is a phosphoprotein and its phosphorylation negatively regulates its DNA binding activity. They cloned a cDNA from *Pennisetum glaucum*, a stress tolerant food grain crop with an open reading frame of 332 amino acids encoding the transcription activation factor *DREB2A*. Phylogenetic tree revealed that *PgDREB2A* is more close to *DREBs* isolated from monocots, though it forms an independent branch. The *PgDREB2A* transcript was up-regulated in response to drought within 1 hour of the treatment, whereas the induction was delayed in response to cold and salinity stress. However, during cold stress, the transcript was induced more as compared to drought and salinity. They purified recombinant PgDREB2A protein using Ni-NTA affinity chromatography and determined its molecular weight. The molecular weight was reported to be 36.6 KDa. By gel mobility shift assays with the purified protein and two *cis* elements of rd29A (responsive to dehydration 29A) gene promoter of *Arabidopsis*, they revealed that *PgDREB2A* binds to drought-responsive element (DRE) ACCGAC and not to GCCGAC. They showed that phosphorylation of *PgDREB2A in vitro* by *P. glaucum* total cell extract occurred at threonine residue(s). The phosphorylated PgDREB2A did not bind to the DREs. On the basis of these results, they interpreted that stress induction of genes could occur via post-translational modification by phosphorylation of DREB2A.

Stress Tolerance in *Gossypium*

Huang et al., (2008) isolated a cDNA encoding one novel DRE-binding protein, GhDBP2 from cotton (*Gossypium hirsutum*) seedlings. Based on multiple sequence alignment and phylogenetic characterization, they classified it into the A-6 group of DREB subfamily. Using semi-quantitative RT-PCR, they found that the GhDBP2 transcripts were greatly induced by drought, NaCl, low temperature and ABA treatments in cotton cotyledons. They also analyzed DNA-binding properties of GhDBP2 by electrophoretic mobility shift assay (EMSA) and showed that GhDBP2 successfully binds to the DRE cis-element as well as the promoter region of the LEA D113 gene. Consistent with its role as a DNA-binding protein, GhDBP2 is preferentially localized to the nucleus of onion epidermal cells. In addition, when GhDBP2 is transiently expressed in tobacco cells, it activates reporter gene expression driven by the LEA D113 promoter. Based on the results, they indicated that GhDBP2 is a DRE-binding transcriptional activator involved in activation of the down-stream genes such as LEA D113 expression through interaction with the DRE element, in response to environmental stresses as well as ABA treatment. The same group

(Huang and Liu, 2006) also cloned a novel cDNA encoding DRE-binding transcription factor designated as *GhDBP3* from cotton (*Gossypium hirsutum*). Based on multiple sequence alignment and phylogenetic characterization, this protein was classified into A-4 group of *DREB* subfamily. Semi-quantitative RT-PCR showed that *GhDBP3* was expressed in the leaves, cotyledons, roots and stems of 2-week-old cotton seedlings under non-stress conditions and was greatly induced in the cotton cotyledons by drought, NaCl, low temperature and ABA treatment. EMSA revealed that *GhDBP3* was able to bind to the *DRE cis*-element *in vitro*. Transient assay using the particle bombardment method showed that *GhDBP3* was a transcriptional activator, capable of activating expression of a reporter gene driven by the LEA D113 promoter containing a DRE like sequence in tobacco cells. Based on the results, they indicated that *GhDBP3* could be a new member of DRE-binding transcription factor family and may play an important role in response to ABA and environmental stresses.

Stress Tolerance in *Lolium*

Xiong and Fei, (2006) showed that dehydration-responsive element binding proteins (*DREB1*)/C-repeat (CRT) binding factors (CBF) function as transcription factors and bind to the *DRE/CRT* cis-acting element (core motif: G/ACCGAC) commonly present in cold-regulated (COR) genes and subsequently up-regulate the expression of such genes in *Arabidopsis*. They identified a *DREB1A/CBF3*-like gene, designated LpCBF3, from perennial ryegrass (*Lolium perenne* L.) by using RT-PCR and RACE (rapid amplification of cDNA end). The *LpCBF3* gene contains all the conserved domains known to exist in other CBF genes. A comprehensive phylo-genetic analysis using known and computationally identified CBF homologs revealed that all monocot CBF genes are separately clustered from eudicot CBF genes and the *LpCBF3* is the ortholog of rice *OsDREB1A/CBF3* gene. Similar to other *DREB1A/CBF3* homologs, expression of the *LpCBF3* is induced by cold stress, but not by abscisic acid (ABA), drought, or salinity. Over-expression of the *LpCBF3* cDNA in *Arabidopsis* induced expression of the *Arabidopsis DREB1A/CBF3* target COR genes, *COR15a* and *RD29A*, without cold acclimation. Ion leakage in leaves of the over-expression transgenic plants was significantly reduced, an indication of enhanced freezing tolerance. The data demonstrated that *LpCBF3* not only resembles *DREB/CBF* genes of *Arabidopsis*, but is also capable of functioning as a transcriptional regulator in *Arabidopsis*, a species distant to the grass family.

Stress Tolerance in *Physcomitrella*

Cuming et al., (2007) showed that dehydration tolerance is an adaptive trait necessary for the colonization of land by plants, and remains widespread among bryophytes: the nearest extant relatives of the first land plants. They carried a genome-wide analysis of water-stress responses in the model moss, *Physcomitrella patens* to identify stress-responsive genes. They also used oligonucleotide microarray for transcriptomic analysis of *Physcomitrella* treated with abscisic acid, or subjected to osmotic, salt and drought stress. Using bioinformatic analysis of the *Physcomitrella* genome, they identified the responsive genes, and a number of putative stress-related cis-regulatory elements. In protonemal tissue, 130 genes were induced by dehydration, 56 genes by abscisic acid, but only 10 and eight genes, respectively, by osmotic and salt stress. Fifty-one genes were induced by more than one treatment. Seventy-six genes, principally encoding chloroplast proteins, were drought down-regulated. They showed that many abscisic acid and drought-responsive genes are homologues of angiosperm genes expressed during drought stress and seed development. These genes include those encoding a number of late embryogenesis abundant (LEA) proteins, a *DREB* transcription factor and a Snf-related kinase homologous with the *Arabidopsis* abscisic acid signal transduction component 'OPEN STOMATA 1'. They concluded that evolutionary capture of conserved stress-regulatory transcription factors by the seed developmental pathway probably accounts for the seed-specificity of desiccation tolerance among angiosperms. Liu et al., (2007) isolated DRE bindling factor1 containing a conserved AP2/ERF domain (*PpDBF1)* from *Physcomitrella patens*. On the basis of sequence comparison and phylogenetic analysis, they showed that *PpDBF1* belongs to the A-5 group of DREB transcription factor subfamily. They also verified transcriptional activation activity and DNA-binding specificity of *PpDBF1* by yeast one-hybrid and electrophoretic mobility shift assay experiments, and demonstrated its nuclear localization by particle biolisitics. *PpDBF1* transcripts were accumulated under various abiotic stresses and phytohormones treatments in *P. patens*, and transgenic tobacco plants over-expressing PpDBF1 gained higher tolerance to salt, drought and cold stresses. On the basis of these results, they suggested that *PpDBF1* may play a role in *P. patens* as a *DREB* transcription factor, implying that similar regulating systems are conserved in moss and higher plants.

Stress Tolerance in *Populus*

Zhuang et al., (2008) performed genome-wide analysis of the *AP2/ERF* gene family in *Populus trichocarpa*. They showed that *AR2/ERF* is a large family of transcription factors in plant, encoding transcriptional regulators with a variety of functions involved in the developmental and physi-

ological processes. Starting from the database of *Populus* genome, they identified 200 *AP2/ERF* genes by *in silico* cloning method using the *AP2/ERF* conserved domain amino acid sequence of *Arabidopsis thaliana* as probe. Based on the number of *ERF/AP2* domains and the function of the genes, they classified *AP2/ERF* genes from *Populus* into four subfamilies named as *AP2, DREB, ERF, RAV*, and a soloist. Among these genes, the number genes of total *AP2/ERF* family genes, *DREB* subfamily, and *ERF* subfamily from *Populus trichocarpa* were about 1.4-1.6-fold than those from *A. thaliana*. The rates were very similar for the putative homologs between *Populus* and *Arabidopsis*.

Stress Tolerance in *Avena*

Bräutigam et al., (2005) reported generation and analysis of 9792 EST sequences from cold acclimated oat (*Avena sativa*). Oat is an important crop in North America and northern Europe. In Scandinavia, yields are limited by the fact that oat cannot be used as a winter crop. It is realized to develop such a crop with more knowledge about mechanisms of cold tolerance in oat. Therefore, they obtained 9792 single-pass EST sequences from an oat cDNA library. The library was prepared from pooled RNA samples isolated from leaves of four-week old oat plants incubated at 4°C for 4, 8, 16 and 32 hours. Exclusion of sequences shorter than 100 bp resulted in 8508 high-quality ESTs with a mean length of 710.7 bp. Clustering and assembly identified a set of 2800 different transcripts denoted the *Avena sativa* cold induced UniGene set (AsCIUniGene set). Taking advantage of various tools and databases, they assigned putative functions to 1620 (58%) of these genes. Of the remaining 1180 unclassified sequences, 427 appeared to be oat-specific since they lacked any significant sequence similarity (Blast E values > 10(-10)) to any sequence available in the public databases). Of the 2800 UniGene sequences, 398 displayed significant homology (BlastX E values < or = 10(-10)) to genes previously reported to be involved in cold stress related processes. 107 novel oat transcription factors were also identified, out of which 51 were similar to genes previously shown to be cold induced. The CBF transcription factors have a major role in regulating cold acclimation. Four oat CBF sequences were found, belonging to the monocot cluster of *DREB* family ERF/AP2 domain proteins. Finally in the total EST sequence data (5.3 Mbp) approximately 400 potential SSRs were found, a frequency similar to what has previously been identified in *Arabidopsis* ESTs. They proposed to use the AsCIUniGene set to fabricate an oat biochip, to perform various expression studies with different oat cultivars incubated at varying temperatures, to generate molecular markers and provide tools for various genetic transformation experiments in oat.

PCR Primer Design

Selective amplification of nucleic acid molecules, that are initially present in minute quantities, provides a powerful tool for analyzing nucleic acids. The polymerase chain reaction is an enzymatic reaction which follows relatively simple, predictable and well understood mathematical principles. However the scientist often relies on intuition to optimize the reaction. To make PCR an efficient and cost effective tool, components of PCR such as Taq DNA polymerase, assay buffer, deoxynucleoside triphosphates (dNTPs), stabilizing agents, DNA template and oligonucleotide primers are important. Efficacy and sensitivity of PCR largely depends on the efficiency of primers. The ability for an oligonucleotide to serve as a primer for PCR is dependent on the following factors:

1. The kinetics of association and dissociation of primer-template duplexes at the annealing and extension temperatures.

2. Duplex stability of mismatched nucleotides and their location.

3. The efficiency with which the polymerase can recognize and extend a mismatched duplex.

The primers which are unique for the target sequence to be amplified should fulfill certain criteria such as primer length, GC %, annealing and melting temperatures, 5' end stability, 3' end specificity.

Although DNA template quality/ purity is not particularly significant for amplification, DNA should not contain inhibitor of Taq DNA Polymerase. DNA isolation may be carried out by using any of the known methods (Murray and Thompson, 1980; Sambrook et al., 1989; Kaneko et al., 1989; Kawasaki, 1990; Green et al., 1991; Klebe et al., 1996; Singh and Naik, 2000).

Taq DNA polymerase plays an important role. Taq DNA polymerase from different suppliers may behave differently because of the different formulations, assay conditions and /or unit definitions. Recommended concentration ranges between 1-2.5 units/ 50-100 ul reaction, (Lawyer et al., 1989) when other parameters are optimal.

Most of the reviews on PCR optimization (Erlich et al., 1991; Dieffenbach et al., 1995; Roux, 1995) consider different parameters of PCR but generally do not discuss basic concepts of PCR primer design.

The design of primers is the most critical parameter for a

successful PCR amplification. All other components being equal, a poorly designed primer may result non-amplification in a PCR reaction. The primer sequence determines several characteristics such as the length of the product, its melting temperature and ultimately the yield. A poorly designed primer may result in little or no product due to non-specific amplification and / or primer-dimer formation which may become competitive enough to suppress product formation.

The sequences of the primers used for PCR amplification may have a major effect on the specificity and sensitivity of the reaction. When choosing two PCR amplification primers, the following guidelines should be considered:

1. Primer Length: Specificity; and the temperature and time of annealing are at least partly dependent on primer length. For broad – spectrum studies, primers of typically 18-30 nucleotides in length give better amplification. Primers should be at least 18 nucleotides in length to minimize a secondary hybridization site on the vector or insert. Primers with long runs of a single base should generally be avoided. It is especially important to avoid 4 or more G's or C's in a row.

2. Melting Temperature (Tm): The optimal melting temperature for a primer should be in the range of 52-58^{0}C. The primers with melting temperature of 52-58^{0}C generally produce better results than primers with lower melting temperatures. Primers with melting temperatures above 65^{0}C should also be avoided because of potential for secondary annealing. It is then advisable to do the sequencing reaction with annealing and extension at 60^{0}C. A good working approximation of this value (generally valid for oligos- in the 18-30 base range) can be calculated using the following formula of Wallace et al., (1979):

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

Using improved nearest- neighbor thermodynamic values given by SantaLucia et al., (1996), an estimate for melting temperature may be obtained for oligonucleotide analysis.

3. GC Content (Tm and Ta are interrelated): GC% is an important characteristic of DNA and provides information about the strength of annealing. Primers should have a GC contents between 45 and 60 percent (Dieffenbach et al., 1993). For primers with GC contents of less than 50%, it may be necessary to extend the primer sequence beyond 18 bases to keep the melting temperature above the recommended lower limit of 50^{o}C. GC contents, melting temperature and annealing temperature are strictly dependent on one another (Rychlik et al., 1990).

4. 3'-End Sequence: It is well established that the 3' terminal position in PCR primers is essential for the control of mis-priming (Kwok et al., 1990). Primers should be "stickier" on their 5' end than on their 3' ends. A "sticky" 3' end as indicated by a high G C content could potentially anneal at multiple sites on the template DNA. A "G" or "C" is desirable at the 3' end but the first part of this rule should apply. This GC clamp reduces spurious secondary bands (Sheffield et al., 1989).

5. Dimers and False Priming Cause Misleading results: Primers should not contain complementary (palindromes) sequences with in themselves. There should not be any hairpin with in the primer. If a hairpin exists, the primer will fold back on itself resulting in an unproductive priming event that decreases the overall signal obtained (Breslauer et al., 1986). Hairpins if formed below 50^{0}C temperature do not generally show such a problem. Primers should also not contain sequences of nucleotides that would allow one primer molecule to anneal on itself or on to other primer used in PCR reactions (primer dimer formation).

6. Specificity: As mentioned above, 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 oligos. However, 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 on amplification of genomic DNA. However, the same primer may give a single band if a single clone from a genomic library is amplified.

7. Degenerate Primers: Degeneracy in primer sequence should also be taken into consideration. Degenerate primers based on the amino acid sequence of conserved regions are also used to search for members of a gene family (Wilks et al., 1989). Computer programs have also been developed specifically for degenerate primer design (Chen and Zhu, 1997).

8. Complementary Primer Sequences: Primers need to be designed with absolutely no intra-primer homology beyond 3 base pairs. If a primer has such a region of self-homology, "snap back" may occur. Besides, inter – primer homology in the middle regions of two primers may interfere with hybridization. If the homology occurs at the 3' end of either primer, primer dimer formation will occur.

Secondary Structure

An important factor to consider when designing a primer

is the presence of secondary structures. It greatly reduces the number of primer molecules available for bonding in the reaction. It is well established that under a given set of conditions, the relative stability of a DNA duplex structure depends on its nucleotide sequences (Cantor and Schimmel, 1980). More specifically, the stability of a DNA duplex appears to depend primarily on the identity of the nearest – neighbor nucleotides. The overall stability and melting behavior of any DNA duplex structure can be predicted from its primary sequence if the relative stability (Go) and the temperature dependent behavior (Ho, Cpo) of each DNA's nearest – neighbor interaction is known (Marky and Breslauer, 1982). Tinoco et al., (1971, 1973) and Uhelenbeck et al., (1973) have predicted stability and melting behavior of RNA molecules for which they and others have determined the appropriate thermodynamic data. However, to the best of our knowledge, no experimental data is available to support the prediction of the thermodynamic properties of hairpin structures, an important factor to consider when designing a primer. Single stranded nucleic acid sequences may have secondary structures due to the presence of complementary sequences within the primer length e.g. hairpin loops and primer-dimer structures. It has been shown experimentally that hairpin loops, if present, can greatly reduce the efficiency of the reaction by limiting primer availability and the ability to bind to the target site (Singh et al., 2000). The effect of primer- template mismatches on the PCR has been studied earlier in a Human Immunodeficiency Virus (HIV) model (Kwok et al., 1990). Studies have also been performed for the characterization of hairpins (Marky et al., 1983, 1985), cruciforms (Marky et al., 1985), bulge and interior loops (Patel et al., 1982, 1983).

Know Your Product Before Amplification

PCR product length is directly proportional to inefficiency of amplification (Wu et al., 1991). Primers should be designed so that only small regions of DNA (150-1000bp) can be amplified from fixed tissue samples or purified plasmid or genomic DNA. The product is ideal for probe hybridization studies (Schowalter and Sommer, 1989). For reverse transcriptase polymerase chain reaction (RT-PCR) as described by Kawasaki, (1990a), primers should only be designed in exons taking care that both primers should be on different exons of mRNA to avoid spurious product amplified from contaminating DNA in the mRNA preparation, if any. If the desired restriction enzyme site is not available within the amplified product, it may be incorporated within the primer (Ponce and Micol, 1992; Jung et al., 1990).

Mismatch to Improve Sensitivity and Specificity

There is a good and a bad aspect to mismatches in primers. Single mismatches at or near the terminal 3' nucleotide of a primer are known to affect both oligonucleotide stability and efficiency of polymerase reaction; mismatches in the primer at or near 3' terminal end affect PCR more dramatically than mismatches at other positions (Petruska et al., 1988). Generally, mismatches at the 3'end terminal nucleotide reduce or inhibit efficiency of amplification (Kwok et al., 1994) but studies have shown that a mismatch 3-4 bases upstream of the 3'end of a primer used for the ARMS study actually increases specificity. A mismatch may therefore be deliberately created while designing a primer for ARMS PCR (Old, 1991).

Nested PCR

Nested PCR is often successful in reducing unwanted products while dramatically increasing sensitivity (Albert and Fenyo, 1990). It is used when the actual quantity of target DNA is very low or when the target DNA is impure. Nested PCR reduces background amplification thereby enhancing target detection. The technique is especially helpful for amplification of low copy number targets (<100 molecules) and while doing quantitative PCR (Haff, 1994). The process involves one PCR reaction followed by the next PCR extension, which amplifies the first PCR product. Two sets of primers are designed. PCR is first carried out using outer primers and subsequently with inner primers positioned within the product obtained in the first extension. It is also possible to perform a nested PCR reaction in a single sample without dilution between the two PCR reactions (Erlich et al., 1991). When designing primers for nested PCR, care must be taken to eliminate potential primer dimers and cross dimers within and between inner and outer primer sets.

Multiplex PCR

This technique involves co-amplification of two or more target sequences within a single sample (Chamberlain et al., 1991; Edward and Gibbs, 1994). A unique pair of primers for each target is preferred but primers can be designed so that a single primer can amplify different regions with two or more counterparts (Varawalla et al., 1991a; 1991b). While designing primers for muiltiplex PCR systems, the basic rule is to have similar annealing temperatures and similar GC% of the primers (Nicodeme and Steyaret, 1997). Product length should also be taken into consideration when designing primers so that they can be effectively separated and studied by electrophoresis. Multiplex PCR may be used for detection of genetic disorders (Old et al., 1990; Shuber et al., 1991). Zhu and Clark, (1996) demonstrated that addition of competitive primers may dramatically increase PCR

amplification efficiency.

Universal Primers

Molecular biologists are well aware of the exponential increase in the DNA sequence databanks with several thousands bases added every day. Many genes of varied importance have been sequenced in several species. However, the scientific community may require information on such genes in other species, which are used as experimental models. Researchers are often forced to re-sequence genes for new species in order to conduct expression level or other PCR related studies of the gene (Kain et al., 1991). Bulat et al., (1992) demonstrated the application of universal primers. Universal primers facilitated the rapid study of novel genes in new models. Rose et al., (1998) demonstrated a new primer design strategy for PCR amplification of unknown targets that are related to multiple-aligned protein sequences. Universal primers are designed in the conserved region of the sequences (Singh et al., 2000). Universal primers should be designed from amino-acid sequences in the regions of lowest degeneracy using a multiple sequence alignment (Nomenclature Committee of the International Union of Biochemistry, 1985). Universal primers have been used for differential display of eukaryotic mRNAs by PCR (Liang and Pardee, 1992). A universal primer set for detection of parasitic genomes has also been designed using *Dirofilaria immitis* as a test sample (Nagano et al., 1996), whereas Venta et al., (1996) designed gene-specific universal primers for the canine genome. These were used for developing a genetic map of dog-based markers. Universal primers may be used for amplification as well as sequencing in one reaction (Berg and Olaisen, 1994).

Web-based Resources for 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 acid sequences. 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 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.

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 program eases quantitation of primers by calculating primer molecular weight and optical activity. 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.

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.

Primer Designing for DREB Genes in *Arabidopsis sp*

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 (Etheylene 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.

Many orthologous genes of *DREB*1*/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, 2006). Overexpression of the *Arabidopsis DREB*1*/CBF* genes in transgenic *Brassica napus* or tobacco plants induced expression of orthologs of *Arabidopsis DREB*1*/CBF*-targeted genes and increased the freezing and drought tolerance of transgenic plants. Constitutive overexpression of *DREB*1B*/CBF1* in transgenic tomato increased drought, chilling, and oxidative stress tolerance (Yamaguchi-Shinozaki and Shinozaki, 2006; references cited therein).

Primers for *DREB*1A

Garg et al., (2008) designed one forward and one reverse primer for *DREB1*A genes using seventeen nucleotide sequences. Sequences were retrieved from RefSeq, UniGene, GenBank and EMBL nucleotide sequence databases. All the seventeen sequences were from *Arabidopsis Thaliana* group. Primers were designed using a conserved region of 286 base pairs present in all the seventeen DREB1A sequences. ClustalX software was used for identifying the conserved region by performing Multiple Sequence Alignment. Primer3 software designed two forward and three backward primers corresponding to the conserved region that were scrutinized for the presence of secondary structures using Premier Biosoft's NetPrimer tool. At last authors finalized one forward primer having optimum primer parameters (%GC, Tm etc) and absence of secondary structures (Hairpin, dimmer, loop etc). No optimum backward primer was identified using this process. Thereafter, authors used SCPrimer tool for designing backward primer. SCPrimer is web-based software for designing degenerate primers. SCPrimer designed 757 potential backward primers. On secondary structure validation, only one backward primer was found suitable for amplification. Authors finally used BLAST tool for validating the sensitivity and specificity of the designed primers against *DREB1*A gene.

Primers for *DREB2*A

Garg and Kumar, (2006) designed eight forward and eight backward primers for *DREB2*A gene using Primer3 and Web Primer software. Primers were designed using cDNA clone sequences of DREB2A gene of *Glycine max* (soybean), a known drought resistant gene. The properties of these primers were determined using Gene runner software and OLREA software was used for determining various restriction endonuclease cutting sites in selected primers. Out of all the primers tested, only four primers were found not to have hairpin structure. Furthermore, authors isolated, purified and amplified genomic DNA of 20 different varieties of soybean using designed forward and backward primers in PCR. Universal primer for soybean was also amplified for confirming the PCR amplification protocol. Only DNA of HARDEE, SL 96 and MAUS 71 varieties of soybean got amplified with the designed primers. These varieties of soybean were known drought resistant varieties thus designed primers were suitable for checking drought resistance due to DREB2A gene in soybean.

Perspectives of Primer Designing

Now a days, *In silico* analysis of genomic data is of utmost importance. The primer designing helps in fast annotation of genomic data that is a significant disadvantage of

various wet lab protocols. Furthermore, hypothesis put forward by *In silico* analysis can be validated by wet lab experiments. Primer designing using *In silico* approach is an important area in bioinformatics. It has manifold significance in molecular biology experiments. Firstly, PCR primers aid in selective amplification of a part of the genome often a gene that is very much required especially when we have small quantity of DNA sample. Secondly, PCR primers can also be used as a probe for determining the relative presence or absence of a gene of interest in the genome. This may help in developing diagnostic kits in medical field and genetic engineering experiments for designing plant varieties that can sustain in adverse environmental conditons like drought, flood, extreme temperature and salinity. With increasing population and depleting food resources, genetically engineered plants are a requirement of the day. The present review discussed about the various stress resistant genes present in plant species and the primer designing protocols and bioinformatics methods for validating these primers. These designed primers may be used for amplification of the gene in wet lab experiments. The designed primers may also be used for determining the presence of that specific gene in a plant variety using wet lab approaches.

Conclusion

Response to critical stress conditions like cold, drought or flood has been a critical feature of various plant species. Genes like *RD29A, COR78, LTI78* in *Arabidopsis* are activated by either ABA-dependent or ABA-independent gene expression pathways. Various studies till date have shown that a major transcription system controls ABA-independent gene expression in response to dehydration and low temperature. The system includes *DRE/CRT* (dehydration-responsive element/C-repeat) cis-acting element and its DNA-binding protein *DREB/CBF* (*DRE*-binding protein/C-repeat binding factor), which has an AP2 domain. The *DRE/CRT* is present in the promoter region of *DREB/CBF* genes and controls their expression. 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 subclasses of *DREB/CBF*, *DREB1/CBF* and *DREB2* have been identified. Former is induced by cold and later is induced by dehydration. The primer designing for *DREB1/CBF* and *DREB2* was discussed in this review. Primers were designed using various online available databases and softwares. The application of BLAST (Basic Local Alignment Search Tool) in determining the specificity of four forward and four reverse primers designed for each subclass of *DREB/CBF* was also discussed.

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