Isolation and functional characterization of a stress-inducible transcription factor *VuDREB2A* from cowpea

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by

AYAN SADHUKHAN
Roll: 09610616

Under the supervision of

DR. LINGARAJ SAHOO
Professor

DEPARTMENT OF BIOTECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI
GUWAHATI-781 039, ASSAM, INDIA

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INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

Department of Biotechnology

Guwahati-781 039

CERTIFICATE

It is certified that the work described in this thesis, entitled ***“Isolation and functional characterization of a stress-inducible transcription factor VuDREB2A from cowpea”***, carried out by Mr. Ayan Sadhukhan for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work under my supervision in the Department of Biotechnology, Indian Institute of Technology Guwahati, and this work has not been submitted elsewhere for a degree.

June, 2014

Dr. Lingaraj Sahoo
Professor
(Thesis Supervisor)

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

Department of Biotechnology

Guwahati-781 039

STATEMENT

I do hereby declare that the matter embodied in this thesis is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, India, under the guidance of Dr. Lingaraj Sahoo.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

June, 2014

Ayan Sadhukhan

Dedicated to my mother

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Date: Ayan Sadhukhan

CONTENTS

ABSTRACT

Cowpea (*Vigna unguiculata* L. Walp) is an important legume crop grown in Africa, southern Europe, Central and South America, Australia and South Asia, which adapts better than other legumes in arid and hot climatic conditions. However, the molecular mechanisms involved in the drought-tolerance of this crop are far from known. The Dehydration-Responsive Element-Binding Protein 2A (DREB2A) group of transcription factors plays key roles in plant responses to drought, salinity and heat. However, no DREB ortholog has been reported from cowpea so far.

In this study, a *DREB2*-type gene was isolated from cowpea by degenerate oligonucleotide-primed PCR and RACE approaches, which encoded a protein of 377 amino acids having a single DNA-binding Apetala 2 (AP2) domain. Further *in silico* analyses established the gene to be a canonical *DREB2A* ortholog and hence, it was named *VuDREB2A*. In cowpea, *VuDREB2A* transcript accumulation was highly induced by desiccation, heat and salt but only slightly by exogenous abscisic acid (ABA) treatment. The *VuDREB2A* promoter was isolated by inverse-PCR and scanned by evaluation of octamers, which were overrepresented in promoters of stress-responsive genes in *Arabidopsis*. This method predicted *cis*-regulatory elements in the *VuDREB2A* promoter viz. the ABA-responsive element (ABRE), its coupling element 3 (CE3) and heat stress-responsive element (HSE), which could account for the expression profile of the gene.

VuDREB2A was expressed in *E. coli* as a Histidine-tagged protein and purified using Ni-NTA-agarose affinity chromatography. The recombinant protein showed specific binding to synthetic oligonucleotides with dehydration-responsive element (DRE) sequences from *Arabidopsis* in electrophoretic mobility shift assay.

Heterologous expression of *VuDREB2A* in *Arabidopsis* improved drought- and heat-resistance of the transgenic plants. Additionally, overexpression of a truncated version of *VuDREB2A*, after removal of a putative negative regulatory domain (a serine/threonine-rich region between amino acids 132 to 182) led to a dwarf phenotype. Microarray and quantitative PCR analyses of *VuDREB2A* overexpressing *Arabidopsis* revealed up-regulation of stress-responsive genes having DRE overrepresented in their promoters.

In summary, *VuDREB2A* was isolated from cowpea that encoded a functional protein having the ability to bind to DRE *in vitro* and conferred drought tolerance in transgenic *Arabidopsis* by inducing downstream stress-responsive genes. VuDREB2A possessed a functional negative regulatory domain similar to AtDREB2A. The *VuDREB2A* promoter conserved *cis*-elements related to stress and phytohormones. Hence the gene was strongly induced by heat, salt and desiccation stress. This effort in isolating and characterizing *VuDREB2A* will be a significant step towards the genetic improvement of drought-resistance in cowpea or other Asiatic grain legume species through molecular breeding and/or transgenic approaches.

ABBREVIATIONS

ABA	Abscisic acid
ABRE	ABA-responsive element
AFLP	Amplified fragment length polymorphism
AP2	Apetala 2
ASM	Allele specific marker
APX	Ascorbate peroxidase
CAMBIA	Center for Application of Molecular Biology to International Agriculture
cAPX	Cytosolic ascorbate peroxidase
CaMV	Cauliflower Mosaic Virus
CaMV polyA	Cauliflower Mosaic Virus strain Cabb B-D polyadenylation signal
CBF	Cold binding factor
cDNA	Complimentary DNA
CDPK	Calcium dependent protein kinase
CGIAR	Consultative Group on International Agricultural Research
cGR	Cytosolic glutathione reductase
CPRD	Cowpea clones responsive to dehydration
CTAB	Cetyltrimethylammonium bromide
C-terminal	Carboxyl (COOH)-terminal
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
DRE	Dehydration-responsive element
DREB	Dehydration-responsive element-binding protein
dtGR	Dual-targetted glutathione reductase
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
ERF	Ethylene response factor
ExPASy	Expert Protein Analysis System
FAO	Food and Agriculture Organisation (of the United Nations)
GR	Glutathione reducatse
GSP	Gene-specific primer
GST	Glutathione S-transferase
HS	Heat shock
IITA	International Institute of Tropical Agriculture
LB	Luria-Bertani
MAPK	Mitogen activated protein kinase
MEGA	Molecular Evolutionary Genetic Analsysis
MeJA	Methyl jasmonate
miRNA	microRNA
mRNA	messenger RNA
MS	Murashige and Skoog’s medium
MYB	Myeloblastosis oncogene
MYC	Myelocytomatosis oncogene
NaCl	Sodium chloride

NCBI	National Center for Biotechnology Information
NCED	9-cis-epoxycarotenoiddioxygenase
N-terminal	Amino terminal
*npt*II	neomycin phosphotransferase II gene
ORF	Open reading frame
PAP	Phosphatidate phosphatases
pAPX	Peroxisomal ascorbate peroxidase
PAT	Patatin
PCR	Polymerase Chain Reaction
PLD	Pathogenesis related protein-1
PR-1	Phospholipase D
qRT-PCR	quantitative Reverse transcription-Polymerase chain reaction
QTL	Quantitative trait loci
RACE	Rapid Amplification of cDNA Ends
RIL	Recombinant inbred line
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-Polymerase chain reaction
ROS	Reactive oxygen species
SA	Salicylic acid
sAPX	Stromatic ascorbate peroxidase
SDS	Sodium dodecyl sulphate
SMART	Simple Modular Architecture Research Tool
SSH	Suppression subtractive hybridization
Ta	Annealing temperature
tAPX	Thylakoidal ascorbate peroxidase
TBE	Tris borate-EDTA buffer
T-DNA	Transferred-DNA
TF	Transcription factor

UNITS

°C	degree Celsius
cm	centimeter
d	days
h	hours
µg	microgram
bp	base pair
kb	kilo base/ kilo base pair
kDa	kilodalton
mg	milligram
ml	milliliter
min	minute
µg/ml	micrograms per milliliter
mg/l	milligrams per liter
mM	millimolar
µL	microliter
µM	micromolar
nM	nanomolar
pH	negative log of of H^+ ion
rpm	revolutions per minute
s	second
t/ha	metric ton/hectare
v/v	volume/volume (concentration)

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Abiotic stresses such as drought, salinity and extreme temperature are serious threats to sustainable crop production as they reduce the crop yield as high as 70 % (Acquaah 2007). The UN Food and Agriculture Organization (FAO) has predicted that world food consumption is likely to double over the next 40 years mostly in drought-prone parts of the world (Avery 2011). Drought is the most serious factor among various stresses limiting crop productivity in arid regions in Africa (Li *et al.* 2009) and South Asia (Li *et al.* 2011). Understanding and improving plant tolerance to drought stress is therefore of prime importance for sustenance of agriculture in these regions. Some crop plants historically cultivated in these regions show relatively enhanced tolerance to drought stress and they could be useful models to study the molecular mechanisms of drought tolerance. For example, cowpea (*Vigna unguiculata* L. Walp) is a legume crop grown in different parts of the world, viz. Africa, South Asia, USA and Australia, under a wide range of climatic conditions such as semiarid to sub-humid and is well adapted to heat and drought (Hall 2012). The ability of cowpea to withstand drought and its small nuclear genome (~620 Mb) make it an ideal crop model to study the molecular mechanisms of drought tolerance (Agbicodo *et al*. 2009).

A number of genes associated with drought response have been identified earlier in cowpea including genes encoding the stress responsive molecules: old yellow enzyme, group 2 LEA proteins, dihydroflavonol-4-reductase (DFR) and vestitone reductase (VR) (Iuchi *et al*. 1996), cystatins (Diop *et al*. 2004), ascorbate peroxidase (D'Arcy-Lameta *et al*. 2006), glutathione reductase (Contour-Ansel *et al*. 2006), glutathione-S-transferase (Gazendam and Oelofse 2007) and putative phosphoinositide-specific phospholipases (El-Maarouf *et al*. 2001). These genes have been reported to be up-regulated under water-stressed conditions, and among them *VuNCED1*, a key gene involved in abscisic acid (ABA) biosynthesis encoding 9-*cis*-epoxycarotenoid dioxygenase, conferred drought resistance in transgenic *Arabidopsis* and bent grass (Iuchi *et al*. 2001; Aswath *et al*. 2005) suggesting manipulation of ABA level could be a key event in the drought tolerance of cowpea.

To develop crop plants with enhanced tolerance to drought stress, a basic understanding of physiological, biochemical and gene regulatory networks is essential (Hussain *et al.* 2011). In the signal transduction network that leads from the perception of stress signals to the expression of stress-responsive genes, transcription factors play an essential role. Because transcription factors, as opposed to most structural genes, tend to regulate multiple pathways, they have emerged as powerful tools for the manipulation of complex metabolic pathways in plants, including abiotic stress tolerance. Numerous stress-induced genes known to promote stress tolerance are regulated by transcription activation factors that interact with *cis*-acting elements in the promoters of those genes. One such class of transcription factors is Dehydration-Responsive Element-Binding Proteins (DREBs) that binds to dehydration responsive *cis*-acting elements (DRE). The DREBs are members of the APETALA2/Ethylene Responsive-Element Binding Factor (AP2/ERF) family of transcription factors, which follow the ABA-independent signal transduction pathway and recognize the core sequence motif A/GCCGAC, DRE, in the promoters of stress-inducible genes (Yamaguchi-Shinozaki and Shinozaki 1994). Among the *DREB* gene family, the A-2 sub-group including *DREB2A* is specifically involved in the response to drought, salinity and heat stress in *Arabidopsis* (Liu *et al*. 1998; Sakuma *et al*. 2006). This signal transduction process includes the up-regulation of *DREB2A* itself and activation of various genes involved in stress tolerance mechanisms in various plant species (Lata and Prasad 2011b; Mizoi *et al.* 2012). However, the signal transduction process involving *DREB2A* is not known in cowpea.

Overexpression of the genes that regulate the transcription of a number of down-stream drought responsive genes such as *DREB2A* seems to be a promising approach in the development of drought-tolerant transgenic plants when compared to engineering individual functional genes (Bartels and Hussain 2008). Therefore, it is important to identify *DREB2A* from cowpea, a drought-hardy crop and enhance regulatory ability of this important transcription factor that activates the expression of many target genes controlling correlated characters for use in manipulating crops for drought tolerance.

In the present study, the cDNA and promoter of cowpea ortholog of *DREB2A* were cloned by degenerate oligonucleotide-primed polymerase chain reaction (Telenius *et al.* 1992) and inverse PCR (Ochman *et al.* 1988). Bioinformatic analyses of these sequences were carried out to compare them with previously characterized *DREB2A* orthologs [e.g. *Arabidopsis* (Liu *et al.* 1998) and soybean (Mizoi *et al.* 2013)], and the gene expression was profiled under various stress conditions that were used in previous studies for *DREB2A* orthologs (Gupta *et al.* 2010, Lata *et al*. 2011; Mizoi *et al.* 2013). Furthermore, the isolated gene was expressed in *E. coli* as a Histidine-tagged protein and the purified recombinant protein was tested for its ability to interact with dehydration-responsive elements *in vitro*. The gene was subsequently characterized using heterologous expression in *Arabidopsis*, a model plant frequently used in plant functional biology to characterize gene orthologs. In summary, a *DREB2A* ortholog from cowpea was identified, which conserved the basic functionality and mode of regulation of *DREB2A* in *Arabidopsis.*

1.2 Objectives

This thesis encompasses the following objectives:

1) Isolation of *VuDREB2A* gene from cowpea.

2) Studies on the transcriptional regulation of *VuDREB2A* under different abiotic stresses.

3) Assessment of *in vitro* DNA-binding ability of VuDREB2A.

4) Functional study of *VuDREB2A* in transgenic *Arabidopsis*.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Impact of drought, salinity and heat on agriculture

Global climate change poses serious threat to the growth and yield of crop plants worldwide. Primitive anthropogenic practices are also instrumental in deteriorating the quality of arable land by increasing desertification, salinization and acidification apart from raising global temperatures and CO_2 concentration in the atmosphere. The principal cause of reduction in growth and productivity of crops world-wide is abiotic stress which reduces the average yields of major crops by 70 % (Acquaah 2007). Abiotic stress, in a broad sense, include both meteorological factors like (1) high and low temperatures, (2) dehydration and flooding and (3) intense radiations as well as pedological factors like (4) salinity, (5) acidity, (6) heavy metal (HM) abundance and (7) nutrient deficiency. While many of these stress factors, individually or in combination, may trigger a common detrimental effect on plants, in some cases, exposure to one type of stress may render the plant vulnerable to damage by others. But drought alone is the most serious factor among various stresses reducing crop yields by 50 % (Bray *et al.* 2000). Drought severely limits crop productivity in Africa (Li *et al.* 2009) and South Asia (Li *et al.* 2011). Salinization of soil is again another threat to agriculture affecting almost one million hectares worldwide (FAO estimates in 2008) limiting productivity of crops (Türkan and Demiral 2009). Additionally, drought occasionally accompanied by heat stress in the field, causes more severe effects on the growth and production of crops than either stress singly (Mittler 2006). Each crop has an optimum temperature for vegetative growth as well as seed production. Increased temperatures hence cause severe reduction in productivity (Karl *et al.* 2009). Many rainfed crops in Africa and Latin America are near their maximum temperature tolerance, so their yields fall sharply over small temperature changes. A fall in agricultural productivity of up to 10-30 % due to changes in temperatures over the 21st century is projected (Lobell *et al.* 2008).

2.2 Molecular mechanisms of plant responses to drought, salinity and heat stress

Drought, cold and high salt all cause dehydration stress for the cell whereas both dehydration and heat stress are ultimately decoded as cellular osmotic stress by plants (Boudsocq and Lauriere 2005; Shinozaki and Yamaguchi-Shinozaki 2007). Plants respond and adapt to these stress conditions with a long-term evolutionary manifestation of developmental and morphological changes as well as short-term biochemical and physiological alterations. Generally, osmotic stress leads to leaf wilting and abscission, reduction in area of leaf, stimulation of root growth, changes in relative water content, electrolytic leakage, generation and accumulation of reactive oxygen species and free radicals leading to lipid peroxidation and damage/malformation of proteins and membranes (Bartels and Sunkar 2005; Lata and Prasad 2011). Multiple signaling pathways regulate the stress responses of plants and there exists an overlap between the patterns of expression of genes that are induced in response to different stress factors (Seki *et al*. 2001; Chen *et al*. 2002). Although most of the components of signaling and response seem to be conserved across land plants, the wiring of these decision-making circuits, the weights assigned to each input value, and the threshold values for arriving at different decision states are quite variable, leading to wide range of variation across different species in tolerance (Geisler 2010).

Numerous stress-induced genes at the transcription level have been identified in several plant species. The products of these genes are thought to promote stress tolerance and to regulate gene expression through signal transduction pathways. Transcriptome analysis using microarray technology (Bohnert *et al*. 2001; Seki *et al*. 2001; Zhu *et al*. 2001) has revealed that genes induced by stress can be categorized into two groups according to the functions of their products. The first group consists of functional proteins such as membrane proteins (water channels and ion transporters) that maintain water/ion movement through membranes; key enzymes for osmolyte biosynthesis (proline, glycine betaine, soluble sugars); detoxification enzymes scavenging reactive oxygen species (ROS) to prevent damage to cellular structures (glutathione *S*-transferase, glutathione reductase, hydrolase, catalase, superoxide dismutase, ascorbate peroxidase); proteins for the protection of macromolecules (LEA protein, osmotin, antifreeze proteins, chaperones, mRNA binding proteins, etc.); enzymes like proteinase inhibitors, and fatty acid metabolism and lipid transfer proteins. The second group comprises regulatory proteins, i.e. transcription factors of different categories like basic leucine zipper (bZIP), myelocytomatosis oncogene (MYC), myeloblastosis oncogene (MYB), dehydration-responsive element binding protein (DREB), zinc finger homeodomain (ZF-HD), NAM, NAC, ATAF, heat shock transcription factor (HSF) etc.; protein kinases like mitogen activated protein kinase (MAPK), calcium dependent protein kinase (CDPK), receptor protein kinase, ribosomal-protein kinase and transcription-regulation protein kinase etc.; protein phosphatase and proteinases (phosphoesterases, phospholipase C etc.) involved in the regulation of signal transduction and gene expression (Sinozaki and Yamaguchi-Shinozaki 2007).

Molecular responses to abiotic stress in general including drought, salinity and heat involve stress perception, signal transduction via secondary messengers and phosphoprotein cascades to transcription factors, and triggered gene expression leading to metabolic changes towards stress adaptation (Fig. 2.1). Abiotic stresses may be sensed by the plant by different means. Though the existence of unique sensors for different abiotic stresses is speculated (Chinnusamy *et al*. 2004), there is not much information on the nature of the biochemical receptor/ ligand interactions. The sensor molecule could be physically altered by the stress, for example, conformation of a membrane protein receptor (e. g. the thylakoid membrane) could be changed by changes in membrane fluidity/rigidity (Murata *et al.* 1997), cytoskeletal reorganization (Őrvar *et al.* 2000) or phosphorylation-dephosphorylation due to temperature changes (Plieth 1999). In contrast, the sensor could be activated as a consequence of abiotic stress leading to metabolic changes, secondary signals (accumulation of ROS or Ca^{2+} influx), release of ATP, or reduced energy levels (Jaspers and Kangäsjarvi 2010; Gill and Tuteza 2010). Sensory kinases like histidine kinase (ATHK1; Urao *et al*. 1999), receptor-like protein kinases (RLKs) (Tamura *et al.* 2003), photoreceptors (Mazzella *et. al.* 2001), G-protein coupled receptors (GPCRs) as well as membrane transporters have been implicated in stress perception. Plants relay the extracellular stress-specific signals through various second messengers which could be calcium, ROS, nitric oxide or membrane phospholipids. A number of studies have shown that cellular calcium levels rise in response to drought and also heat (Larkindale and Knight 2002) and that it functions as a messenger in modulating diverse physiological processes that are important for stress adaptation. Various sensors/ responders and calcium-binding proteins sense increased cellular Ca^{2+} levels which can activate many calcium-dependent protein kinases (CDPKs) and thereby change gene expression patterns in response to abiotic stresses. Ca^{+} influx and activation of CDPKs are closely related to expression of heat shock proteins (HSPs) in case of heat stress (Sangwan and Dhindsa 2002). Ca^{+} binding activates calmodulin (CaM), which induces a regulatory event cascade including HSP genes (Liu *et al*. 2003). Many Ca^{2+} and Ca^{2+}/ CaM-binding transcription factors have also been identified in plants (reviewed by Reddy *et al*. 2011). The role of phospholipids in signal perception and signal relay/transduction is evident as changes in phospholipid composition occur during osmotic stress (reviewed by Munnik *et al*. 1998). Besides calcium and phospholipids, ROS also acts as a key second messenger mediating stress-specific cellular responses. These harmful ROS moieties can denature proteins, DNA and can damage lipids. To combat the toxic effects of these ROS, scavenging components of the cell come into play, thereby maintaining equilibrium between ROS production and scavenging. This equilibrium gets disturbed in when plants are exposed to various abiotic and biotic stresses. In this case these ROS moieties in the cell act as signaling molecules and lead to expression of many genes to combat the effect of various stresses (Mittler *et al*. 2004). From the secondary messengers, the signals are relayed through phosphoprotein cascades involving serial phosphorylation of downstream signaling proteins. MAPKs and CDPKs are major components. MAP Kinases: AtMPK4 and AtMPK6 which were activated by cold, drought, saline stress, and sorbitol have been reported in *Arabidopsis* detached leaves in a general response to hyperosmotic stresses (Ichimura *et al*. 2000). In addition, another MAPKK: AtMKK1 was also activated by drought and high salinity (Matsuoka *et al*. 2002) which phosphorylated and activated AtMPK4 *in vitro* (Huang *et al*. 2000). A heat shock activated MAPK (HAMK) triggered by changes in membrane fluidity coupled with cytoskeletal remodeling has also been reported (Sangwan and Dhindsa 2002). The SOS (Salt Overly Sensitive) signaling pathway is one of the best characterized pathways in salinity stress. In this pathway, cytosolic calcium is triggered by excess intracellular or extracellular Na^+ (Zhu 2000). SOS1, 2 and 3 form the major components of SOS mediated pathway. SOS2 and SOS3 are soluble cytoplasmic proteins (protein kinase and calcium-binding protein respectively) involved in regulating the expression level of *SOS1* which is a salt tolerance effector gene encoding a plasma membrane Na^+/H^+ antiporter which excludes Na^+ outside the cell (Shi *et al*. 2000; Qiu *et al*. 2002).

The stress signals relayed through the second messengers and phosphoprotein cascades ultimately reach the transcription factors (by phosphorylation) which interact with *cis*-elements present in the promoters of various stress-related genes and thus up-regulating their expression resulting in stress alleviation. The heat shock transcription factors bind the heat-responsive heat shock elements (consensus: nGAATTnnTTCn/ nTTCnnGAAn) in the promoters of heat shock genes (Nover and Scharf 2001; Schöffl *et al.* 1998) after trimerization, resulting in enhanced expression of HSPs which leads to protection of protein homeostasis.

ABA is an important phytohormone specifically involved in osmotic stress. It mainly exerts its role in stress by regulation of transpiration through control of stomatal closure (thereby reducing the photosynthetic rate and increasing the water use efficiency), and activation of stress responsive genes. The investigation of dehydration-induced genes in *Arabidopsis* has revealed both ABA-dependent and ABA-independent signal transduction pathways (Yamaguchi-Shinozaki and Sinozaki 2005). In *aba* (ABA deficient) and *abi* (ABA insensitive) mutants, several genes are induced by dehydration indicating that these genes do not require ABA for their expression under drought conditions. Some genes are induced in response to exogenous ABA treatment but some are not (Shinozaki *et al.* 2003; Yamaguchi-Shinozaki and Sinozaki 2005). This multiple expression pattern involves at least two *cis*-acting elements in the promoter of such genes. The 9-base pair direct repeat (TACCGACAT), termed the dehydration responsive element (DRE), functions in the initial rapid response to dehydration, salt, or cold. The slower ABA response is mediated by another *cis*-element known as ABA-responsive element (ABRE; consensus: C/TACGTGGC; Yamaguchi-Shinozaki and Shinozaki 1993, 1994). The MYC/MYB and the ABA-responsive element-binding protein/ABA-binding factor (AREB/ABF) regulons (group of genes controlled by a particular type of transcription factor) work in ABA-dependent manner, whereas the DREB/cold-binding factor (CBF), NAC, ZF-HD regulons exert their control in an ABA-independent manner. Recent studies with the *DREB2A* promoter (Kim *et al.* 2011) however, have thrown light on the additional ABA-dependent regulation of the *DREB2A* gene. The ABA-dependent and independent pathways considerably overlap and there exists cross-talk between them, forming a highly complex gene network, also evident from microarray analyses (Fowler and Thomashaw 2002; Umezawa *et al.* 2006).

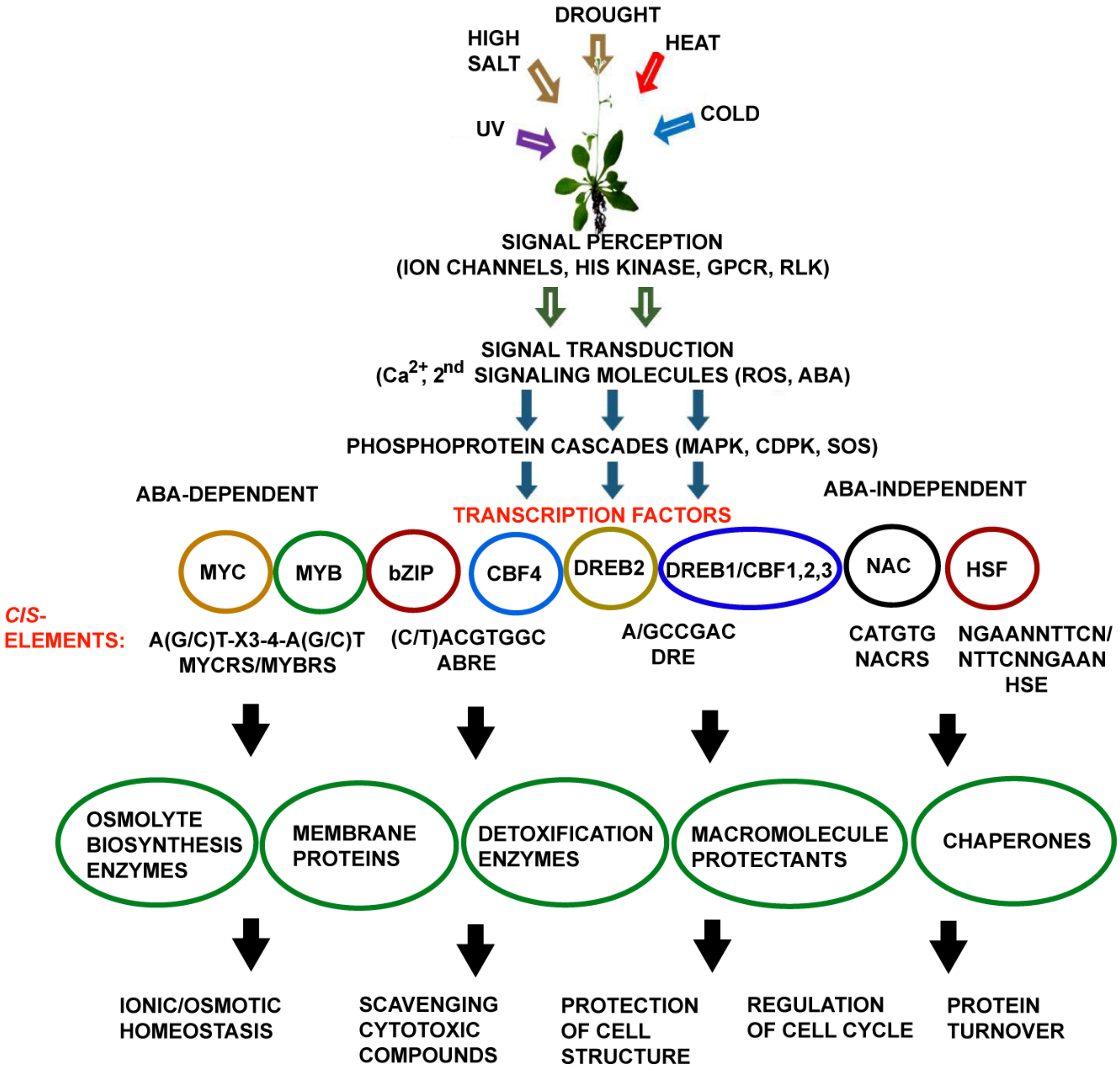


Figure 2.1 Schematic representations of plant molecular responses to abiotic stress.

2.3 *DREB2* genes and their roles in stress tolerance of plants

The transcription factors of the DREB2 group are well-characterized master regulators of plant responses to drought, heat and osmotic stresses.

2.3.1 Structure and phylogeny of DREB2 proteins

All DREB proteins belong to the larger AP2 superfamily of transcription factors, which are characterized by the presence of the DNA-binding AP2 domain of 60-70 amino acids (Weigel 1995). This domain is generally plant-specific but evidence of this domain in homing endonucleases of cyanobacteria, ciliates and viruses have been reported, hypothesizing horizontal gene transfer from bacteria or viruses into plants leading to the origin of the AP2 domain proteins in plants (Magnani *et al.* 2004). The 3D-structure of the AP2 domain (AtERF1; PDB ID: 1GCC; Allen *et al.* 1998) consists of an N-terminal three-stranded ß-sheet (first 2 strands known as YRG element, rich in basic and hydrophilic residues) and one C-terminal amphiphatic α-helix (core of a broader RAYD element) running almost parallel to the ß-sheet. The domain interacts with DNA via Arg and Trp residues in the ß-sheet, whereas, the conserved A37 in the α-helix might play a crucial role in the DNA binding or the stability of the AP2/EREBP domain (Liu *et al.* 2006). The main function of RAYD element is to regulate the special binding activity of DREB transcription factors by influencing the conformation of the YRG element or interacting with other proteins (Okamuro *et al.*1997).

The AP2 superfamily is classified into 2 families: the AP2 family, which contains 2 AP2 domains, and the AP2/Ethylene-responsive element-binding protein (AP2/EREBP) family (transcription factors implicated in plant development and hormone-dependent gene expression) having a single AP2 domain. The AP2/EREBP family is again subdivided into five sub-families (in *Arabidopsis*) viz. DREB, ERF, AP2, RAV and others based on sequence characteristics of the

AP2 domain. The DREB sub-family contains conserved valine and glutamic acid residues at the 14th and 19th position, respectively of the AP2 domain, determining specificity of target *cis*-element recognition (Wang *et al*. 2008; Sakuma *et al*. 2002). DREB transcription factors can identify and bind specifically to the dehydration responsive element (DRE; TACCGACAT) originally isolated from the promoter of the *RD29A* gene of *Arabidopsis* and is involved in both cold- and dehydration-inducible gene expressions via an ABA-independent pathway (Yamaguchi-Shinozaki and Shinozaki 1994). The sequence CCGAC within the DRE is the minimal motif for binding, and C4, G5, and C7 are necessary for specific interaction (Hao *et al*. 1998; Sakuma *et al*. 2002).

The DREB sub-family is further divided into six sub-groups from A-1 to A-6 (Sakuma *et al*. 2002). The A-2 and A-3 subgroups are characterized by a conserved nuclear localization signal at the N-terminal end of the protein, RKxPAKGSKKGCMxGKGGPENxx and RKxxxxKGGPx-NxKF (containing a conserved motif CMIV-1; Nakano *et al*. 2006) closely upstream to the AP2/EREBP domain in the upstream (but different from the A-1 sub-group in absence of a conserved motif close to the C-terminal of the AP2 domain). DREB2 proteins belonging to the A-2 subgroup DREBs are specifically involved in response to drought, heat and salinity/ osmotic stresses but not by cold (Dubouzet *et al*. 2003; Liu *et al*. 1998; Nakashima *et al*. 2000) with some exceptions. The first DREB2 proteins, DREB2A and DREB2B was isolated by a yeast one hybrid screening technique from a cDNA library prepared from dehydrated *Arabidopsis* (Liu *et al*. 1998).

Since then, *DREB2* orthologs have been reported from various crop plants, viz. *OsDREB2A*, *B, C* and *E* from rice (Dubouzet *et al*. 2003; Matsukura *et al*. 2010), *TaDREB1* from wheat (Shen *et al*. 2003a), *AhDREB1* from halophyte *Atriplex hortensis* (Shen *et al.* 2003b), *HvDRF1* from barley (Xue and Loveridge, 2004), *ZmDREB2A* from maize (Qin *et al*. 2007), *PgDREB2A* from pearl millet (Agarwal *et al.* 2007), *SiDREB2* from foxtail millet (Lata *et al.* 2011a), *LeDREB2* from tomato (Guo and Wang 2011), *ORCA1* from periwinkle (Menke *et al*. 1999), *HaDREB2* from sunflower (Díaz-Martín *et al.* 2005), *GmDREB2A;1* and *2* from soybean (Mizoi *et al*. 2013).

The DREB transcription factors also contain another structural feature apart from the DNA-binding domain, an acidic region at the C-terminal end which was shown to act as a transcriptional activation domain in *Arabidopsis* protoplasts (Sakuma *et al*. 2006). This region contains a conserved sequence CMIV-3 (Nakano *et al*. 2006). Some DREB2 orthologs are truncated proteins having short C-terminal activation domains, e.g. AtDREB2E and AtDREB2H from *Arabidopsis*, GmDREB2B and GmDREB2C homologs from soybean (Li *et al*. 2005), CAP2 from chickpea (Shukla *et al*. 2006) and EsDREB2B from a desert legume *Eremosparton songoricum* (Li *et al*. 2014). These truncated proteins contain a specific motif CMIV-4 (Nakano *et al*. 2006).

Table 2.1 *DREB2*-type genes identified from different plants.

Gene	Accession	Plant	Stress response	Reference
DREB2A	AB007790	*Arabidopsis thaliana*	Drought, salt, ABA	Liu *et al*. 1998
DREB2B	AB016571	*Arabidopsis thaliana*	Drought, salt	Nakashima *et al*. 2000
DREB2C	NM_129594	*Arabidopsis thaliana*	Salt, Mannitol, Cold	Lee *et al*. 2010
OsDREB2A	AF300971	*Oryza sativa*	Drought, Salt, Cold, ABA	Dubouzet *et al*. 2003
OsDREB2B	JF915843	*Oryza sativa*	Heat, Cold	Matsukura *et al*.2010
OsDREB2C	AK108143	*Oryza sativa*	-	Matsukura *et al*.2010
WDREB2	BAD97369	*Triticum aestivum*	Drought, Salt, Cold, ABA	Egawa *et al*. 2006
HvDRF1	AY223807	*Hordeum vulgare*	Drought, Salt, ABA	Xue and Loveridge 2004
TaDREB1	AAL01124	*Triticum aestivum*	Cold, Dehydration, ABA	Shen *et al*. 2003
ZmDREB2A	AB218832	*Zea mays*	Drought, Salt, Cold, Heat	Qin *et al*. 2007
PgDREB2A	AAV90624	*Pennisetum glaucum*	Drought, Salt, Cold	Agarwal *et al*. 2007
SbDREB2	ACA79910	*Sorghum bicolor*	Drought	Bihani *et al*., 2011
SiDREB2	HQ132744	*Setaria italica*	Drought, salt	Lata *et al*. 2011a
CaDREB-LP1	AY496155	*Capsicum annum*	Drought, salt, wounding	Hong and Kim 2005

AhDREB1		*Artiplex hortensis*	Salt	Shen *et al*. 2003
GmDREBa	AY542886	*Glycine max*	Drought, salt, cold	Li *et al*. 2005
GmDREBb	AY296651	*Glycine max*	Drought, salt, cold	Li *et al*. 2005
GmDREBc	AY244760	*Glycine max*	Drought, salt, ABA	Li *et al*. 2005
GmDREB	AF514908	*Glycine max*	Drought, salt	Shiqing *et al*. 2005
GmDREB2	ABB36645	*Glycine max*	Drought, salt	Chen *et al*. 2007
GmDREB2A;1, *GmDREB2A;2*	JX440386, JX440387	*Glycine max*	Drought, salt, cold, heat	Mizoi *et al*. 2013
MtDREB2A	DQ908959	*Medicago trancatula*	Drought, salt	Chen *et al*. 2009
PsDREB2A	HM229349	*Pisum sativum*	Dehydration	Jovanovic *et al*. 2013
CAP2	DQ321719	*Cicer arietinum*	Drought, salt, ABA, auxin	Shukla *et al*. 2006
PeDREB2	EF137176	*Populus euphratica*	Drought, salt, cold	Chen *et al*. 2009
SbDREB2A	GU592205	*Salicornia brachiata*	Drought, salt, heat	Gupta *et al*. 2010
ORCA1	AJ238739	*Catharanthus roseus*	Jasmonate	Menke *et al*. 1999
LeDREB2	AF500012	*Lycopersicon esculentum*	Salt, ABA, H_2O_2, methyl viologen	Guo and Wang 2011
HaDREB2	AY508007	*Helianthus annuus*	-	Díaz-Martín *et al*. 2005
DvDREB2A	EF633987	*Dendronthema × moriforlium*	Drought, salt, heat, cold, ABA	Liu *et al*. 2008
BnDREB	AY444875	*Brassica napus*	-	Zhao *et al*. 2007
GhDREB	AF509502	*Gossypium hirsutum*	Drought, salt, cold	Gao *et al*. 2009
EsDREB2B	ADV57357	*Eremosparton songoricum*	ABA, drought, salt, cold, heavy metal, oxidative stress	Li *et al*. 2014

2.3.2 Regulation of *DREB2* genes

The DREB2 genes are controlled precisely to be expressed only under stress conditions and not under normal conditions. This control is exerted both at the transcriptional and post-transcriptional levels.

2.3.2.1 Transcriptional regulation

The DREB2 genes are regulated by various abiotic stress conditions at the transcript level.

2.3.2.1.1 Expression patterns of *DREB2* genes

The DREB2 genes have been generally observed to be inducible to dehydration, heat and osmotic stresses in a very quick manner, but not by cold or exogenous ABA treatment, with some exceptions. The expression of *Arabidopsis DREB2A* and *DREB2B* and were induced by dehydration (within 6 h) and high salt stress (within 20 min), but not by cold stress and weakly by exogenous ABA treatment (Liu *et al*. 1998; Nakashima *et al*. 2000; Kim *et al*. 2011).

DREB2C expression was induced only by high salt treatment (Lee *et al*. 2010). *OsDREB2A* transcript was highly induced within 24 h of dehydration and high salt stress but only weakly in ABA and cold treatments. In general, the high levels of *DREB2* transcripts were found to be maintained till 24 h of treatment. In sorghum, however, *SbDREB2* transcripts were highly induced within 1h of drought but dropped to basal levels afterwards (Gupta *et al*. 2010).

Wheat *TaDREB1* and *WDREB2*, maize *ZmDREB2A*, pearl millet *PgDREB2*, *Populus euphratica PeDREB2* and soybean *GmDREB2A* homologs showed response to cold stress as well (Dubouzet *et al*. 2003; Shen *et al*. 2003a; Egawa *et al*. 2006; Agarwal *et al*. 2007; Qin *et al*. 2007; Chen *et al*. 2009; Mizoi *et al*. 2013). The *OsDREB2B* transcript level increased significantly after 24 h of low temperature, as well as after 20 min of high temperature (Matsukura *et al*. 2010; Dubouzet *et al*. 2003). *ZmDREB2A* transcripts also responded to high temperature (Qin *et al.* 2007). The transcript level of chickpea *CAP2* increased under ABA and auxin treatments apart from dehydration and salt (Shukla *et al*. 2006). Chrysanthemum *DvDREB2A* was highly induced by heat, low temperature, and ABA apart from drought and salt treatments (Liu *et al.* 2008). In the desert plant *Eremosparton songoricum*, *EsDREB2B* transcripts were also upregulated by exogenous ABA, heavy metal, oxidative stress and wounding within 6 h of treatments other than osmotic, salt, cold, heat stresses (Li *et al*. 2014). *LeDREB2* from tomato (Guo *et al*. 2011) was induced by various oxidative stresses.

DREB2 transcripts have been found normally in all plant organs, viz. stem, leaves and roots. For example, *AtDREB2A* accumulated in roots, stems, and leaves under normal growth conditions (Liu *et al.* 1998), whereas *DREB2C* expression was observed in mature embryo and the cotyledons of germinating seedlings as well (Lee *et al.* 2010). *AhDREB1* from *Artiplex hortensis* (Shen *et al.* 2003) was inducible to salt only in the roots. The highest transcript accumulation of Chrysanthemum *DvDREB2A* was reported in flowers (Liu *et al.* 2008). *SiDREB2*, of foxtail millet was expressed in young and mature spikelets also, other than foliage, suggesting its role in developmental pathways (Lata *et al.* 2011).

2.3.2.1.2 Structure of the *DREB2A* promoter

The *AtDREB2A* promoter was functionally characterized by evaluating transcriptional activities of base-substituted variants. The osmotic stress responsiveness of the *DREB2A* gene was shown to be regulated by the ABRE *cis*-element and its coupling element CE3 approximately -100 bp from the transcription start site (TSS) in the *DREB2A* promoter (Kim *et al*. 2011). Yeast one-hybrid and chromatin immunoprecipitation (ChIP) assays showed that ABRE-binding protein 1 (AREB1), AREB2 and ABRE-binding factor 3 (ABF3) bind to and activate the *DREB2A* promoter. These recent observations have thrown light on the fact in addition to an ABA-independent pathway, the ABA-dependent pathway also has considerable role in regulation of the *DREB2A* system. The heat stress response of *DREB2A* was caused by another *cis*-element, the heat shock element (HSE) at approximately -150 bp from the TSS in the promoter where several *Arabidopsis* heat shock factors (HSFs) were shown to bind (Yoshida *et al.* 2011).

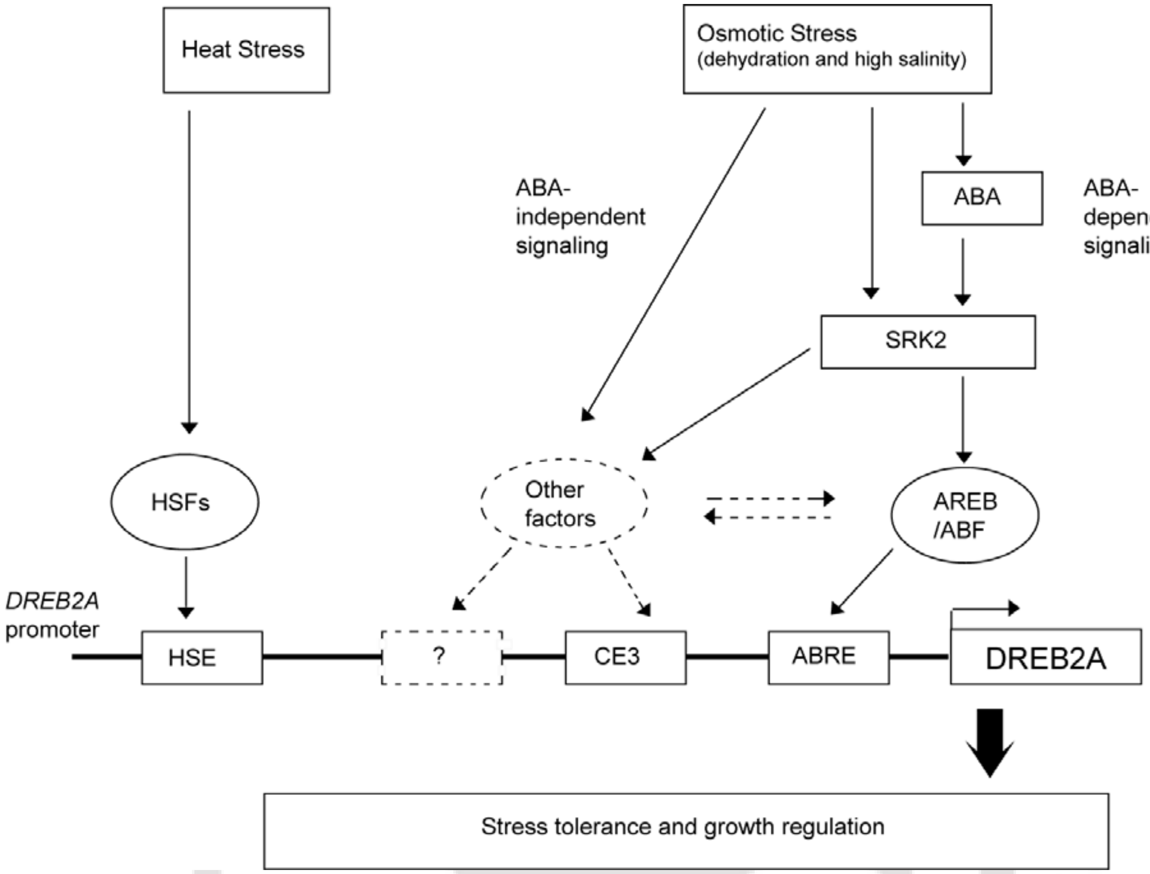


Figure 2.2 Transcriptional regulation of *DREB2A* in osmotic and heat stresses (modified from Kim *et al*. 2011). Osmotic stress (dehydration and salinity) signals are transmitted in both ABA-dependent and independent manner to *cis*-elements in the *DREB2A* promoter. SRK2, a SnRK2 member (signaling kinase), receives osmotic signals and relay them via ABA-binding factors (AREB/ABF) binding to the ABRE or some unknown factors (probably WRKY, MYB or other bZIP family factors) binding to the CE3/some other motifs in the *DREB2A* promoter. The solid lines represent identified and the dotted lines unknown/speculated signaling pathways/*cis*-elements. Heat stress signals are relayed via HSFs which bind the HSE on the *DREB2A* promoter.

2.3.2.2 Post-transcriptional regulation

In the grass family (Poaceae), DREB2 genes contain introns, and are regulated post-transcriptionally, by stress-induced alternative splicing. Wheat *WDREB2* produced three transcript forms: α, β, γ through alternative splicing with the α and γ forms transiently increased under drought and salt stress and the β form contained a insertion leading to premature stop codon and hence, a truncated protein without the AP2 domain (Egawa *et al*. 2006). *ZmDREB2A* from maize had two transcription forms, *ZmDREB2A-L* and *ZmDREB2A-S* (Qin *et al*. 2007). *ZmDREB2A-L* contained a 53 bp insertion in contrast with *ZmDREB2A-S*. This insertion resulted in a frame shift and a premature stop in translation and a shorter protein. Similar observations were made in barley *HvDRF1* (Xue and Loveridge 2004) and rice (Mutsukura *et al*. 2010)

2.3.2.3 Post-translational regulation

The region downstream to the AP2 domain in DREB2A was shown to act as a negative regulatory domain (NRD) which destabilizes the protein, and removal of this domain transformed *DREB2A* into a constitutively active form (Sakuma *et al*. 2006). This NRD contains a PEST sequence which targets the protein for proteasome-mediated degradation (Qin *et al*. 2008, Nakashima and Yamaguchi-Shinozaki 2010). Removal of regions corresponding to the NRD in MtDREB2A of *Medicago truncatula* led to enhanced activity in yeast (Chen *et al*. 2009) and in GmDREB2A;2 higher stability and activity in *Arabidopsis* and soybean protoplasts, and transgenic *Arabidopsis* (Mizoi *et al*. 2013). Higher levels of stable DREB2 proteins (without the NRD) led to higher induction of downstream target genes and hence a growth penalty in the transgenic plants, in all cases. The NRD region also contains stretches of serine and threonine residues, which are targets of phosphorylation. It was shown that phosphorylated PgDREB2A cannot bind to the DRE (Agarwal *et al*. 2007). The N-terminal region of DREB2A (containing the negative regulatory domain) was found to interact with DREB2A-Interacting Protein 1 (DRIP1) and DRIP2, which were shown to function as E3 ubiquitin ligases, ubiquitinating DREB2A to target it for 26S proteasome-mediated degradation (Qin *et al*. 2008). These observations suggest role of post-translational modification in the regulation of DREB2 proteins and plants use such strategies to switch off the DREB2 gene under non-stressed conditions to minimize metabolic burden.

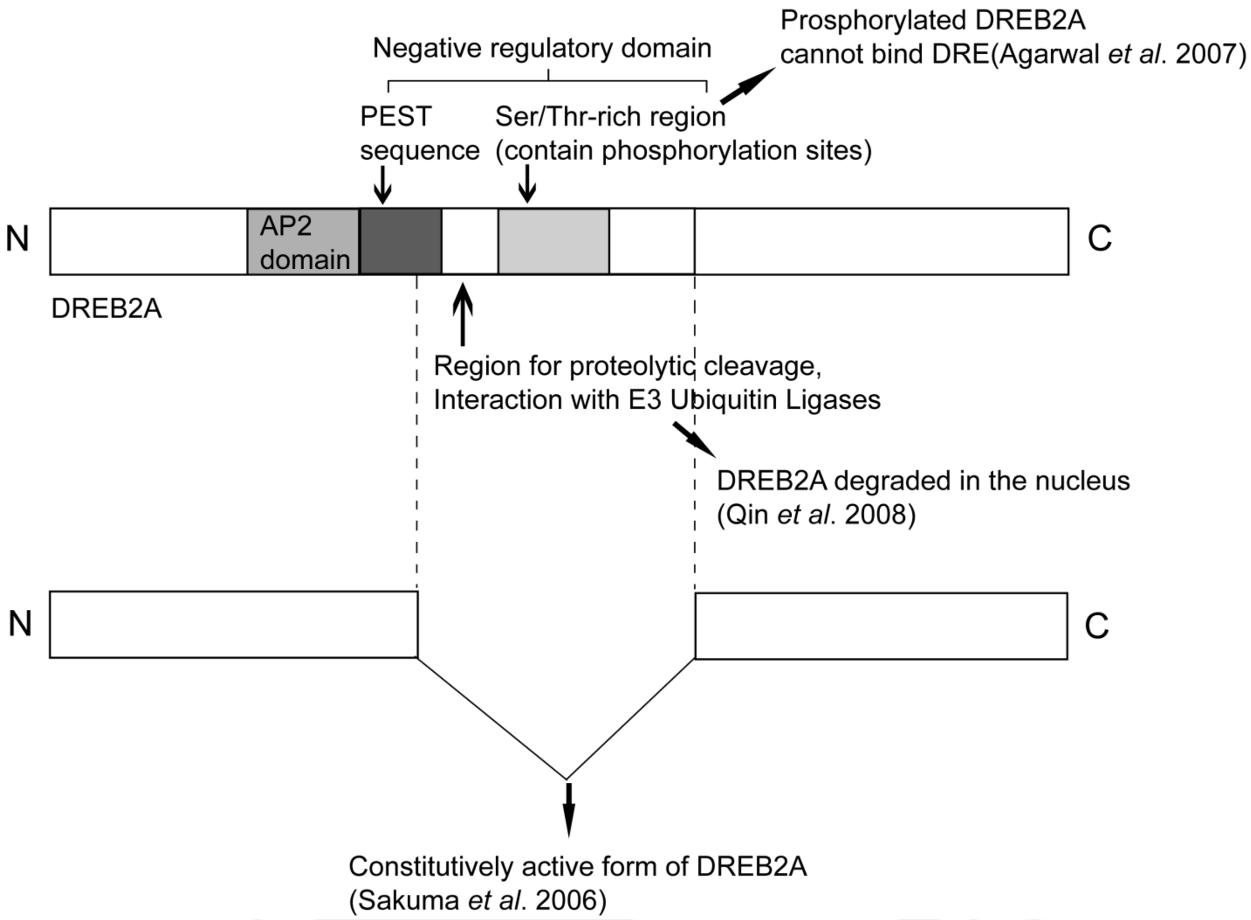


Figure 2.3 Post-translational regulation of DREB2A.

2.3.3 Application of *DREB2* genes

2.3.3.1 Genetic engineering

Genetic engineering via *DREB2* overexpression has been demonstrated in recent years to improve the drought, salinity and heat tolerance of plants or lower organisms like yeast and bacteria. Sakuma *et al* (2006) showed that overexpression of active form of *AtDREB2A* (without the NRD) up-regulated the downstream drought and salt inducible genes and significantly improved drought stress tolerance of *Arabidopsis* but with growth retardation of the transgenic plants. Similarly Chen *et al* (2009) showed that a constitutive form, viz., *MtDREB2a* of *M. truncatula DREB2A* resulted in enhanced transcriptional activation ability in transgenic Medicago but associated with similar growth penalty. Sugar-sensitive dwarf phenotypes were reported in case of overexpression of constitutively active forms of soybean *GmDREB2A;2* (Mizoi *et al*. 2013). Overexpression of wheat *WDREB2* and maize *ZmDREB2A* also caused such dwarf phenotypes in transgenic *Arabidopsis* and tobacco (Dubouzet *et al.* 2003; Kobayashi *et al.* 2007; Qin *et al.* 2007). The constitutive expression of sorghum *SbDREB2* led to pleiotropic effects in transgenic rice without any seed setting (Bihani *et al*. 2011). However, these dwarf phenotypes were avoided by use of the stress-inducible rd29A promoter in almost all cases. Recently, the constitutively active form of *AtDREB2A* was expressed in soybean under the control of the rd29A promoter without any growth penalty. Highest expression levels of the transgene were found in the roots under water deficit stress (Engels *et al.* 2013).

Overexpression of intact *ZmDREB2A-S* (functional form of two transcription forms S and L) enhanced drought stress tolerance without post translational modification and microarray analyses revealed that many stress-inducible genes were upregulated in the transgenic plants under non-stressed control conditions. *AhDREB1* gene conferred salt and drought tolerance in the transgenic tobacco plants (Shen *et al.* 2003b). Overexpression of *OsDREB2B* in rice significantly improved tolerance to water deficit stress (Chen *et al.* 2008). Overexpression of *GhDREB* (Gao *et al*. 2009) from cotton improved drought tolerance of transgenic wheat plants. *PgDREB2A* from *Pennisetum glaucum* engineered hyperionic and hyperosmotic stress tolerance in tobacco plants (Agarwal *et al*. 2010). Overexpression of *ZmDREB2A* in *Arabidopsis* resulted in an enhanced tolerance to drought stress (Qin *et al.* 2007).

Additional induction of heat shock genes in the *Arabidopsis DREB2A* overexpressors resulted in thermotolerance of the transgenic plants also (Sakuma *et al*. 2006). Over-expression of *DREB2C* was also found to induce the expression of many heat shock stress inducible genes, resulting in thermotolerance of transgenic *Arabidopsis* (Lim *et al.* 2007). OsDREB2B in transgenic *Arabidopsis* showed improved heat shock tolerance (Matsukura *et al.* 2010). In *Arabidopsis*, HsfA3, an HS TF regulates expression of many heat-inducible genes downstream of the DREB2A stress-regulatory system and functions under the control of the DREB2A transcriptional cascade (Schramm *et al*. 2008; Yoshida *et al*. 2008). The constitutively overexpressing sunflower *HaDREB2* alone could not increase thermotolerance. However, on *HaDREB2* and *HaHSFA9* (sunflower Heat Shock Factor A9) overexpressed together, positive effects on seed longevity were observed, apart from thermotolerance, suggesting functional interaction between DREB2 and HSF (Almoguera *et al*. 2009). Expression of chickpea *CAP2* cDNA in yeast also enhanced heat tolerance, with increased expression of the gene for heat shock factor 1 (Hsf1) and its target yeast heat shock protein 104 (Hsp 104) suggesting strong evolutionary conservation of the stress response mechanisms (Shukla *et al.* 2009).

The role of *SbDREB2A* from *Salicornia brachiata* in abiotic stress was studied in *E. coli* BL21 (DE3). The recombinant *E. coli* cells exhibited better growth in basal LB medium as well as in LB supplemented with NaCl, PEG and mannitol probably due to the regulation of stress-regulated functional genes and/or interactions with transcriptional network in the bacterial cells (Gupta *et al.* 2010). *EsDREB2B* from desert plant *Eremosparton songoricum* led to tolerance to osmotic, salt, heat, cold and oxidative stress in yeast and in transgenic tobacco (Li *et al*. 2014).

Table 2.2 Engineering stress-tolerance through overexpression of *DREB2*-type genes.

DREB2-type gene	Transgenic plant	Stress tolerance	Reference
AtDREB2A	*Arabidopsis*	None	Liu *et al*. 1998
AtDREB2A-CA	*Arabidopsis*	Drought	Sakuma *et al*. 2006
AtDREB2C	*Arabidopsis*	Heat	Lim *et al*. 2007
OsDREB2A	*Arabidopsis*	None	Dubouzet *et al*. 2003
OsDREB2B	*Arabidopsis*	Drought and heat	Matsukura *et al*. 2010
ZmDREB2A	*Arabidopsis*	Drought and heat	Qin *et al*. 2007
PgDREB2A	Tobacco	Ionic stress	Agarwal *et al*. 2010
AhDREB1	Wheat	Drought, salt	Shen *et al*. 2003a
GmDREB	*Arabidopsis*	Freezing	Shiqing *et al*. 2005
GhDREB1	Tobacco	Drought, salinity and freezing	Shan *et al*. 2007
PpDBF1	Tobacco	Drought and salinity	Liu *et al*. 2007
CAP2	Tobacco	Salinity	Shukla *et al*. 2006
PeDREB2	*Arabidopsis*	Salinity	Chen *et al*. 2009
HvDREB1	*Arabidopsis*	Drought	Xu *et al*. 2009
EsDREB2B	Tobacco	Salt, heat, cold, oxidative	Li *et al*. 2014

2.3.3.2 Marker assisted selection

Conventional crop breeding for introgression of drought adaptive traits into desired cultivars is hugely accelerated by marker-assisted selection (MAS). With the assistance of molecular markers it is easier to trace valuable alleles either in segregating or natural populations. Of the DNA markers, the most powerful and stable ones are the single nucleotide polymorphisms (SNPs). Research efforts have been directed towards discovering SNPs associated with *DREB2* genes. A SNP associated with dehydration tolerance was detected at the 558th base pair (an A/G transition) in the *SiDREB2* gene in a core set of 45 foxtail millet accessions used. Based on the identified SNP, three primers were designed to develop an allele specific marker (ASM) for dehydration tolerance. The ASM produced a 261 bp fragment in all the tolerant accessions and produced no amplification in the sensitive accessions (Lata *et al*. 2011a). With the help of this marker, dehydration-tolerant cultivars could be selected in foxtail millet. In other studies, eight

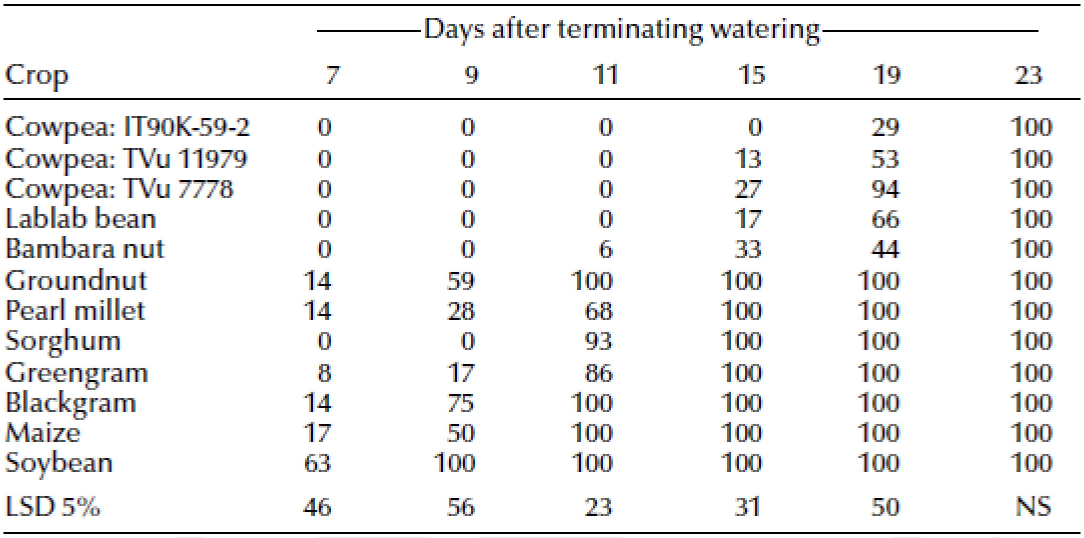
SNPs were found in the common bean *DREB2A*, indicating two distinct haplotypes, whereas a single SNP was observed in chickpea *DREB2A* (Nayak *et al.* 2009).

2.4 Cowpea, a drought-tolerant legume

Cowpea is one of the most ancient crops, originated and domesticated in prehistoric Africa, along with sorghum and pearl millet. It is widely grown in different parts of the world for both food and feed purposes e.g. dry beans are used as grains in West Africa, India, and the USA, as a green vegetable in East Africa and as a fodder crop for livestock in Australia. The grains are rich in proteins (25 %), several vitamins and minerals. An estimated 14.5 million hectares of land is used for cowpea cultivation each year worldwide (CGIAR). Global production of dried cowpeas in 2010 was 5.5 million metric tons (7.56 million tons on 17.76 million hectares, according to FAO estimates). Africa was responsible for 94 % of total world production. Nigeria is the largest producer and consumer of cowpea, producing 2.2 million metric tons of dried grain in 2010. Cowpeas are grown under a wide range of climatic conditions ranging from semiarid to subhumid as a dryland crop and are reported to have good tolerance to both heat and drought (Agbicodo *et al.* 2009).

The relative drought tolerance of 10 major crops grown in the semiarid tropics was compared by Singh *et al* (1999). The overall ranking of the crops in the increasing order of drought tolerance was soybean followed by blackgram, greengram, groundnut, maize, sorghum, millet, bambara nut, lablab, and cowpea.

Table 2.3 Relative drought tolerance of different crops in the semiarid tropics. Proportion of dead plants at different intervals after termination of watering (Source: Singh *et al*. 1999).



	Days after terminating watering					
Crop	7	9	11	15	19	23
Cowpea: IT90K-59-2	0	0	0	0	29	100
Cowpea: TVu 11979	0	0	0	13	53	100
Cowpea: TVu 7778	0	0	0	27	94	100
Lablab bean	0	0	0	17	66	100
Bambara nut	0	0	6	33	44	100
Groundnut	14	59	100	100	100	100
Pearl millet	14	28	68	100	100	100
Sorghum	0	0	93	100	100	100
Greengram	8	17	86	100	100	100
Blackgram	14	75	100	100	100	100
Maize	17	50	100	100	100	100
Soybean	63	100	100	100	100	100
LSD 5%	46	56	23	31	50	NS

Pusa Komal, an Indian cowpea variety released by the Indian Agricultural Research Institute, Pusa, New Delhi, is a product of crosses between photoinsensitive line P85-2 and photosensitive line P426, was tested at 9 sites throughout India during 1977 to 1984 and released in 1986. It had a higher green pod yield (up to 17.2 t/ha) than the controls Pusa Dofasli and Pusa Barsati. It was resistant to bacterial blight (*Xanthomonas* [*campestris* pv.] *vignicola*) under artificial testings, flowered early and bore good quality pods. This cultivar has been classified as drought-tolerant by the Department of Agriculture and Co-operation, Ministry of Agriculture, Govt. of India (Contingency Agriculture Plan for Kharif 2010).

2.5 Mechanisms of drought tolerance and avoidance in cowpea

In cowpea, drought tolerance at the seedling stage has been described to be of 2 types (Mai-Kodomi *et al*. 1999) indicating evolution of different mechanisms in different cowpea genotypes to cope with prolonged drought encountered in the semi-arid regions, the places of their origin. In the type 1 drought tolerant cultivars (e. g. TVu 11986 and TVu 11979) growth stops after the onset of drought stress and turgidity is maintained uniformly at the beginning, then declines gradually in all plant parts over 2 weeks. The type 2 drought-tolerant cultivars (e. g. Dan Ila and Kanannado) remains green for a longer time and continues slow growth of the trifoliates under drought stress, while the lower unifoliates wither out fast. With continued moisture stress for about 4 weeks, however plants die. Closure of stomata to reduce transpirational water loss and reduced growth rate seems to be the mechanism for type 1 drought avoidance (Lawan 1983) while type 2 employ three mechanisms; stomatal regulation (partial opening), osmotic control and selective mobilization of photosynthetic reserves between upper and lower leaves (Mai-Kodomi *et al*. 1999). Muchero *et al* (2008) found significant genetic variation in response to drought stress in 14 genotypes of cowpea at the seedling stage. Genotypes, IT93 K-503-1and IT98 K-499-39 were found to be the most tolerant whereas CB46 and Bambey 21 were most susceptible. The more productive genotypes of cowpea have a higher photosynthesis rate due to higher internal carbon dioxide concentration in their leaves, determined by measuring carbon isotope discrimination (Hall *et al*. 1997; Condon and Hall 1997). This was probably due to more open stomata in cowpea leading to higher rates of photosynthesis or beneficial effects on the plant from greater evaporative cooling (Lu *et al*. 1998). Cowpea resists vegetative-stage drought by its characteristic limited decrease of leaf water potential even under extreme drought. The lowest leaf water potential recorded for cowpea is -18 bar (-1.8 MPa) (Turk and Hall 1980). Another drought avoidance mechanism in cowpea is the orientation of leaflets parallel to the sun’s rays when subjected to soil drought, causing them to be cooler and thus transpire less, minimizing water loss (Shackel and Hall 1979).

2.6 Studies on drought tolerance genes in cowpea

Cowpea has been proposed to be an ideal model to study the molecular mechanisms of drought tolerance in crops for its natural drought-tolerance and relatively small nuclear genome size (estimated at ~620 Mb) (Arumuganathan and Earle 1991). Various attempts have been made to understand the molecular mechanisms of drought tolerance in cowpea. Recently, Muchero *et al* (2009) reported the mapping of 12 quantitative trait loci (QTL) associated with seedling drought tolerance and maturity in a cowpea recombinant inbred (RIL) population. 127 F8 RILs developed from a cross between varieties IT93K503-1 and CB46 were screened through amplified fragment length polymorphisms (AFLPs). The population was phenotyped for maintenance of stem greenness and recovery dry weight after drought stress, in greenhouse, and drought stress-induced premature senescence in field experiments. Differential screening of mRNA has been used to isolate several cowpea candidate genes responsive to dehydration stress. Genes coding for protease inhibitors, enzymes responsible for degradation of membrane lipids, ROS scavenging enzymes and enzymes involved in ABA biosynthesis have been cloned from cowpea under water stress conditions (Table 2.4). Cowpea orthologs of these genes showed differential regulation under stress in cultivars contrasting in drought tolerance.

One of the adaptive mechanisms for drought stress is the employment of protease inhibitors (e.g. cystatin) for the regulation of protein degradation. Diop *et al* (2004) employed Northern and Western blot analysis to study expression of leaf cystatins in cowpea cultivars subjected to controlled drought stress and exogenous ABA treatment. Two cystatin transcripts were found in the leaves of stressed plants, which translated into two polypeptides. The polypeptide with the lowest molecular weight was identified to be the deduced polypeptide of the cowpea cystatin, *VuC1* cDNA. In cowpea seeds, other minor cystatin-like polypeptides were also identified (Flores *et al*. 2001). This multiplicity of forms in cowpea cystatins was supposedly related to multiple biological roles.

Severe drought is associated with a massive degradation of membrane lipids (Monteiro de Paula *et al*. 1993). El-Maarouf *et al* (1999) found that Phospholipase D (VuPLD1), the main enzyme responsible for the drought-induced degradation of membrane phospholipids, was highly induced by drought stress in a susceptible cowpea cultivar (1,183) but remained almost unchanged in a tolerant cultivar (EPAT-1). Similar mode of regulation was observed in case of *VuPAT1*, a putative patatin-like gene encoding for galactolipid acyl hydrolase, which degrades the chloroplast membrane under drought stress (Matos *et al*. 2001). The susceptible cultivar (1,183) showed a rapid accumulation of *VuPAT1* at mild drought stress while the tolerant (EPAT-1) maintained lower levels of transcripts. Phosphatidate phosphatases (PAPs) find place in the enzymatic cascade leading to membrane lipid degradation under stress (Todd *et al*. 1992, Sahsah *et al*. 1998). Of the identified cowpea orthologs, *VuPAP-a* transcripts remained very low during drought treatment, but was strongly induced on rehydration; while expression of *VuPAP-b* did not change in plants subjected to water stress, but increased rapidly in air desiccated leaves (Marcel *et al*. 2000).

Stress conditions induce the production of ROS like H_2O_2 mainly in the chloroplasts and mitochondria which cause major cell damage. Enzymes like glutathione reductase (GR) and ascorbate peroxidase (APX) play key roles in scavenging and detoxifying the ROS to prevent cell damage. cDNAs encoding dual-targeted (dtGR) and cytosolic GR (cGR) were cloned from cowpea (Contour-Ansel *et al*. 2006). In both drought tolerant (EPACE-1) and sensitive (1,183) cowpea cultivars the cGR showed up-regulation under drought, whereas the dtGR was down-regulated under progressive drought treatment in the tolerant cultivar but up-regulated in the sensitive one. Suppression subtractive hybridization (SSH) on a drought tolerant (IT96D-602) and a susceptible (TVu7778) cowpea cultivar was used to clone genes encoding glutathione-S-transferase (GST) and pathogenesis related protein-1 (PR-1) (Gazendam and Oelofse 2007). D’Arcy-Lameta *et al* (2006) isolated four cowpea cDNAs encoding putative cytosolic (VucAPX), peroxisomal (VupAPX), chloroplastic (stromatic VusAPX) and thylakoidal (VutAPX) ascorbate peroxidases. Transcript levels of VucAPX and VupAPX increased after 2 h of ABA treatment and after 30 min of desiccation in sensitive cultivar 1,183, while in tolerant cultivar EPACE-1 no changes were observed in the same isoforms. The chloroplastic isoforms were strongly induced in the tolerant cultivar even at low levels of stress whereas in the sensitive one, they were stimulated much later, at severe water deficits.

Several cDNAs were cloned from drought-tolerant cowpea variety IT84S-2246-4 by a differential screening method, representing ten genes collectively named CPRD (cowpea clones responsive to dehydration) (Iuchi *et al*. 1996a). Nine of these genes were induced by drought, of which five (CPRD8, CPRD14, CPRD22, CPRD12 and CPRD46) were further characterized. These coded for old yellow enzyme, group 2 LEA proteins, dihydroflavonol-4-reductase (DFR) and vestitone reductase (VR). The CPRDI2 and CPRD46 cDNAs encoded putative proteins related to nonmetallo-short-chain alcohol dehydrogenase (CPRD12) and chloroplastic lipoxygenase (CPRD46) (Iuchi *et al.* 1996b). These genes were induced by dehydration, high-salinity stress and exogenous abscisic acid, but not by cold stress. The CPRD46 gene was also found to be responsive to heat stress, methyl jasmonate and salicylic acid. Another gene, *VuNCED1* was cloned from the same variety which encoded a 9-cis-epoxycarotenoid dioxygenase that catalyzes the key step in ABA biosynthesis (Iuchi *et al*. 2000). Expression of *VuNCED1* was strongly induced by drought stress in cowpea and its ectopic expression conferred drought resistance in transgenic *Arabidopsis* (Iuchi *et al*. 2001). Restriction fragment length polymorphism (RFLP) markers linked to twelve known drought responsive cDNAs in cowpea were correlated with seedling drought tolerance phenotypes (Muchero *et al*. 2008). Putative fragments generated from CPRD12, CPRD46, galactolipid acylhydrolase, phospholipase D, and 9-cis-epoxycarotenoiddioxygenase showed strong correlations with drought related phenotypes.

Forty four drought-associated micro RNAs (miRNAs) were identified from two cowpea genotypes (CB46, drought-sensitive, and IT93K503-1, drought-tolerant) by deep-sequencing, of which thirty were upregulated in drought condition and fourteen were down-regulated (Barrera-Figueroa *et al*. 2011). Among these, nine miRNAs were predominantly or expressed only in one of the two genotypes while eleven were drought-regulated in only one genotype, but not in the other.

The final validation of a candidate gene is accepted only by physiological analyses, and genetic transformation. Though the genes isolated from cowpea (discussed above) were found to be inducible in drought stress, however, clear evidence that these genes affect drought tolerance through transgenic analyses has not been reported so far (with the exception of the *VuNCED1* gene). So, there lies immense possibility of discovering other candidate genes inferred from studies in related crops and model plants. An interesting group of genes are transcription factors that are involved in the drought response including MYB, WRKY, ERF and DREB genes. Sequencing and analysis of the gene-rich, hypomethylated areas of the cowpea genome has been reported (Timko *et al*. 2008) generating over 250,000 gene-space sequence reads (GSRs). Sixty-two out of sixty four well characterized plant transcription factor gene families are represented in the cowpea GSRs again pointing to the importance of such master regulators in this plant.

Table 2.4 Drought tolerance genes identified from cowpea.

Symbol	Accession	Gene	Function	Reference
VuC1	AF278573	Cystatin	Regulation of protein degradation during stress	Diop *et al.* 2004
VuPLD1	U92656	Phospholipase-D	Drought-induced degradation of membrane phospholipids	El-Maarouf *et al.* 1999
VuPAT1	AF193067	Patatin (galactolipid acyl hydrolase)	Degradation of the chloroplast membrane under drought stress	Matos *et al.* 2001
VuPAP	AF165891, AF171230	Phosphatidate phosphatase	Membrane lipid degradation under stress	Marcel *et al.* 2000
dtGR, *cGR*	DQ267474, DQ267475	Dual-targeted and cytosolic glutathione reductase	Scavenging and detoxifying ROS under stress	Contour-Ansel *et al.* 2006
VuGST, *VuPR-1*		Glutathione-S-transferase and pathogenesis related protein-1	Scavenging and detoxifying ROS under stress	Gazendam and Oelofse 2007
VucAPX, *VupAPX*, *VusAPX*, *VutAPX*	U61379, AY466858, AY484493, AY484492	Cytosolic, peroxisomal, chloroplastic, stromatic and thylakoidal ascorbate peroxidase	Scavenging and detoxifying ROS under stress	D'Arcy-Lameta *et al.* 2006
CPRD8, 14, 22	D83970, D83971, D83972	Cowpea clones responsive to dehydration	Dehydration response	Iuchi *et al.* 1996a
CPRD12	D88121	Nonmetallo-short-chain alcohol dehydrogenase	Dehydration response	Iuchi *et al.* 1996b
CPRD46	D88122	Chloroplastic lipoxygenase	ABA biosynthesis	Iuchi *et al.* 1996b
VuABA1	AB030295	Zeaxanthin epoxidase	ABA biosynthesis	Iuchi *et al.* 2000
VuNCED1	AB030293	9-*Cis*-epoxycarotenoid dioxygenase	ABA biosynthesis	Iuchi *et al.* 2000

CHAPTER 3

MATERIALS AND METHOS

3.1 PCR-based cloning of the *VuDREB2A* gene

3.1.1 Cowpea growth conditions and stress treatment

Seeds of cowpea (*Vigna unguiculata* L. Walp) cultivar Pusa Komal procured from National Seeds Corporation, New Delhi were used in this study. Cowpea seeds were sown on a nylon mesh over modified MGRL media (Fujiwara *et al*. 1992) (1/50 strength with inorganic phosphate eliminated and calcium concentration adjusted to 200 µM, pH 5.5; Appendix Table A5) under hydroponics at 25 °C under a 12 h day (with photon flux density of 30 µmol m^{-2} s^{-1})/12 h night cycle. One week old cowpea seedlings were treated for 6 h by dipping the roots in 200 mM NaCl.

3.1.2 RNA isolation from cowpea and RT-PCR

Total RNA was isolated from salt-stressed cowpea shoots by RNAqueous® Kit (Ambion, USA) following manufacturer's instructions. Five µg of total RNA was used for one cDNA synthesis reaction using the First strand cDNA synthesis kit (Fermentas, Hanover, MD, USA) following manufacturer's instructions.

3.1.3 Degenerate oligonucleotide-primed PCR

Reported *DREB2* sequences of legumes in NCBI (www.ncbi.nlm.nih.gov) were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). From the conserved regions, degenerate primers were designed (cf. Fig. 4.1; Appendix Table A7). Using 400 ng cDNA as template, 200 nM primers, 200 µM each of dATP, dGTP & dCTP, 100 µM [^{3}H]-dTTP and 1.25 U TaKaRa ExTaq™ DNA polymerase, a 50 µL PCR was carried out under conditions: 94 °C, 5 min, 1 cycle; 94 °C, 30 s; 59 °C, 30 s and 72 °C, 30 s, 28 cycles; 72 °C, 10 min 1 cycle. Gels were visualized using the Gel Doc™ XR+ Imaging system (Bio-Rad Laboratories, Hercules, CA, USA). The amplified product was purified from agarose gel and sequenced at a commercially available facility at Delhi University South Campus, New Delhi, India. The 386 bp sequence was subjected to NCBI BLAST search (www.ncbi.nlm.nih.gov/BLAST) for assessment of homology with reported *DREB2* genes.

3.1.4 Rapid Amplification of cDNA Ends (RACE)

RACE experiments were conducted using 5’ and 3’ RACE systems version 2.0 (Invitrogen, USA) following manufacturer’s instructions. The 386 bp partial *VuDREB2A* sequence was used for design of gene-specific primers (GSP). The first strand cDNA was synthesized using reverse 5'RACE_GSP1. The purified poly dC-tailed first -strand cDNA was used as template for PCR amplification with reverse 5'RACE_GSP2 and kit provided Abridged Anchored Primer using an annealing temperature (Ta) of 63 °C. Hundred times diluted product of the previous reaction was used for subsequent PCR amplification with nested reverse 5'RACE_GSP3 and Abridged Universal Amplification Primer (AUAP) of the kit at Ta of 60 °C. The amplification product was purified from agarose gel and sequenced. For 3’ RACE, cDNA was synthesized using the Adaptor Primer (AP) of the kit. The first 3’ RACE PCR was carried out using AUAP and forward 3'RACE_GSP1 at a Ta of 60 °C. The product was diluted 100 times and used for the second PCR reaction with forward 3'RACE_GSP2 and AUAP at a Ta of 61 °C. The amplfied products of 3’ and 5’ RACE were purified from agarose gel and sequenced. These sequences were used for design of primers at the ends *VuDREB2A*_FL_1 and *VuDREB2A*_FL_2. The full length *VuDREB2A* sequence was obtained by PCR from cowpea cDNA with 200 nM each of primers *VuDREB2A*_FL_1 and *VuDREB2A*_FL_2, TaKaRa Primestar Max^{TM} DNA polymerase (having proofreading activity) under conditions: 94 °C, 5 min for 1 cycle; 94 °C, 1 min; 55 °C, 0.5 min and 72 °C, 1.5 min for 28 cycles. Primer sequences are provided in Appendix Table A7.

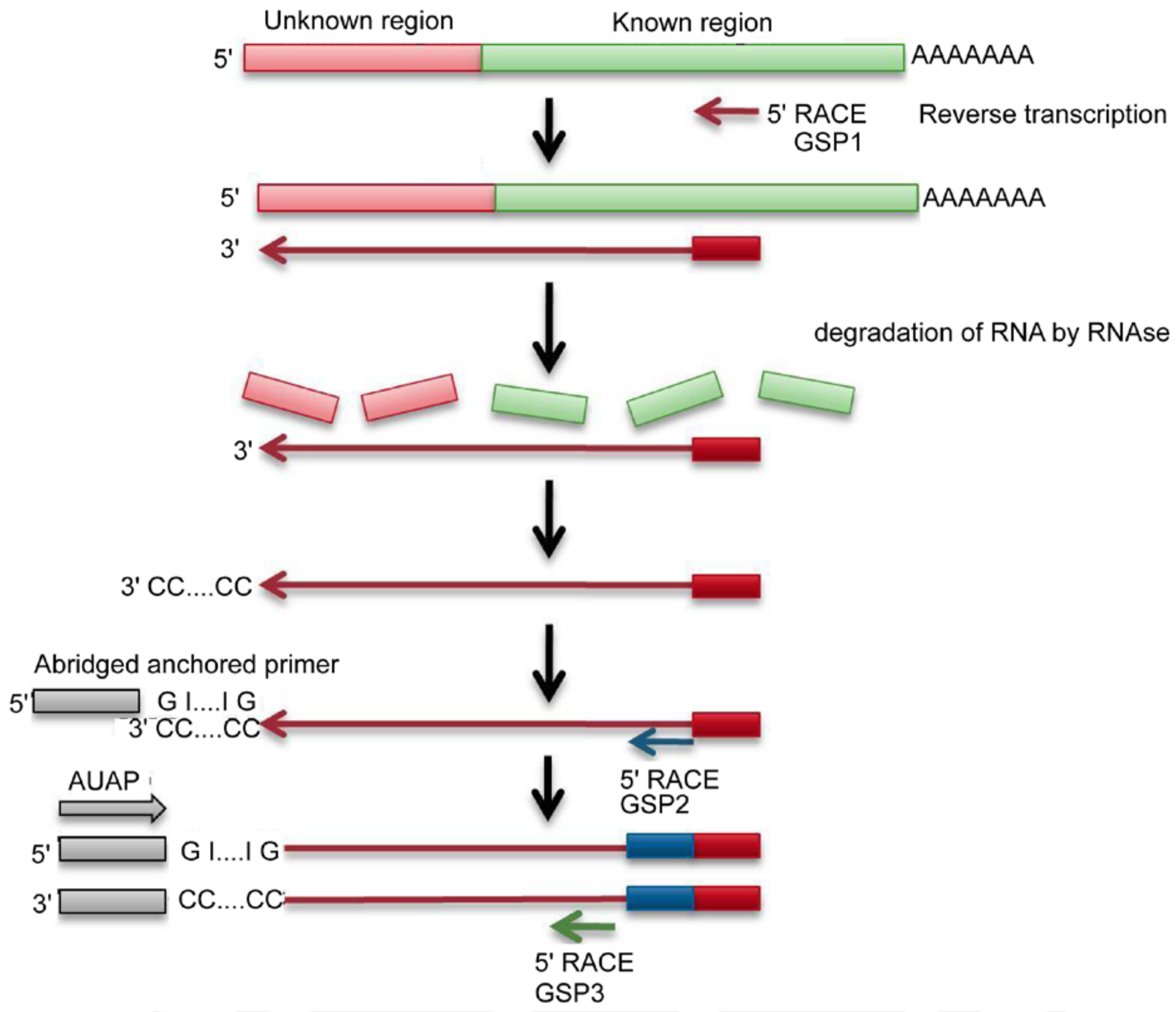


Figure 3.1 Scheme for 5’ RACE of *VuDREB2A* (adapted from Invitrogen 5’ RACE system user manual).

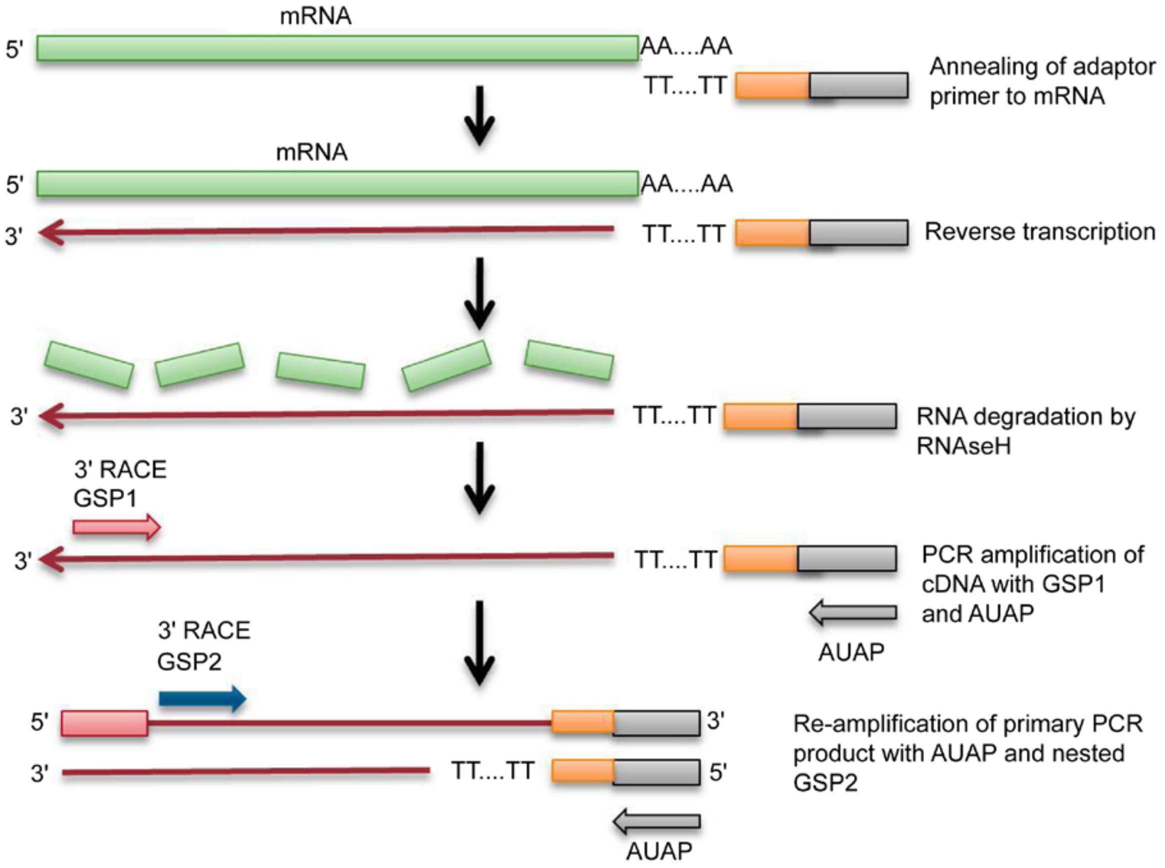


Figure 3.2 Scheme for 3' RACE of *VuDREB2A* (adapted from Invitrogen 3' RACE system user manual).

3.1.5 Genomic DNA isolation and PCR

Genomic DNA was extracted from 500 mg of young leaves of cowpea by the cetyl trimethyl ammonium bromide (CTAB) method (Roger and Bendich 1988). The primers *VuDREB2A*_FL_1 and *VuDREB2A*_FL_2 were used for amplifying *VuDREB2A* from cowpea genomic DNA under similar conditions as that from cDNA. Primer sequences are provided in Appendix Table A7.

3.1.6 *In silico* analysis of the *VuDREB2A* gene

The nucleotide sequence of VuDREB2A was conceptually translated using the translate tool from ExPASy (http://web.expasy.org/translate/). SMART program (http://smart.embl-heidelberg.de) was used for detection of potential protein domains. Multiple amino acid alignment was performed by using CLUSTALW (Larkin *et al*. 2007) and GENETYX ver. 11 (http://www.genetyx.co.jp/) softwares. Protein secondary structures were predicted by PSSpred (Protein Secondary Structure prediction) server (http://zhanglab.ccmb.med.umich.edu/PSSpred/). Phylogenetic tree was constructed using Molecular Evolutionary Genetic Analysis (MEGA) v6.0.5 (Tamura *et al*. 2013). The distances between branches were calculated by the neighbor-joining method based on the Jones-Taylor-Thornton model (Jones, Taylor and Thornton 1992) with 1,000 bootstrap replicates. The phosphorylation sites within the serine/threonine-rich region of VuDREB2A were predicted by NetPhosK1.0 Server (http://www.cbs.dtu.dk/services/NetPhosK/). PEST sequences were predicted using ePestfind program (http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind).

3.2 Expression analysis of *VuDREB2A* and cloning of the *VuDREB2A* promoter

3.2.1 Stress treatments of cowpea

One-week-old cowpea plants grown hydroponically in modified MGRL medium (Fujiwara *et al*. 1992) (1/50 strength with inorganic phosphate eliminated and calcium concentration adjusted to 200 µM; Appendix Table A5) were given the following treatments: for salinity stress, plants were dipped in 250 mM NaCl in modified MGRL medium and kept at 25 °C with a 12 h photoperiod; for desiccation stress, plant roots were wrapped dry in tissue paper and kept at 25 °C with a 12 h photoperiod; for heat stress, plants were kept in an incubator maintained at 50 °C with a 12 h photoperiod; for cold stress, plants were kept at 4 °C in a refrigerator under dim light; ABA, methyl jasmonate and salicylic acid treatments were given both by dipping roots in and spraying shoots with 100 µM (+)-*cis*, *trans*-ABA, 100 µM (in 0.1 % v/v ethanol) methyl jasmonate (MeJA) and 500 µM salicylic acid (SA), respectively. For the salicylic acid treatment plants were kept at 25 °C in the dark. Other phytohormone treatments were carried out at 25 °C with a 12h photoperiod. To avoid the effect of volatile MeJA on plants subjected to other treatments, MeJA treatment was performed in a separate growth chamber under identical conditions. At different time points, stressed cowpea tissues were immediately frozen in liquid nitrogen and stored in -80 °C before RNA extraction.

3.2.2 RNA isolation from cowpea and qRT-PCR

RNA was isolated and cDNA prepared as described in 3.1.2. Real-time PCR was carried out using Takara SYBR Green PCR master mix II and a Takara Thermal Cycler Dice Real time system II (Takara-Bio, Japan). Gene-specific primers for real-time PCR analyses were designed using the Primer3, version 4.0 software (Koressar *et al.* 2007). Primer sequences are provided in Appendix Table A7. All qRT-PCR quantifications were performed by the standard curve method using a cDNA dilution series (Bustin *et al.* 2009), and transcript levels of particular genes were relatively quantified using an internal standard, the homolog of the *UBQ1* gene in cowpea (GenBank accession: FG859491). All RNA samples were estimated using Nanovue (GE Healthcare Bio-sciences Corp., Piscataway, NJ, USA). Graphs were generated by Sigmaplot 10 (Systat Software, San Jose, CA, USA) and Microsoft Excel (Microsoft, Tokyo, Japan).

3.2.3 Inverse PCR

Five µg of cowpea genomic DNA was digested with *Nde*I for 12 h at 37 °C, the product extracted with phenol-chloroform, precipitated using isopropanol and resuspended in TE buffer. Approximately 0.5 µg of digested DNA was ligated in a total volume of 500 µl O/N with 5 µl T4 DNA ligase at 15 °C. A PCR reaction was carried out using 5 µl of the ligation reaction and 200 pmol of each primer in a final volume of 25 µl using the primers F1 and R1. Hundred times diluted product of the above reaction was used as a template for nested amplification using the flowing set of primers F2 and R2. Primer sequences are provided in Appendix Table A7. The resultant 1.9 kb product was cloned in TA vector and sequenced.

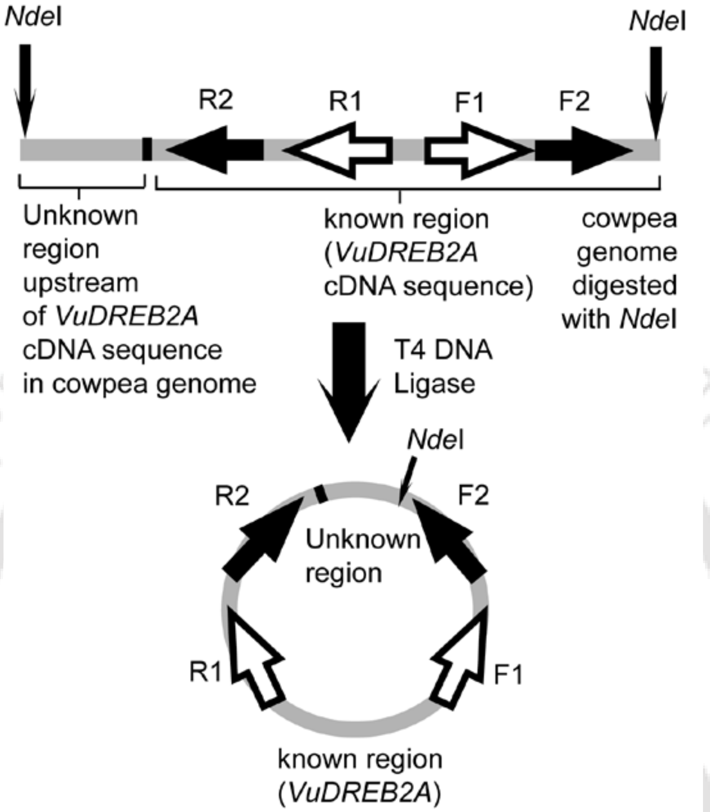


Figure 3.3 Scheme of inverse PCR for isolation of the *VuDREB2A* promoter. The cowpea genome was digested with *Nde*I which had a single recognition site inside the known region (cDNA sequence) of the *VuDREB2A* gene. The primers F1, F2, R1, R2 were designed inside the known region as shown in figure. The *Nde*I-digested genomic DNA fragment was then ligated to form a circular structure as shown in figure, which was used as template for PCR with F1 and R1, and another nested PCR with F2 and R2 (Appendix Table A7) to amplify the unknown region upstream of the *VuDREB2A* cDNA sequence in the cowpea genome.

3.2.4 *In silico* analysis of the *VuDREB2A* promoter

The transcription start site (TSS) of the VuDREB2A promoter was predicted using the TSSP web-program (http://linux1.softberry.com/berry.phtml?topic=tssp&group=programs&subgroup=promoter) (Shahmuradov *et al*. 2003).

The *VuDREB2A* promoter was scanned in 1 bp steps for presence of 8 nucleotide stretches (octamers) which were overrepresented in promoters of stress-regulated genes in *Arabidopsis*. Promoter sequences of *Arabidopsis* genes, which were > 3 fold up-regulated in the following treatments: 3 h ABA (643 genes); 3 h cold (327 genes); 3 h osmotic stress (327 genes); 1 h dehydration stress (294 genes) and 1 h H_2O_2 (329 genes) were used for the calculation. Promoter sequence from -1000 to -1 relative to the major transcription start site of each gene, determined by large scale TSS-tag sequencing (Yamamoto *et al.* 2009) was taken into consideration. A relative appearance ratio (RAR) was calculated for each octamer occurring in the *VuDREB2A* promoter, which was defined as follows:

Frequency of the octamer in the promoters of a set of stress-inducible genes (ABA; 645 genes/ cold; 327genes/ osmotic stress; 327genes/dehydration; 294 genes/H_2O_2; 329 genes)

RAR= --

Frequency of the octamer in the promoters of total 24956 *Arabidopsis* genes

Home-made Excel macros (Microsoft) following the method developed by Yamamoto *et al* (2011) were used for the calculations. The results were subjected to a two-sided Fischer's exact test. The RAR values were plotted against the promoter positions (Figure 4.10) to indicate locations of overrepresented octamers. The PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) web-programs were also used to locate *cis*-acting motifs within the promoter.

3.3 Assessment of DNA-binding ability of VuDREB2A

3.3.1 Cloning of *VuDREB2A* in bacterial expression vector

VuDREB2A was amplified using proofreading Takara Primestar Max DNA polymerase (Clonetech Laboratories Inc.) and cloned in pET28a expression vector (Novagen, USA; vector map in Appendix) between *Bam*HI and *Xho*I sites. The positive clones were sequenced.

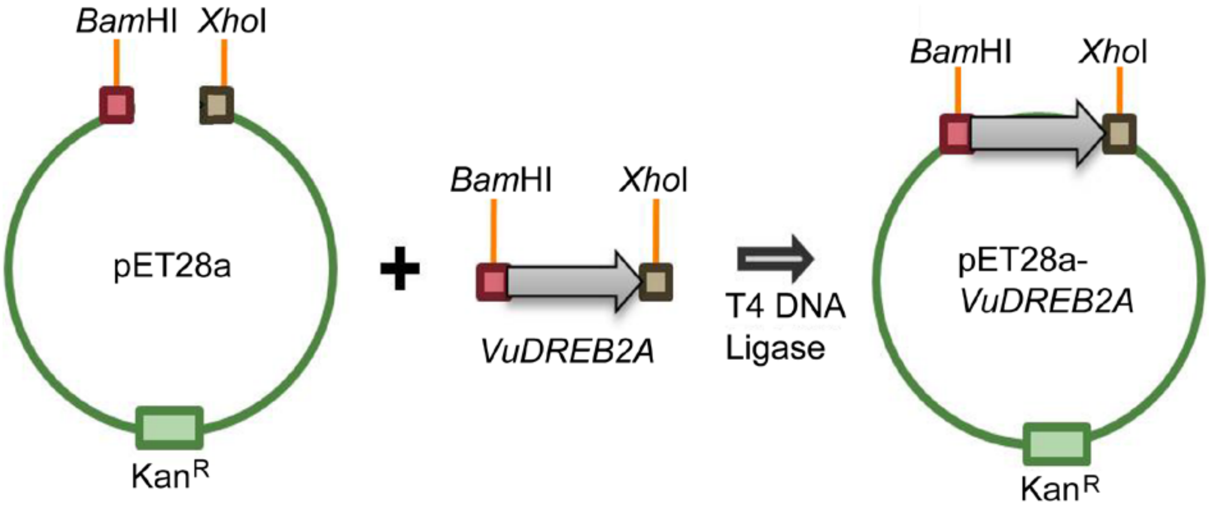


Figure 3.4 Scheme for cloning of *VuDREB2A* into bacterial expression vector. *VuDREB2A* ORF (without the stop codon) was first PCR amplified from cDNA using primers having *Bam*HI and *Xho*I sites at the ends, digested by *Bam*HI and *Xho*I and ligated with bacterial expression vector (pET28a) also digested by the same enzymes. Kan^R: kanamycin resistance gene.

3.3.2 Bacterial expression strains and mobilization

The positive pET28a clones carrying *VuDREB2A* were mobilized to chemically competent *E. coli* BL21 (DE3) cells by 42 °C heat shock for 90 s and subsequent cooling on ice for 30 min (Chung *et al.* 1989).

3.3.3 Induction and purification of recombinant VuDREB2A protein

Expression of recombinant protein was induced in *E. coli* BL21 (DE3) cells with 1 mM isopropyl-ß-D-1-thiogalactoside at 28 °C for 12 hours with shaking (150 rpm). *E. coli* cells were pelleted down by centrifugation at 5000 rpm for 5 min and resuspended in lysis buffer and lysed by sonication using UP200S Ultrasonic processor (Hielscher Ultrasound Technology, Germany) at amplitude of 25 % and cycles of 0.5 s for 5 min. The protein was purified under native conditions using Ni–NTA purification column (GE Healthcare Bio-sciences Corp.) following manufacturer’s instructions. The purity of the proteins was evaluated by SDS-PAGE following Laemmli (1970). Western-blotting analysis was performed following Towbin *et al* (1979) using anti-His×6 mouse monoclonal antibody and horse radish peroxidase-conjugated goat anti-mouse IgG (Abexome Biosciences, Bangalore, India), a Hybond™-P hydrophobic polyvinylidene difluoride (PVDF) membrane (GE Healthcare Bio-sciences Corp.) and a TE77 semi-dry transfer unit (GE Healthcare Bio-sciences Corp.) following the manufacturer’s instructions. All buffer compositions are provided in Appendix Table A6.

3.3.4 Electrophoretic Mobility Shift Assay (EMSA)

For EMSA, the native DRE probes were designed from the *Arabidopsis* rd29A promoter as reported earlier (Agarwal *et al.* 2007) with mutations introduced at various regions in the core motif CCGAC for testing specificity of binding (Fig. 4.12). The EMSA was carried out using EMSA kit (Invitrogen) following manufacturer’s instructions with modified buffer (Agarwal *et al*. 2007) in a reaction volume of 20 µL. Protein-DNA complexes were fractionated on a 12 % polyacrylamide gel with 0.5X TBE buffer containing 5 % glycerol. Densitometric analysis from gel images was performed by the ImageJ software (Schneider *et al*. 2012). Graphs were generated by Sigmaplot 10 (Systat Software).

3.4 Functional analysis of *VuDREB2A* in transgenic *Arabidopsis*

3.4.1 Megaprimer PCR deletion

The region in *VuDREB2A* between residues 394 and 546 was deleted using megaprimer PCR (Kammann *et al.* 1989; Ke and Madison 1997; Chen *et al.* 2009). The ‘megaprimer’ method uses three oligonucleotide primers and two rounds of PCR. One oligonucleotide is mutagenic and brackets the region to be deleted. The mutagenic primer and the forward primer are used in the first PCR to generate and amplify a megaprimer, which is subsequently used in the second PCR in conjunction with the reverse primer to amplify a longer segment without the Ser/Thr-rich region (Fig. 3.5). To generate the megaprimer, the first PCR was performed for 25 cycles of 94 °C for 40 s , 39 °C for 1 min and 72 °C for 1 min using the forward primer ∆394-546_Fw and the mutagenic reverse primer ∆394-546_mRv. The second PCR was performed for another 25 cycles of higher melting temperature at 60 °C after thermostable DNA polymerase and the reverse primer, ∆394-546_Rv, were added to the reaction tube of the first PCR. Primer sequences are provided in Appendix Table A7. The generated products were purified and ligated into TA vector (Fermentas) for sequencing to verify the deletion.

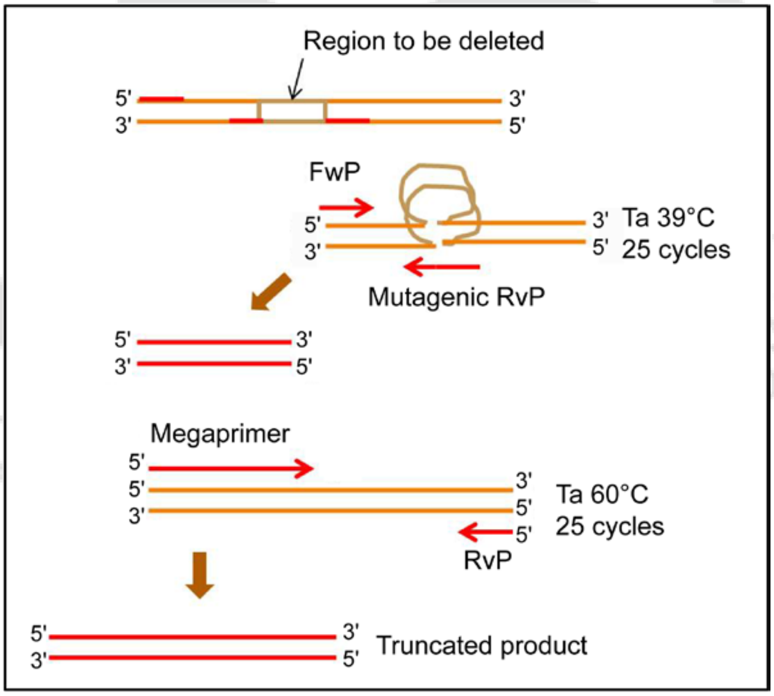


Figure 3.5 Scheme for megaprimer PCR deletion of the putative negative regulatory domain in VuDREB2A. The first step of the process involved 25 cycles of PCR at a low annealing temperature (Ta) of 39 °C with the forward primer (FwP) and a mutagenic reverse primer bracketing the region to be deleted. The amplified product of this reaction acted as a

'megaprimer' along with the reverse primer (RvP) for the next step PCR of 25 cycles at a higher annealing temperature (60 °C).

3.4.2 Preparation of *VuDREB2A* overexpression constructs

The *Eco*RI and *Bam*HI restriction sites flanking the full length and truncated (∆394-546, putative negative regulatory domain removed) ORF were used to subclone into a constitutive Cauliflower Mosaic Virus (CaMV) 35S promoter/CaMV strain Cabb B-D polyadenylation signal cassette, within the intermediate vector pRT101 (Topfer *et al*. 1987). Subsequently the CaMV35S promoter-*VuDREB2A*-35S terminator cassette was moved to the *Pst*I site of the T-DNA of binary vector pCAMBIA2301 (Fig. 3.6). Vector maps are provided in Appendix.

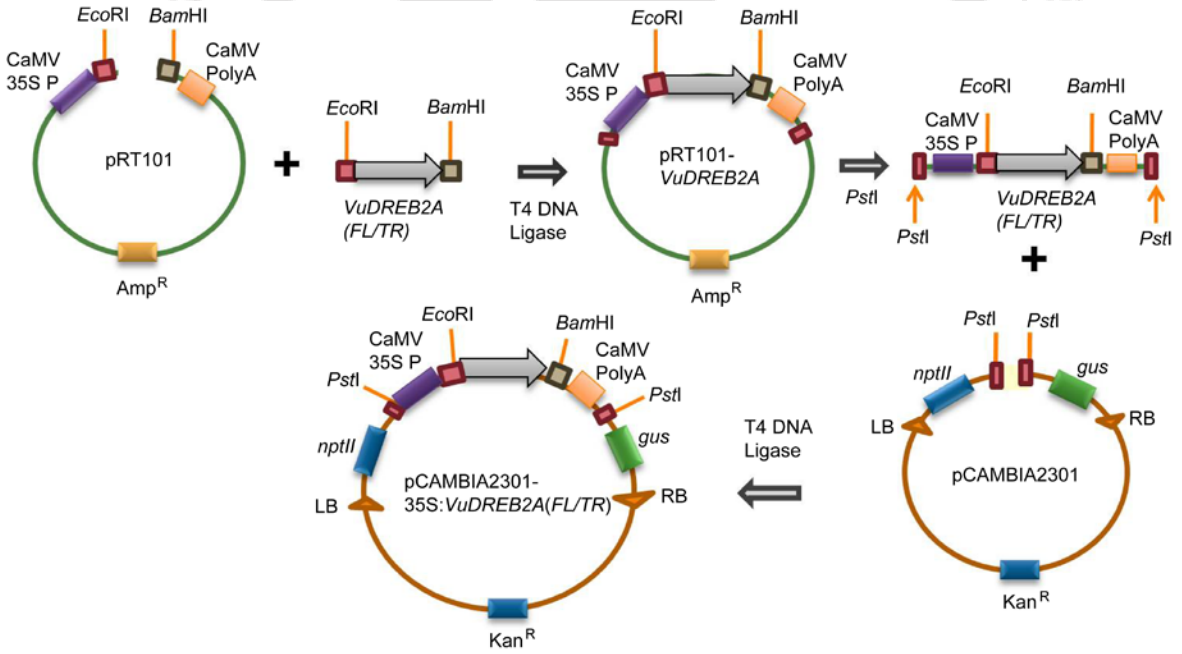


Figure 3.6 Scheme for constructing *VuDREB2A* overexpression vectors for plant transformation. The full length or truncated *VuDREB2A* ORF was first PCR amplified from cDNA using primers having *Eco*RI and *Bam*HI sites at the ends, digested by *Eco*RI and *Bam*HI and ligated with plant expression vector (pRT101) also digested by the same enzymes. The CaMV 35S promoter::*VuDREB2A*::35S polyA cassette was digested out of pRT101 by *Pst*I and ligated into pCAMBIA2031 also digested with *Pst*I. Amp^{R}: ampicillin resistance gene. Kan^{R}: kanamycin resistance gene. CaMV 35SP: Cauliflower mosaic virus 35S promoter; CaMV polyA: Cauliflower mosaic virus strain Cabb B-D polyadenylation signal; *nptII*: neomycin phosphotransferase; *gus*: β-glucuronidase.

3.4.3 *Agrobacterium* strain and floral dip transformation

Constructs were mobilized to *Agrobacterium tumefaciens* strain GV3101 by Electroporation (Nagel *et al.* 1990) and *Arabidopsis* transformation was carried out by the floral dip method (Clough and Bent 1998). The transformants were selected by growing in half strength MS (Murashige and Skoog 1962) medium with 1 % sucrose containing 50 µg/ml kanamycin.

3.4.4 *Arabidopsis* ecotype and growth conditions

Seeds of *Arabidopsis thaliana* L. Heynh ecotype Columbia (Col-0) obtained from RIKEN Bio Resource Centre, Tsukuba, Japan were used in this study. Both wild type and transgenic seeds were sown on ½ MS (Murashige and Skoog 1962) agar plates or soil containing perlite and plants were grown under under a 12 h day (with photon flux density of 30 µmol m^{-2} s^{-1})/12 h night cycle.

3.4.5 Drought- and heat-response test of *VuDREB2A* overexpressing *Arabidopsis*

Drought-response test of transgenic *Arabidopsis* were performed as described earlier (Iuchi *et al*. 2001). Precisely wild type and transgenic plants were grown in ½ MS media (Appendix Table A5) with 1 % sucrose for 15 days and shifted to soil in uniform sized pots, perforated at the bottom, placed on a tray with nutrient solution for 10 days. The solution was removed, and pots kept dry for 18 days. Survival rates were determined after 7 days of recovery following rewatering. Two independent lines of *35S: VuDREB2A full length* (*FL*) and a dwarf *35S:VuDREB2A truncated* (*TR*) line with 10 plants per line (5 in each pot) was used in the test. Wild type Col-0 was used as a control. Plants in pots subjected to progressive drought stress were weighed regularly at the same hour for calculation of water contents according to Vartanian *et al* (1994). Then pots were left to dry for a day in an 80 °C oven for calculation of dry weights. Water contents were calculated according to the formula (weight at day N – dry weight)/ (weight at day 1 – dry weight)*100. Water loss was due to evapo-transpiration and it was assumed that the increase in plant biomass was negligible compared with the water loss.

Heat-response test was carried out with the same transgenic lines. 7 days-old seedlings grown in ½ MS media were transferred to filter paper moistened with ½ MS nutrient solution and grown for additional 2 days. Then the seedlings were given a heat shock of 50 °C for 30 min. Survival rates were determined after 7 d of recovery growth at 22 °C.

3.4.6 RNA isolation from *Arabidopsis* and qRT-PCR

RNA was isolated from three week-old *Arabidopsis* as described earlier (Suzuki *et al.* 2004) and cDNA was prepared from the RNA using ReverTraAce® reverse transcriptase (Toyobo, Japan) following the manufacturer's instructions. The qRT-PCR was carried out by using Takara SYBR Green PCR master mix II and Takara Thermal Cycler Dice Real time system II. The gene-specific primers for real-time PCR analyses were designed using Primer3, version 4.0 (Koressaar and Remm 2007). All primer sequences are provided in Appendix Table A7. Graphs were generated and statistical analyses performed using Microsoft Excel (Microsoft Japan, Tokyo). All data were analyzed using a one-way analysis of variance (ANOVA), performed at the 95 % confidence level. A multiple comparison test (Tukey's test) was then used to compare the significant differences. The data shown are the mean values ± SE of three replicates, and the significance level relative to controls is $P < 0.05$.

3.4.7 Microarray and data analyses

Microarray experiment was performed by the competitive hybridization method using Agilent Microarray system following manufacturer's instructions. Two comparisons including one dye swap were made between biologically independent samples. The arrays were scanned with a G2505C scanner (software version 6.5) and processed using Feature extraction software (version

10.7.3.1). Data were analyzed with GeneSpring 12.6 (Agilent Technologies) and Genevestigator (Hruz *et al*. 2008). The significance of the expression changes was evaluated by the Student's *t*-test. The microarray raw data are available at the NCBI Gene Expression Omnibus with accession code GSE53113.

3.4.8 Promoter analyses of up-regulated genes in *VuDREB2A* overexpressing *Arabidopsis*

The frequency of each octamer sequence in the promoters of top 200 up-regulated genes in *35S:VuDREB2A* plants (determined through microarray) was compared with its frequency in the promoters of total 24956 *Arabidopsis* genes and a RAR was computed for each octamer.

$$\mathrm{RAR}=\frac{\text{Frequency of an octamer in the promoters of top 200 up-regulated genes in } \mathit{35S{:}VuDREB2A}\ (\mathit{FL/TR})\ \mathit{Arabidopsis}}{\text{Frequency of the octamer in the promoters of total 24956 } \mathit{Arabidopsis} \text{ genes}}$$

Those overrepresented octamers (RAR > 3 and $P < 0.05$) containing DRE core motif (CCGAC) or ABRE (ACGT) were tabulated (cf. Table 4.3). Calculations were performed using home-made Excel macros (Microsoft).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation and nomenclature of the *VuDREB2A* gene

4.1.1 Isolation of the *VuDREB2A* gene

A partial cDNA fragment (386 bp) was obtained (Fig. 4.1B) by PCR from 6 h salt-stressed cowpea cDNA using degenerate primers designed from highly conserved regions in legume DREB2-type proteins (Fig. 4.1A). The PCR product was cloned into the TA cloning vector (Fermentas) (Fig. 4.1C) and the insert fragment was confirmed by sequencing. The 386 bp sequence showed highest homology with the DREB2A sequences of model legume, Barrel Medic (*Medicago trancatula*) and pea (*Pisum sativum*) as well as with DREB2C-like (AP2 7 and 8) of soybean (*Glycine max*) in BLAST search. Using 5' and 3' RACE procedures, we obtained amplifications of 491 bp (Fig. 4.2) and 656 bp (Fig. 4.3) respectively of the 5' and 3' ends the cDNA. Each amplified products were separately confirmed by BLAST search for homology with *DREB* genes, and assembled together. The full-length mRNA sequence was amplified (1481 bp), cloned in TA cloning vector (Fermentas) (Fig. 4.4A) and again sequenced by double-stranded primer walking. It contained a CDS of 1134 bp encoding a protein of 377 amino acids with 214 bp of 5' UTR and 157 bp of 3' UTR. The deduced amino acid sequence was found highly homologous to previously isolated DREB2 proteins, closest to a legume DREB2A isolated from soybean (77 % homology) and only 43 % homology with *Arabidopsis* DREB2A (Fig. 4.5) The primers used for amplification of the full-length cDNA were used for amplification from cowpea genomic DNA. The 1891 bp fragment was cloned in TA cloning vector (Fermentas) (Fig. 4.4B) and sequenced. Comparison of the genomic amplicon (GenBank Accession: JQ066264.2) and the full-length cDNA showed that the *VuDREB2A* gene carried an intron of 418 bp in the 5' UTR.

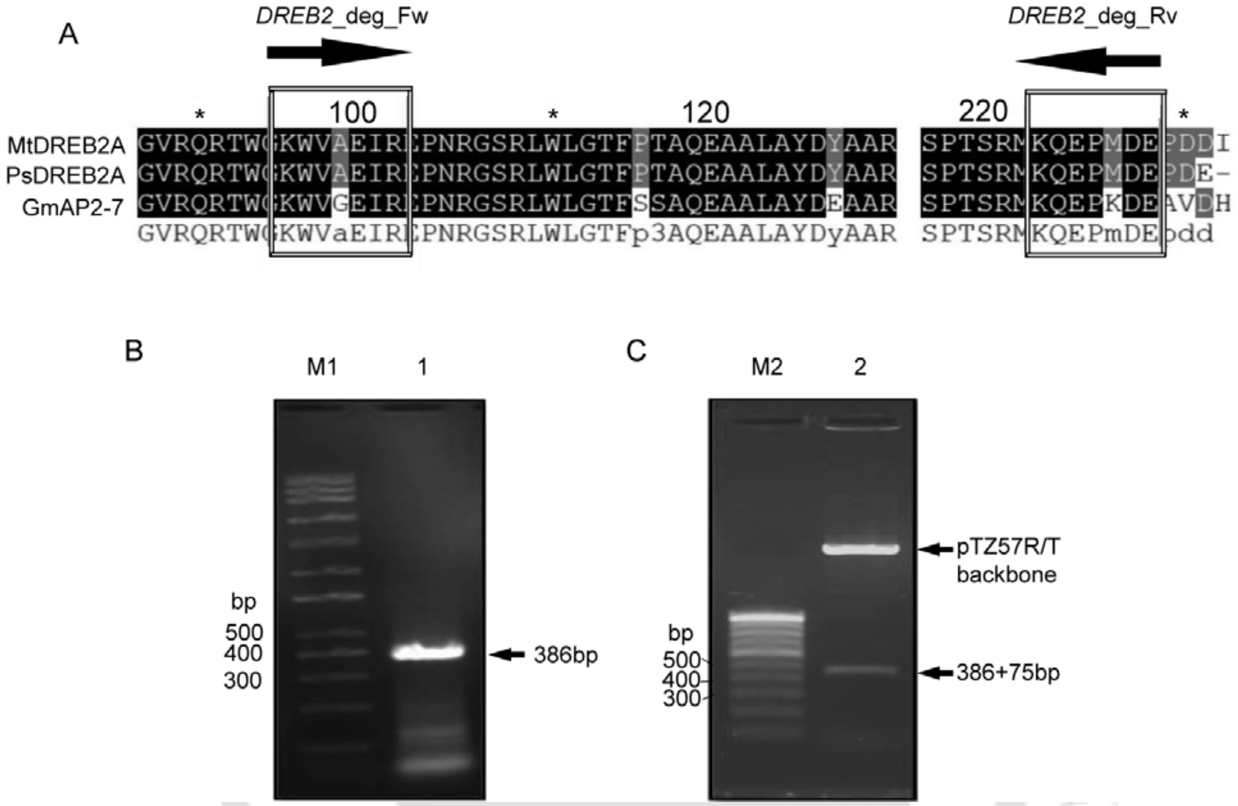


Figure 4.1 Cloning of partial *VuDREB2A* by degenerate oligonucleotide-primed PCR. A Conserved regions in DREB2-type proteins of legumes [*Medicago trancatula* MtDREB2A, GenBank accession: ABJ88942; *Pisum sativum* PsDREB2A, GenBank accession: ADL27988; *Glycine max* GmAP2-7, GenBank accession: NP_001240942] together with regions for design of degenerate primers. **B** 386 bp *VuDREB2A* partial amplification from salt-stressed cowpea cDNA. **C** TA clone of the partial *VuDREB2A* digested with *Eco*RI and *Hind*III to release the insert of 386 bp + 75 bp (distance between *Eco*RI and *Hind*III sites in the vector).

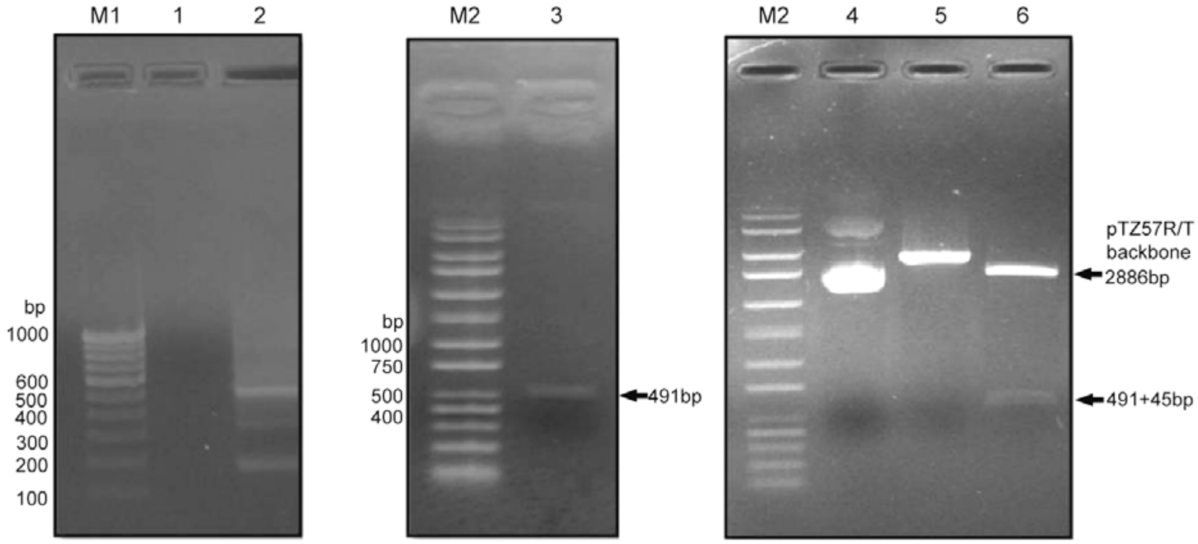


Figure 4.2 Cloning of the 5’ end of the *VuDREB2A* cDNA. Amplifications in all intermediate steps of 5’ RACE (see Methods). M1 and M2: DNA size markers. Lane 1: amplification with 5’RACE GSP2 & AUAP (not detected in gel); lane 2: amplification with 5’RACE GSP3 & AUAP with 100 times diluted PCR product (from lane 1) as template; lane 3: most prominent band (491 bp) from lane 2 purified and reloaded; undigested (lane 4), *Eco*RI digest (lane5) and *Eco*RI and *Bam*HI digest (lane 6) of TA vector with the cloned amplicon (that in lane 3) of 491 bp + 45 bp (distance between *Eco*RI and *Bam*HI sites in the vector).

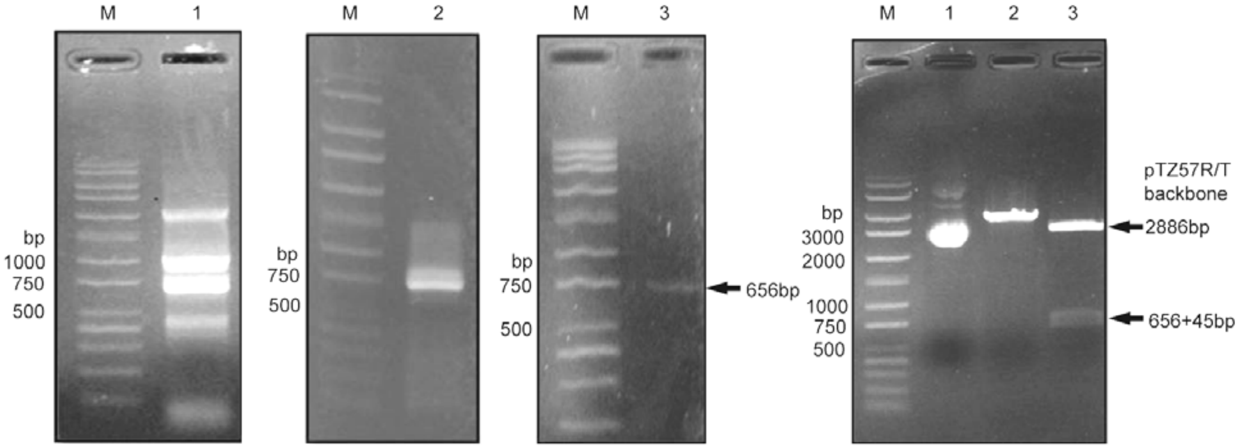


Figure 4.3 Cloning of the 3’ end of the *VuDREB2A* cDNA. Amplifications in all intermediate steps of 3’ RACE (see Methods). M: DNA size marker. Lane 1: amplification with 3’ RACE GSP 1 & AUAP; lane 2: amplification with 3’ RACE GSP 2 and AUAP with 100 times diluted PCR product (from lane 1); 3: most prominent band from lane 2 (656 bp) purified and reloaded; undigested (lane 4), *Eco*RI digest (lane5) and *Eco*RI and *Bam*HI digest (lane 6) of TA vector with cloned amplicon (that in lane 3) of 656 bp + 45 bp (distance between *Eco*RI and *Bam*HI sites in the vector).

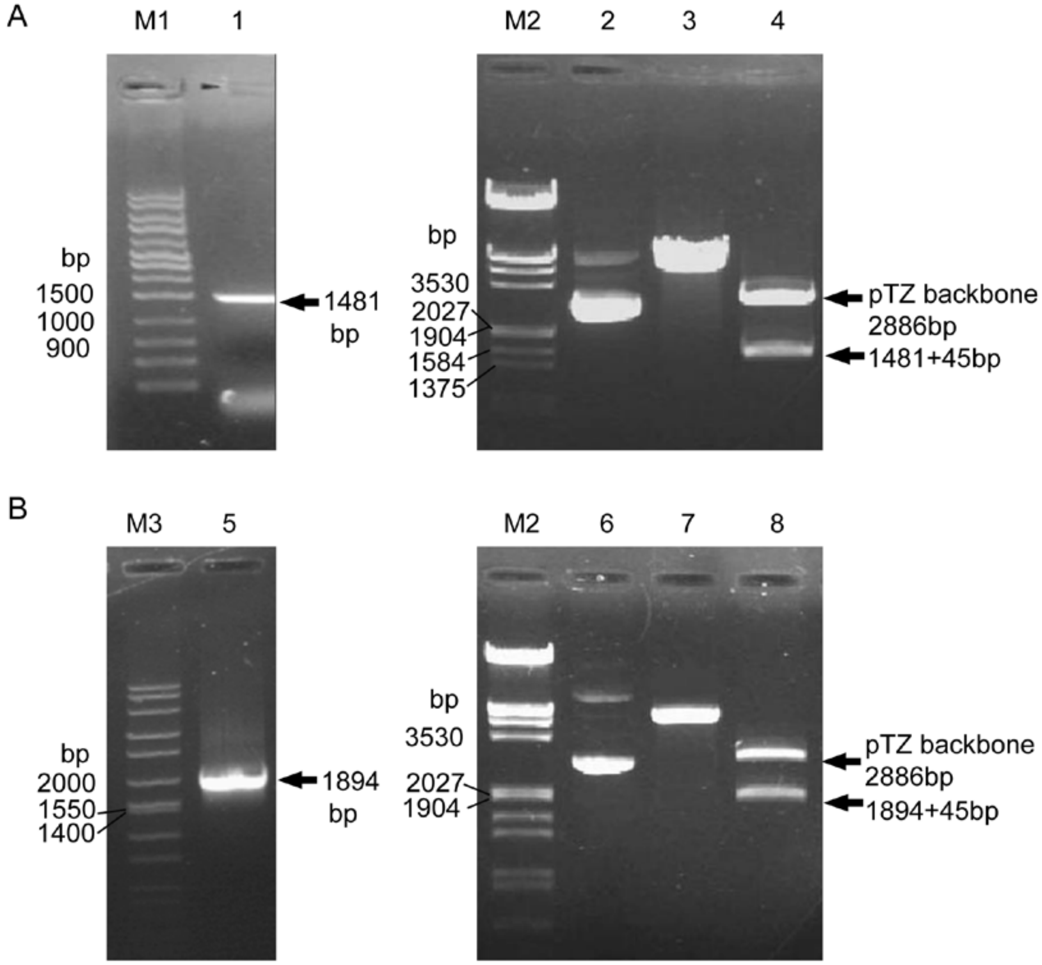


Figure 4.4 Cloning of the full length *VuDREB2A* cDNA and genomic sequence. A Amplification and cloning in TA vector of the full length *VuDREB2A* cDNA. Lane 1: The 1481 bp full length VuDREB2A cDNA; undigested (lane 2), *Eco*RI digest (lane3) and *Eco*RI & *Bam*HI digest (lane 4) of TA vector with cloned amplicon (that in lane 1) of 1481 bp + 45 bp (distance between *Eco*RI and *Bam*HI sites in the vector). **B** Amplification and cloning in TA vector of the full length *VuDREB2A* genomic DNA sequence with same primers used to clone the full length cDNA. Lane 5: The 1894 bp full length VuDREB2A genomic DNA sequence; undigested (lane 6), *Eco*RI digest (lane7) and *Eco*RI & *Bam*HI digest (lane 8) of TA vector with cloned amplicon (that in lane 5) of 1894 bp + 45 bp (distance between *Eco*RI and *Bam*HI sites in the vector). M1-3: DNA size markers.

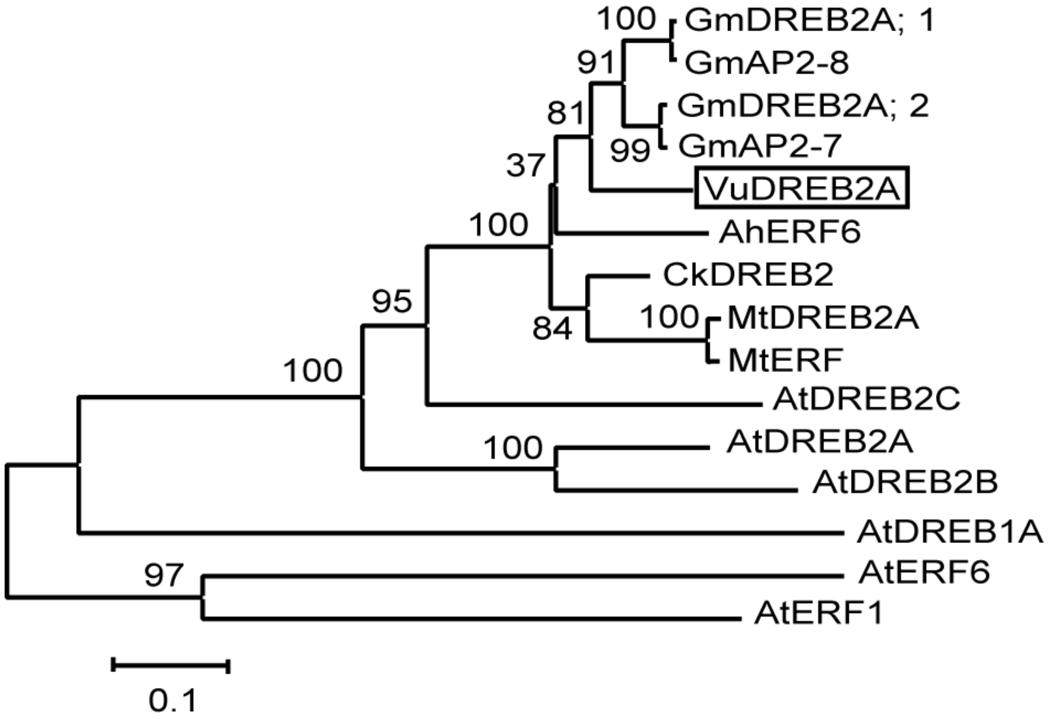
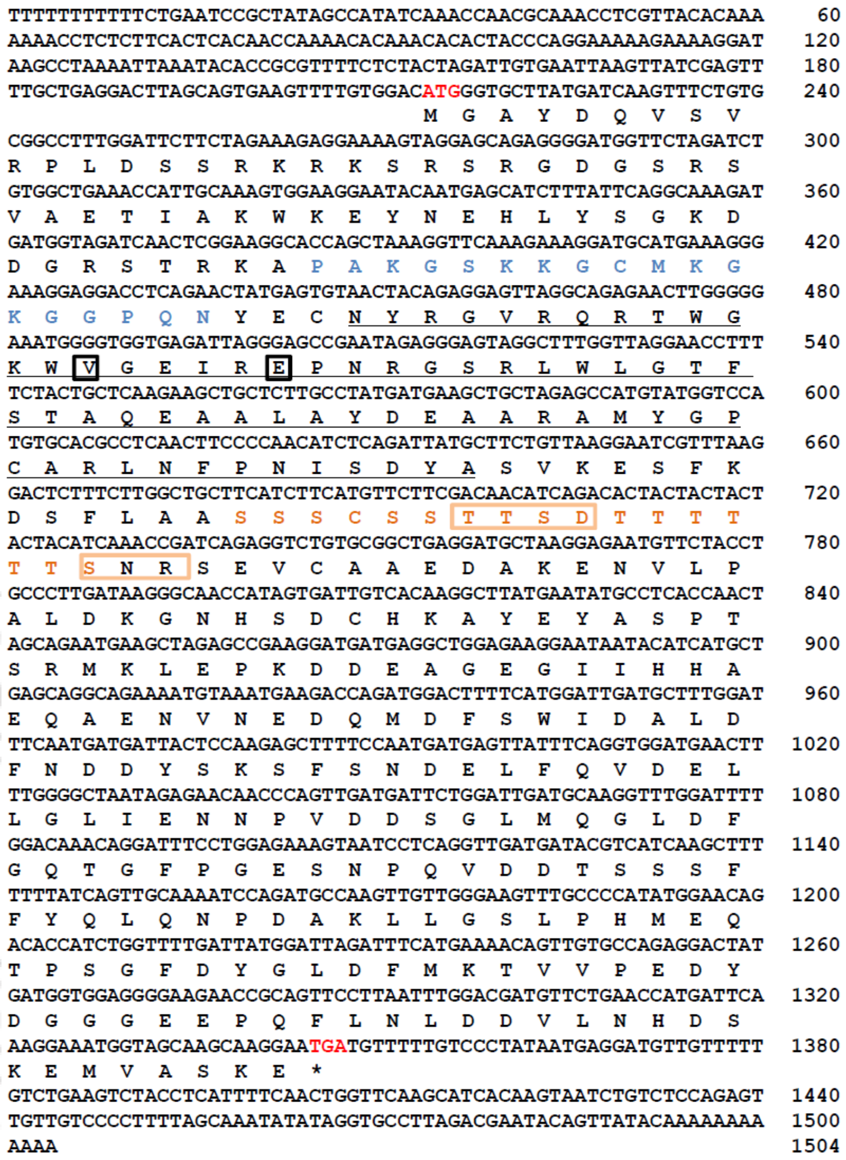


Figure 4.5 Phylogenetic tree of proteins having ≥ 60 % sequence identity with VuDREB2A. *Arabidopsis* DREB and ethylene-responsive element binding factor (ERF) homologs were used as out groups for the analysis. The tree was generated by MEGA 6.0.5 using the Neighbor-Joining method with 1000-replicate bootstrap testing. The numbers against each branch indicate the bootstrap values and the scale bar indicates substitution rate per site. The tree was constructed from a sequence alignment of the following proteins: *Arabidopsis thaliana* AtDREB1A [GenBank: BAA33434], AtDREB2A [GenBank: AAU93685], AtDREB2B [GenBank: BAA36706], AtDREB2C [GenBank: Q8LFR2], AtERF1 [GenBank: AAD03544], AtERF6 [GenBank: NP_567529] *Glycine max* GmDREB2A;1 [GenBank: AFU35562], GmDREB2A;2 [GenBank: AFU35563], GmAP2-7 [GenBank: NP_001240942], GmAP2-8 [GenBank: NP_001240005]; *Vigna unguiculata* VuDREB2A [GenBank: AEY75222]; *Medicago truncatula* MtDREB2A [GenBank: ABJ88942], MtERF [GenBank: XP_003616701]; *Arachis hypogaea* AhERF6 [GenBank: AFU07643]; *Caragana korshinskii* CkDREB2 [GenBank: AGI78251].

4.1.2 Sequence characteristics and nomenclature of the *VuDREB2A* gene

The isolated gene encoded a protein possessing a single DNA-binding AP2 domain, hence it could be categorized into the DREB sub-group of the AP2 superfamily. Further, it conserved the consensus amino acids in the AP2 domain (i.e. the 14th valine and 19th glutamic acid at 79–142 amino acids), and carried a conserved nuclear localization signal (Fig. 4.6), a signature of the A-2 subgroup DREBs (Sakuma *et al.* 2002). It was thus assumed that the gene was the cowpea ortholog of *DREB2A*, and it was deposited to GenBank (named *VuDREB2A*; accession number: JN629045.3, theoretical average molecular mass of 41.76 KDa and pI of 4.76). The protein also had conserved motifs common to DREB2s (Nakano *et al*. 2006), carrying CMIV-1 and CMIV-2 in the N-terminus and CMIV-3 in the C-terminal activation domain (Fig. 4.7). A serine/threonine-rich region immediately downstream of the AP2 domain was conserved in VuDREB2A (Fig. 4.6 and 4.7), which acts as a negative regulatory domain that destabilizes the DREB2A protein in *Arabidopsis* by phosphorylation (Sakuma *et al*. 2006; Agarwal *et al*. 2007) and degrades it by the ubiquitin-proteasome mediated process (Qin *et al*. 2008). Phosphorylation sites (of protein kinase C and casein kinase II) were detected (Fig. 4.6) in the serine/threonine-rich region in VuDREB2A. Some of the DREB2A orthologs also contains PEST motifs for protein degradation (Rechsteiner and Rogers 1996) in this region (e.g., MtDREB2A, Chen *et al*. 2009; GmDREB2A;2, Mizoi *et al*. 2013). Alignment of the protein sequences closest to VuDREB2A revealed conservation of a low score 'poor' PEST sequence in VuDREB2A and very identical sequence to the downstream high-score 'potential' PEST motif in other DREB2A orthologs (Fig. 4.7). The presence of phosphorylation sites in the conserved serine/threonine-rich region and the PEST motifs pointed to similar negative regulation of VuDREB2A as other DREB2A orthologs.



```
TTTTTTTTTTTCTGAATCCGCTATAGCCATATCAAACCAACGCAAACCTCGTTACACAAA    60
AAAACCTCTCTTCACTCACAACCAAAACACAAACACACTACCCAGGAAAAAGAAAAGGAT   120
AAGCCTAAAATTAAATACACCGCGTTTTCTCTACTAGATTGTGAATTAAGTTATCGAGTT   180
TTGCTGAGGACTTAGCAGTGAAGTTTTGTGGACATGGGTGCTTATGATCAAGTTTCTGTG   240
                                 M  G  A  Y  D  Q  V  S  V
CGGCCTTTGGATTCTTCTAGAAAGAGGAAAAGTAGGAGCAGAGGGGATGGTTCTAGATCT   300
R  P  L  D  S  S  R  K  R  K  S  R  S  R  G  D  G  S  R  S
GTGGCTGAAACCATTGCAAAGTGGAAGGAATACAATGAGCATCTTTATTCAGGCAAAGAT   360
V  A  E  T  I  A  K  W  K  E  Y  N  E  H  L  Y  S  G  K  D
GATGGTAGATCAACTCGGAAGGCACCAGCTAAAGGTTCAAAGAAAGGATGCATGAAAGGG   420
D  G  R  S  T  R  K  A  P  A  K  G  S  K  K  G  C  M  K  G
AAAGGAGGACCTCAGAACTATGAGTGTAACTACAGAGGAGTTAGGCAGAGAACTTGGGGG   480
K  G  G  P  Q  N  Y  E  C  N  Y  R  G  V  R  Q  R  T  W  G
AAATGGGGTGGTGAGATTAGGGAGCCGAATAGAGGGAGTAGGCTTTGGTTAGGAACCTTT   540
K  W  V  G  E  I  R  E  P  N  R  G  S  R  L  W  L  G  T  F
TCTACTGCTCAAGAAGCTGCTCTTGCCTATGATGAAGCTGCTAGAGCCATGTATGGTCCA   600
S  T  A  Q  E  A  A  L  A  Y  D  E  A  A  R  A  M  Y  G  P
TGTGCACGCCTCAACTTCCCCAACATCTCAGATTATGCTTCTGTTAAGGAATCGTTTAAG   660
C  A  R  L  N  F  P  N  I  S  D  Y  A  S  V  K  E  S  F  K
GACTCTTTCTTGGCTGCTTCATCTTCATGTTCTTCGACAACATCAGACACTACTACTACT   720
D  S  F  L  A  A  S  S  S  C  S  S  T  T  S  D  T  T  T  T
ACTACATCAAACCGATCAGAGGTCTGTGCGGCTGAGGATGCTAAGGAGAATGTTCTACCT   780
T  T  S  N  R  S  E  V  C  A  A  E  D  A  K  E  N  V  L  P
GCCCTTGATAAGGGCAACCATAGTGATTGTCACAAGGCTTATGAATATGCCTCACCAACT   840
A  L  D  K  G  N  H  S  D  C  H  K  A  Y  E  Y  A  S  P  T
AGCAGAATGAAGCTAGAGCCGAAGGATGATGAGGCTGGAGAAGGAATAATACATCATGCT   900
S  R  M  K  L  E  P  K  D  D  E  A  G  E  G  I  I  H  H  A
GAGCAGGCAGAAAATGTAAATGAAGACCAGATGGACTTTTCATGGATTGATGCTTTGGAT   960
E  Q  A  E  N  V  N  E  D  Q  M  D  F  S  W  I  D  A  L  D
TTCAATGATGATTACTCCAAGAGCTTTTCCAATGATGAGTTATTTCAGGTGGATGAACTT  1020
F  N  D  D  Y  S  K  S  F  S  N  D  E  L  F  Q  V  D  E  L
TTGGGGCTAATAGAGAACAACCCAGTTGATGATTCTGGATTGATGCAAGGTTTGGATTTT  1080
L  G  L  I  E  N  N  P  V  D  D  S  G  L  M  Q  G  L  D  F
GGACAAACAGGATTTCCTGGAGAAAGTAATCCTCAGGTTGATGATACGTCATCAAGCTTT  1140
G  Q  T  G  F  P  G  E  S  N  P  Q  V  D  D  T  S  S  S  F
TTTTATCAGTTGCAAAATCCAGATGCCAAGTTGTTGGGAAGTTTGCCCCATATGGAACAG  1200
F  Y  Q  L  Q  N  P  D  A  K  L  L  G  S  L  P  H  M  E  Q
ACACCATCTGGTTTTGATTATGGATTAGATTTCATGAAAACAGTTGTGCCAGAGGACTAT  1260
T  P  S  G  F  D  Y  G  L  D  F  M  K  T  V  V  P  E  D  Y
GATGGTGGAGGGGAAGAACCGCAGTTCCTTAATTTGGACGATGTTCTGAACCATGATTCA  1320
D  G  G  G  E  E  P  Q  F  L  N  L  D  D  V  L  N  H  D  S
AAGGAAATGGTAGCAAGCAAGGAATGATGTTTTTGTCCCTATAATGAGGATGTTGTTTTT  1380
K  E  M  V  A  S  K  E  *
GTCTGAAGTCTACCTCATTTTCAACTGGTTCAAGCATCACAAGTAATCTGTCTCCAGAGT  1440
TGTTGTCCCCTTTTAGCAAATATATAGGTGCCTTAGACGAATACAGTTATACAAAAAAAA  1500
AAAA                                                          1504
```

Figure 4.6 Sequence characteristics of the *VuDREB2A* gene. The AP2/ERF conserved domain of VuDREB2A is single underlined. Two conserved amino acid residues, valine (V) and glutamic acid (E), in the AP2 domain of the VuDREB2A translated protein sequence are shown by black boxes. The start (ATG) and stop (TGA) codons are in red. The N-terminal nuclear localization signal, shown in blue, is a signature of A-2 subgroup of DREB proteins. The serine/threonine-rich region is shown in orange. Predicted phosphorylation sites are shown by orange boxes. Protein kinase C: TTSD and casein kinase II: SNR.

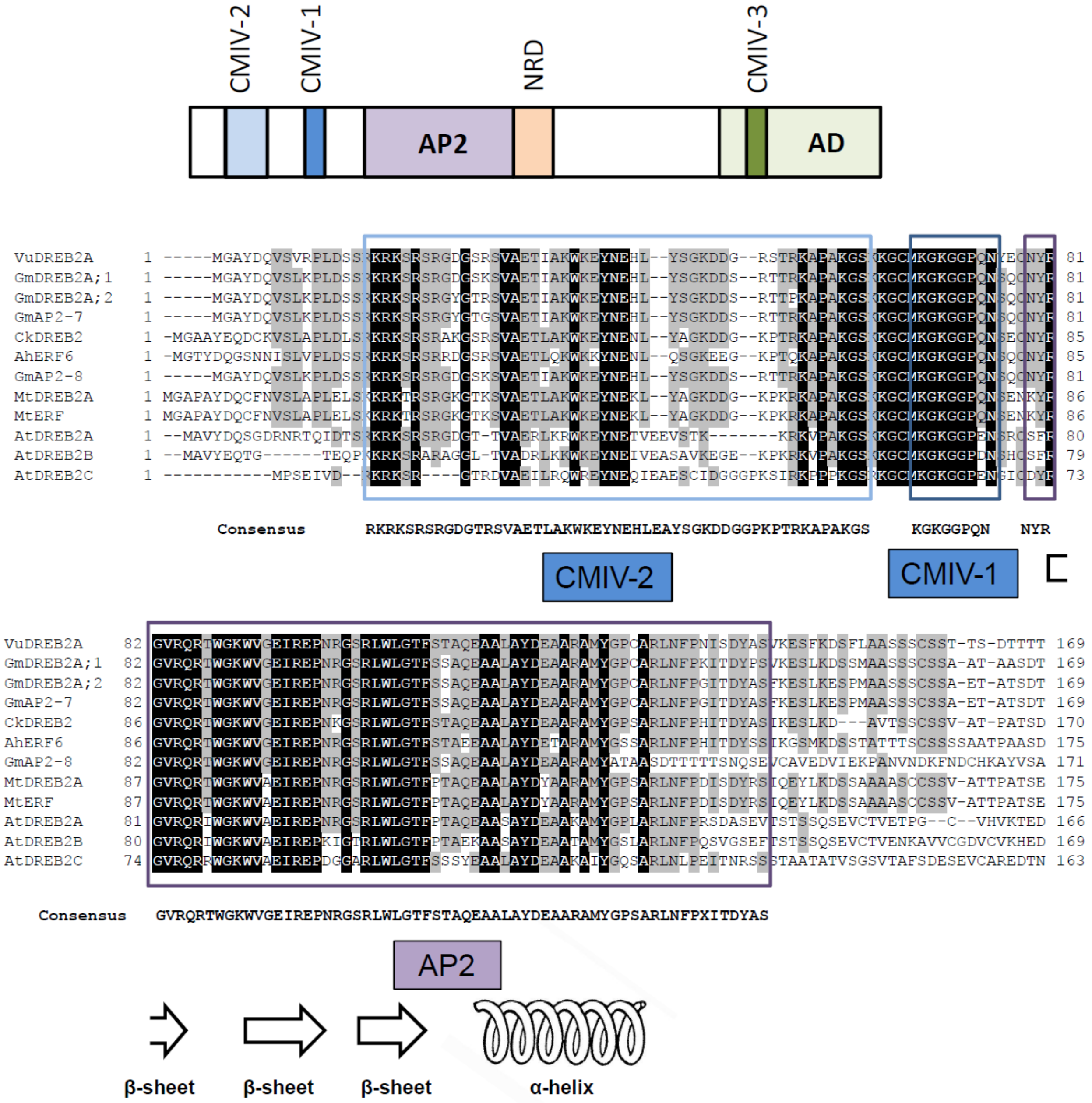


Figure 4.7 Conserved motifs in proteins with ≥ 60 % sequence homology with VuDREB2A, and *Arabidopsis* DREB2 proteins. AP2 and AD are the AP2/ERF-type DNA-binding domain and the activation domain, respectively. CMIV-1 to 3 indicate conserved motifs (as described in Nakano *et al*. 2006). Shading color: black, 100 % conservation and gray, ≥ 50 % conservation of amino acids. The secondary structures: 3 β-sheets (YRGVRQ, WVAEIR and RLWL) and α-helix (AQEAALAYDEAARAM) constitute the DNA binding domain.

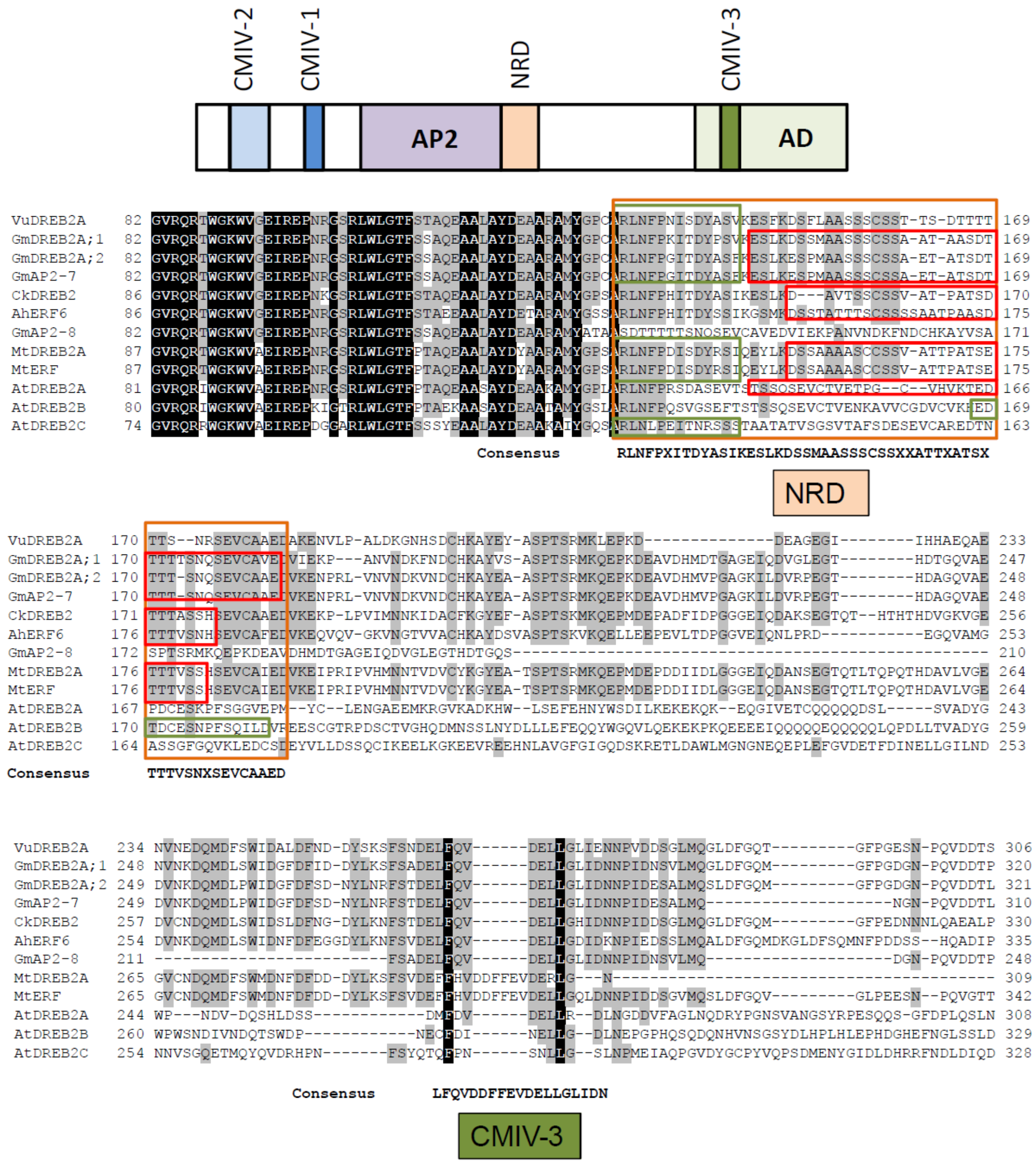


Figure 4.7 (contd.) The negative regulatory domain (NRD) region in legumes has stretches of serine/threonine residues as well as PEST motifs. Red rectangles indicate ‘potential’ (PEST score > 5) while green rectangles ‘poor’ (PEST score < 5) PEST motifs for protein degradation.

4.2 Transcriptional regulation of *VuDREB2A* under abiotic stress

4.2.1 Stress-inducible expression of *VuDREB2A*

After six hours treatment, the *VuDREB2A* transcript was found to be highly induced by heat, salinity and desiccation stresses in cowpea shoots (Fig. 4.8A), similarly to its ortholog in *Arabidopsis* (Liu *et al.* 1998). All these stressors activated the expression very quickly, as identified by the time course analyses (Fig. 4.8B). Exogenous application of ABA only slightly induced *VuDREB2A*, again similar to its *Arabidopsis* ortholog (Kim *et al.* 2011). Similar pattern of expression was also observed in cowpea roots (data not shown).

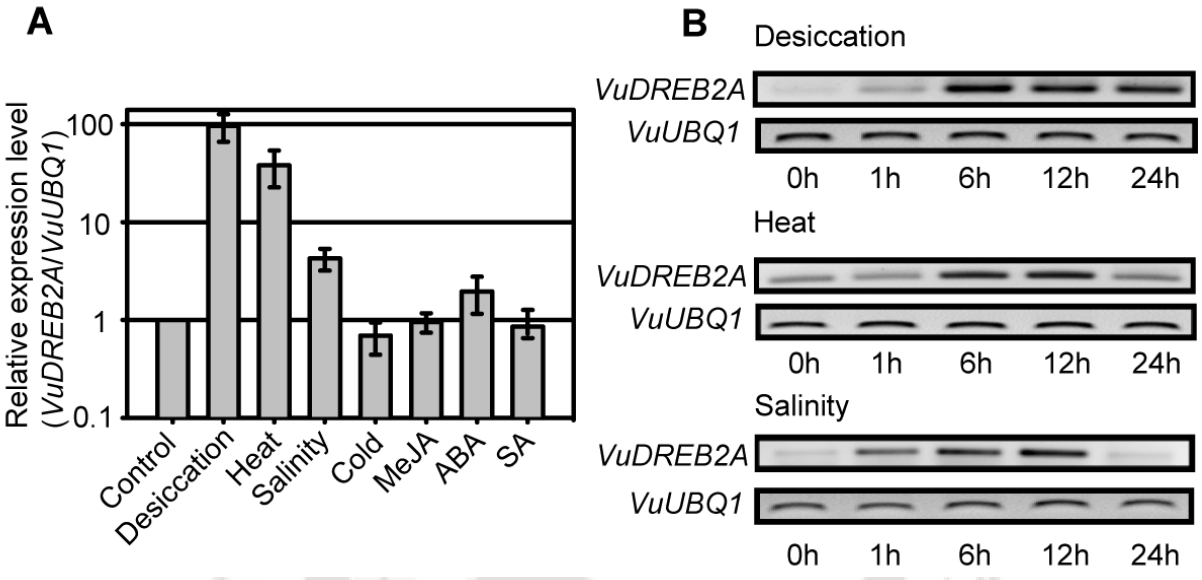


Figure 4.8 Stress inducible expression of *VuDREB2A*. A Transcript levels of *VuDREB2A* in the shoots of cowpea plants grown for one week and then exposed to different stress treatments (desiccation, heat, cold, ABA, MeJA and SA) for 6h. Columns indicate relative expression levels of *VuDREB2A* normalized against levels of *VuUBQ1* as calculated by real-time qRT-PCR (means ± SE of 3 biological replicates). **B** Transcript abundance of *VuDREB2A* at different time points with major inducing stressors viz. desiccation, heat and salinity. Gel photos indicate expression levels in a single experiment.

4.2.2 Isolation of the *VuDREB2A* promoter

The resultant 1.9 kb product of inverse PCR was cloned in TA cloning vector (Fig. 4.9) and sequenced. A 966 bp genomic fragment upstream to the already known *VuDREB2A* sequence was unraveled. This sequence was predicted to contain a promoter with a transcription start site (TSS) at 966 bp from the 5’ end and a TATA box (Joshi 1987) at -32 from the TSS.

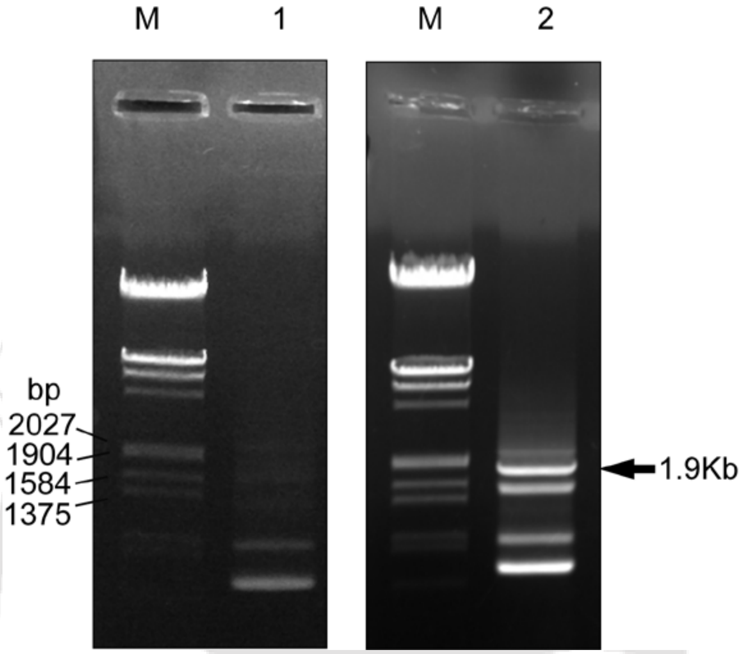


Figure 4.9 Isolation of the *VuDREB2A* promoter by inverse PCR. M: DNA size marker. Lane 1: Amplification with F1 and R1 from the ligated fragments of *Nde*I digested cowpea genomic DNA (see Methods); lane 2: Nested amplification with F2 and R2 taking 100 times-diluted PCR product (that in lane 1).

4.2.3 Stress-responsive *cis*-elements in the *VuDREB2A* promoter

The promoter scanning analysis using the overrepresented octamers in the promoters of stress-responsive genes in *Arabidopsis* (fold change > 3; NaCl, ABA, dehydration and H_2O_2) (Yamamoto *et al.* 2011) predicted 12 *cis*-elements (V1-12) that are related to salinity, dehydration and oxidative-stress, and ABA signaling (Fig. 4.10 and Table 4.1). Of these, 4 elements contained functionally validated promoter motifs from literature: ABA responsive element (ABRE; core motif: ACGT) (Kim *et al.* 2011) in V9 and V12; CGCG-box (consensus: [A/C/G]CGCG[C/T/G]) (Yang *et al.* 2002) and Coupling element 3 (CE3; consensus: [A/C]ACGCG[C/T/G]) (Kaplan *et al.* 2006) in V7 (complete identity) and V10 (partial identity);

a heat shock element (HSE; consensus: NGAANNTTCN/NTTCNNGAAN) (Sun *et al.* 2006, Yoshida *et al.* 2011) in V12; and a CGTCA motif involved in MeJA response (Wang *et al.* 2011) in V9. Few additional *cis*-elements predicted by the PLACE and PlantCARE web-programs are: a low temperature-responsive element (LTE) (Dunn *et al.* 1998), TCA elements involved in SA-response (Reinbothe *et al.* 1994), WRKY factor binding sites (Eulgem *et al.* 2000), a MYB binding site responsive to dehydration (Abe *et al.* 2003) and TC-rich repeats involved in defense and stress-response (Rushton and Somssich 1998) (Appendix Fig. A1).

. The expression profile of the *VuDREB2A* gene was found to be similar to the homologs in *Arabidopsis* and soybean (section 4.2.1). This could be explained by conserved *cis*-elements responsive to these stressors in the promoters of *Arabidopsis*, soybean and cowpea *DREB2* genes (Appendix Figs. A1, A2 and Table A8). Particularly, promoters of *DREB2A* in soybean and cowpea possessed similar clusters of *cis*-elements (V5-12 and G2-10; Appendix Fig. A2) related to the dehydration-response of *Arabidopsis*, with at least 10 bp sequence identity in five cases (V5-G2, V7-G10, V9-G6, V10-G7 and V12-G9). This suggested strong conservation of the *DREB2A* promoter structure within the two legume species. The *VuDREB2A* promoter was found to conserve the ABA-responsive complex including ABRE and its Coupling Element 3 (and the similar CGCG box). These results suggest that in addition to an ABA-independent pathway (the general feature of the DRE-mediated signaling), the ABA-dependent pathway also play role in the transcriptional regulation of *VuDREB2A*. Roles of the ABRE and CE3 in the dehydration-responsiveness of the *AtDREB2A* promoter were demonstrated earlier (Kim *et al.* 2011). Additional roles of the CE3 in calcium signaling were also reported (Kaplan *et al.* 2006). The CGCG box is commonly observed in promoters of genes involved in ABA signaling and are specific targets of calmodulin-binding proteins in *Arabidopsis* (Yang *et al.* 2002). Involvement of similar pathways in the regulation of *DREB2A* in cowpea can be speculated from the localization of the ABRE-CE3 in the *VuDREB2A* promoter. The observation of the heat stress-responsive element in the *VuDREB2A* promoter could be correlated to the strong induction of *VuDREB2A* by heat stress. We could detect some additional *cis*-elements responsive to low temperature, MeJA and SA in the *VuDREB2A* promoter (Fig. 4.10 and Appendix Fig. A1). Consistent with these findings, the *VuDREB2A* gene was found to be slightly induced by exogenous application of MeJA and SA. Cold (4 °C) had an induction effect on the gene after 12 h of treatment (Appendix Fig. A3).

Table 4.1 Predicted *cis*-elements in the *VuDREB2A* promoter

Position from ATG[a]	Octamer[b]	Treatment[c]	RARf[d]	*cis*-element[e]	Sequence	Motif[f]	Reference[g]
-771	TCGAGGGC	H_2O_2	3.53	V12	TCGAGGGCCACGTGCCAGC	HSE	Sun *et al.* 2006
-771	TCGAGGGC	Osmotic stress	3.55				Yoshida *et al.* 2011
-769	GAGGGCCA	Dehydration	3.20			ABRE	Kim *et al.* 2011
-767	GGGCCACG	Dehydration	3.33				
-766	GGCCACGT	Cold	3.06				
-766	GGCCACGT	Dehydration	3.51				
-765	GCCACGTG	ABA	3.53				
-765	GCCACGTG	Dehydration	3.12				
-764	CCACGTGC	Dehydration	3.12				
-764	CCACGTGC	Osmotic stress	3.50				
-763	CACGTGCC	Dehydration	3.29				
-760	GTGCCAGC	ABA	3.23				
-747	CGGGAGTC	Osmotic stress	5.26	V11	CGGGAGTCC		
-746	GGGAGTCC	Osmotic stress	4.13				
-730	ATAACGCG	Cold	3.05	V10	ATAACGCGACTAG	CGCG Box/CE3-like	Yang *et al.* 2002
-730	ATAACGCG	H_2O_2	4.69				Kaplan *et al.* 2006
-727	ACGCGACT	H_2O_2	4.14				
-726	CGCGACTA	H_2O_2	3.45				
-725	GCGACTAG	ABA	3.76				
-713	ACGCCACG	Cold	3.28	V9	ACGCCACGTCAC	ABRE	Kim *et al.* 2011
-713	ACGCCACG	Dehydration	4.84			CGTCA motif (MeJA responsive element)	Reinbothe *et al.*1994
-712	CGCCACGT	ABA	4.34				Wang *et al.*2011
-712	CGCCACGT	Cold	3.62				
-712	CGCCACGT	Osmotic stress	3.20				
-712	CGCCACGT	Dehydration	4.45				
-709	CACGTCAC	ABA	3.84				
-709	CACGTCAC	Osmotic stress	3.02				
-695	GGGGGGCA	Cold	3.22	V8	GGGGGGCA		
-695	GGGGGGCA	Dehydration	7.38				
-494	ATACACCG	H_2O_2	3.48	V7	ATACACCGCGTTTT	CGCG Box/CE3	Yang *et al.* 2002
-492	ACACCGCG	Dehydration	3.40				Kaplan *et al.* 2006
-491	CACCGCGT	Dehydration	3.69				
-490	ACCGCGTT	Cold	6.30				
-490	ACCGCGTT	Dehydration	8.13				
-489	CCGCGTTT	Cold	5.62				
-489	CCGCGTTT	Dehydration	6.44				
-488	CGCGTTTT	Cold	4.61				
-488	CGCGTTTT	Dehydration	4.53				
-421	TGCACGCT	Dehydration	3.92	V6	TGCACGCT		
-386	TGTGCGAT	H_2O_2	3.13	V5	TGTGCGAT		
-225	CTTGGGGG	H_2O_2	3.25	V4	CTTGGGGGTTAC		
-221	GGGGTTAC	Osmotic stress	3.12				
-184	TGGCTACT	Cold	3.04	V3	TGGCTACT		
-164	AGCGTGTT	Cold	3.11	V2	AGCGTGTT		
-21	TAGCAGTG	Osmotic stress	3.47	V1	TAGCAGTG		

[a] Position from start codon of VuDREB2A. [b] Overrepresented octamers in promoters of genes up-regulated > 3 fold in *Arabidopsis* under [c] different treatments (viz., ABA, cold, osmotic stress, dehydration and H_2O_2). [d] Relative Appearance Ratio (RAR) of each octamer = count of the octamer in n genes up-regulated under different treatments/n)/(count of the octamer in total 24956 promoters/24956). n = 643 (ABA)/ 337 (cold)/ 327 (osmotic stress)/ 294 (dehydration)/329 (H_2O_2). RARf = filtered RAR (RAR > 3, $P < 0.05$). [e] Predicted cis-elements that are related to ABA, cold, osmotic stress, dehydration and H_2O_2 (V1-12; Fig. 4.10). [f] Motifs in the predicted cis-elements, based on functional analyses in other plant species . [g] Source of functional analyses.

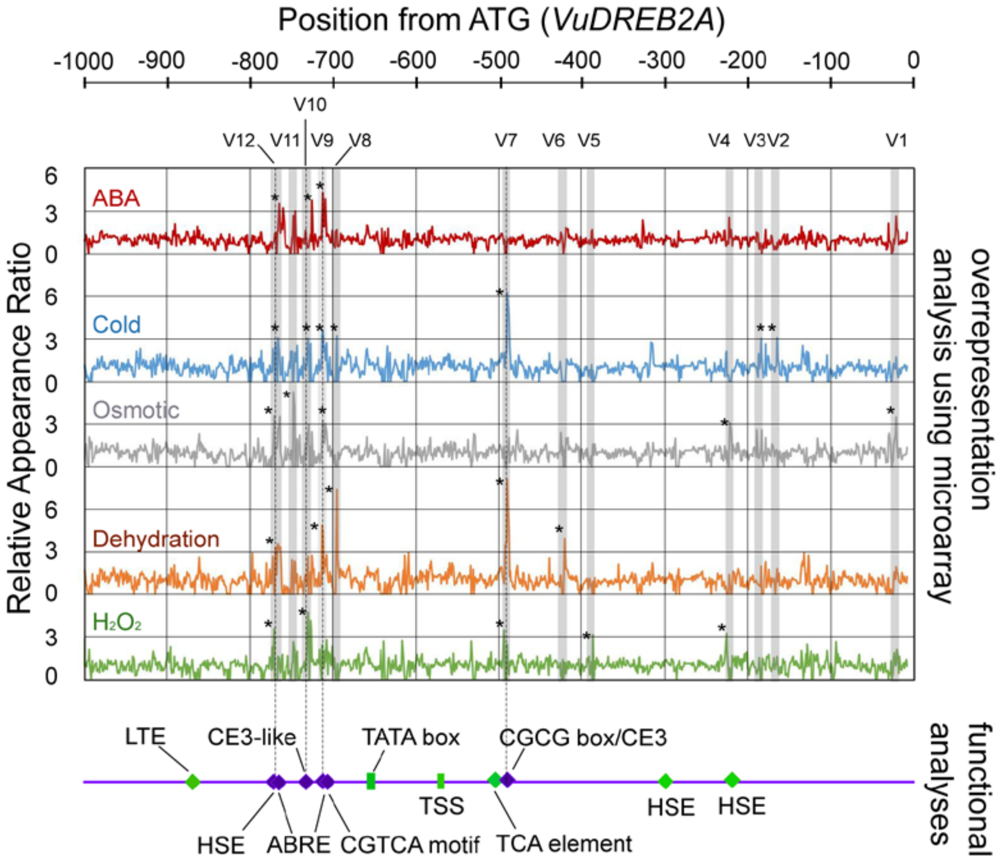


Figure 4.10 *In silico* analysis of the *VuDREB2A* promoter. The *VuDREB2A* promoter and 5'-UTR (1000 bp upstream of ATG) was scanned for *cis*-elements by evaluation of octamers in 1 bp steps based on a Relative Appearance Ratio (RAR) table as described previously (Yamamoto *et al*. 2011). RAR (ratio of octamer in the promoter of stress inducible genes to genome-wide promoters) was calculated from microarray data of *Arabidopsis* under different treatments (ABA, cold, osmotic stress, dehydration and H_2O_2). **A** The graphs show scanning results based on microarray data of the ABA response (red), the cold response (blue), the osmotic response (gray), the dehydration response (orange) and the H_2O_2 response (green) of *Arabidopsis*. Asterisks indicate octamers with RAR > 3 and $P < 0.05$. Locations of predicted *cis*-elements are indicated by gray columns (V1–12; Table 4.2). Promoter motifs determined in other plant promoters by functional analyses, are shown on the purple line. Purple symbols indicate those motifs carried by our predicted *cis*-elements, while green symbols indicate additional motifs detected by the PlantCARE and PLACE signal scan searches. ABRE: ABA-responsive element; CE3: Coupling element 3; HSE: Heat stress-responsive element; LTE: Low temperature-responsive element; TSS: Transcription start site.

4.3 *In vitro* interaction of recombinant VuDREB2A with dehydration-responsive elements

The *VuDREB2A* ORF (minus the stop codon for introducing Hisx6 to C-terminus) was cloned successfully into the pET28a vector (Fig. 4.11). The positive and sequenced clones were used for expression in *E. coli* BL21. Using highly-purified VuDREB2A protein obtained by the *E. coli* expression system [i.e. that gave single band in SDS-PAGE (Fig. 4.12A, lane 4) and in western blotting for the His-tag (Fig. 4.12A, lane 5)], we analyzed the interaction of VuDREB2A with known DRE sequences, which were previously identified in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki 1994), by electrophoretic mobility shift assay (EMSA). Protein–double-stranded oligonucleotide complexes were observed with both ACCGAC and GCCGAC DRE sequences (Fig. 4.12B, lanes 2 and 3). The complexes disappeared when a few random mutations were introduced into the core sequence of CCGAC (Fig. 4.12B, lanes 4–7). At a fixed concentration (0.1 nM) of both ACCGAC and GCCGAC probes, increased binding with increasing concentrations of VuDREB2A protein (0.2–4 µM) was detected (Fig. 4.12C, D), which was also evident by a hyperbolic increase in the fraction of bound DNA (Fig. 4.12E) and a hyperbolic decay in the fraction of free DNA (Fig. 4.12F). These results suggested simple stoichiometric interaction with similar affinity for both DREs. Additionally, no complex was detected when the probes were incubated with BSA (data not shown). From these results, we concluded that VuDREB2A has the capacity to bind with the DRE consensus.

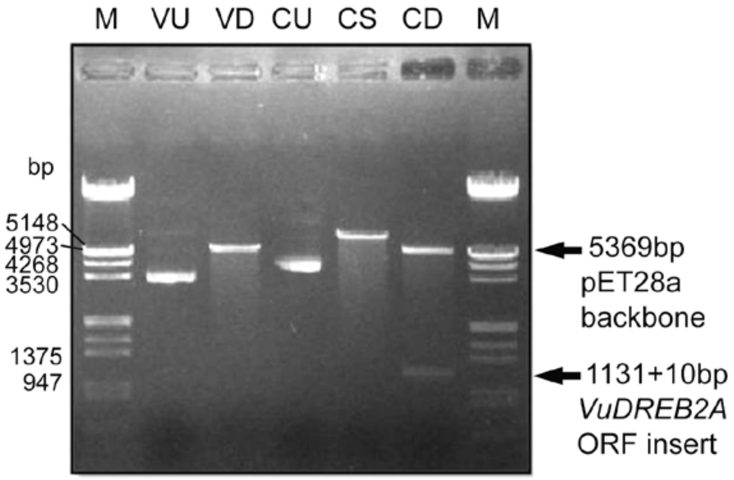


Figure 4.11 Cloning of *VuDREB2A* ORF (without the stop codon) in pET28a. Gel picture shows the confirmation of a ligated clone by re-digestion with *Bam*HI and *Xho*I. M: *Eco*RI & *Hind*III digest of λ DNA (size marker), VU: undigested pET28a, VD: *Bam*HI & *Xho*I digest of pET28a, IU: undigested pTZ57R/T-*VuDREB2A*, ID: *BamH*I & *Xho*I digest of pTZ57R/T-*VuDREB2A*, CU: undigested pET28a-*VuDREB2A*, CS: *Xho*I digest of pET28a-*VuDREB2A*, CD: *Bam*HI & *Xho*I digest of pET28a*VuDREB2A*.

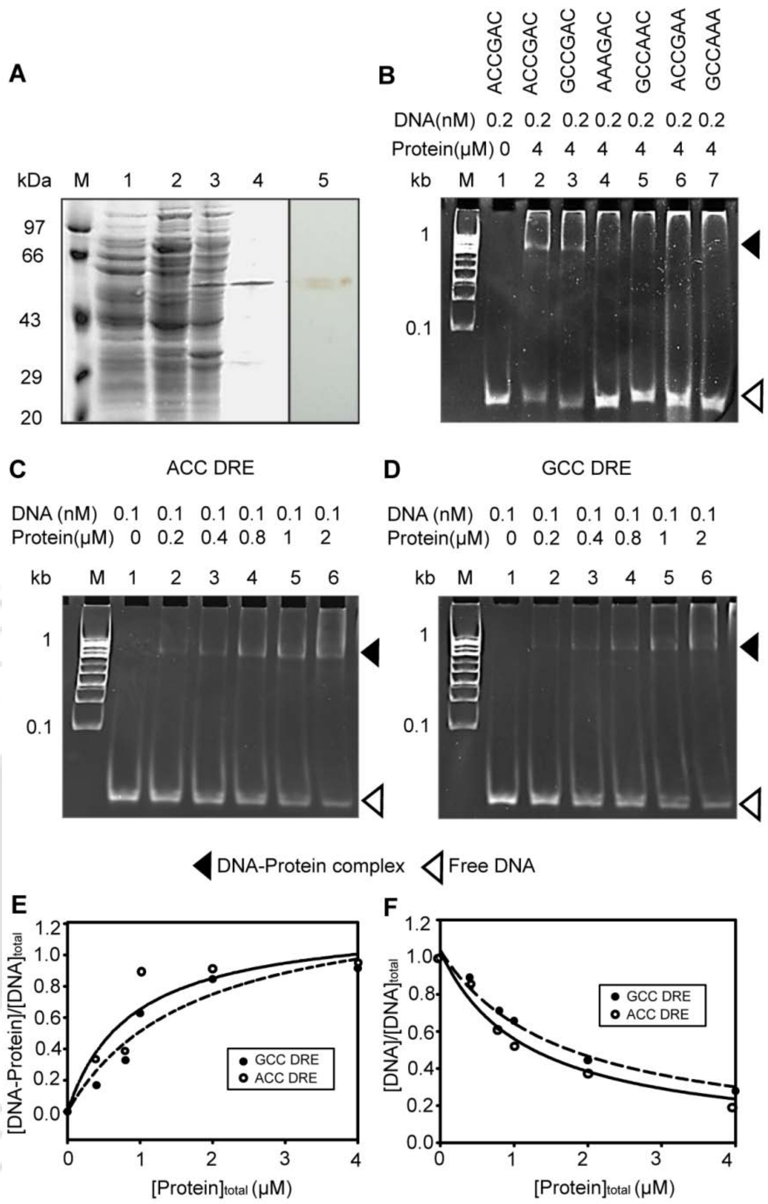


Figure 4.12 Electrophoretic mobility shift assay of VuDREB2A for binding ability to DRE sequences. VuDREB2A protein His×6-tagged at both the N- and C-termini was expressed in *E. coli* BL21 (DE3) cells, purified with a Ni-NTA agarose affinity column, and then checked for quality by SDS-PAGE [**A**; lane M: protein size markers, lane 1: crude extract of BL21(DE3), lane 2: crude extract of BL21(DE3)pET28a, lane 3: crude extract of BL21(DE3) pET28a*VuDREB2A*, lane 4: purified *E. coli* expressing VuDREB2A (with Ni-NTA agarose affinity chromatography)] and western blotting with anti-His antibody (**A**; lane 5). Electrophoretic Mobility Shift Assay in Native-PAGE of recombinant VuDREB2A with oligonucleotides carrying ACCGAC or GCCGAC dehydration-responsive element (DRE) sequences from the *Arabidopsis* rd29A promoter with VuDREB2A protein (**B**). Titration of fixed amounts of ACC (**C**) and GCC (**D**) DRE oligonucleotides with different amounts of recombinant VuDREB2A protein. "M" in all electrophoresis gels indicates molecular mass markers. Black arrow-heads indicate positions of the DNA-protein complexes while white arrow-heads indicate

positions of free DNA. Plots of fraction of bound DNA versus total protein (**E**) and fraction of free DNA versus total protein (**F**) for the ACC and GCC DREs as calculated by densitometry from (**C**) and (**D**).

4.4 Enhancement of drought- and heat-resistance through induction of stress-responsive genes in *Arabidopsis* overexpressing *VuDREB2A*

4.4.1 Improvement of drought- and heat-resistance of the transgenic plants

To further evaluate the function of *VuDREB2A* in plant, a putative negative regulatory domain between amino acid residues 132 and 182 (described in section 4.1.2) was deleted successfully (Fig. 4.13). Both the *35S:VUDREB2A full length (FL)* and *35S:VuDREB2A truncated* (*TR*) genes were cloned into plant expression vectors under control of the CaMV35S promoter (Fig. 4.14) and introduced into the Col-0 wild type *Arabidopsis* generating ten independent lines of each. Drought resistance was analyzed at the T_2 generation of the transgenic lines highly expressing *VuDREB2A* (Fig. 4.15B).

Two *FL* and one *TR* transgenic lines were exposed to drought stress and their survival rates were compared with the wild type Col-0 (Fig. 4.15C). Both types of transgenic lines showed greater than 85 % survival rates following drought recovery, while no more than 30 % of the wild type plants survived (Fig. 4.15C), indicating that *VuDREB2A* could enhance drought-tolerance in *Arabidopsis*. Supporting this observation, the rates of water loss from the transgenic lines were found to be slower than that from Col-0 (Appendix Fig. A4). Similarly, the survival rates of the transgenic plants increased significantly than the wild-type after application of 30 min heat shock (50 °C) (Fig. 4.16).

4.4.2 Dwarf phenotype of *Arabidopsis* overexpressing truncated *VuDREB2A*

In the control (no stress) growth conditions, the *FL* transgenic lines grew comparably to Col-0, while several *TR* transgenic lines showed a typical dwarf phenotype (Fig. 4.17). The *TR* transgenic line had shorter roots on gel medium (Fig. 4.17A, C) and delayed bolting in pot culture (Fig. 4.17B). This suggested that the negative regulatory domain of VuDREB2A is functional since the removal of this domain had a negative impact on growth, as was previously identified in *Arabidopsis* overexpressors (Sakuma *et al*. 2006; Mizoi *et al*. 2013).

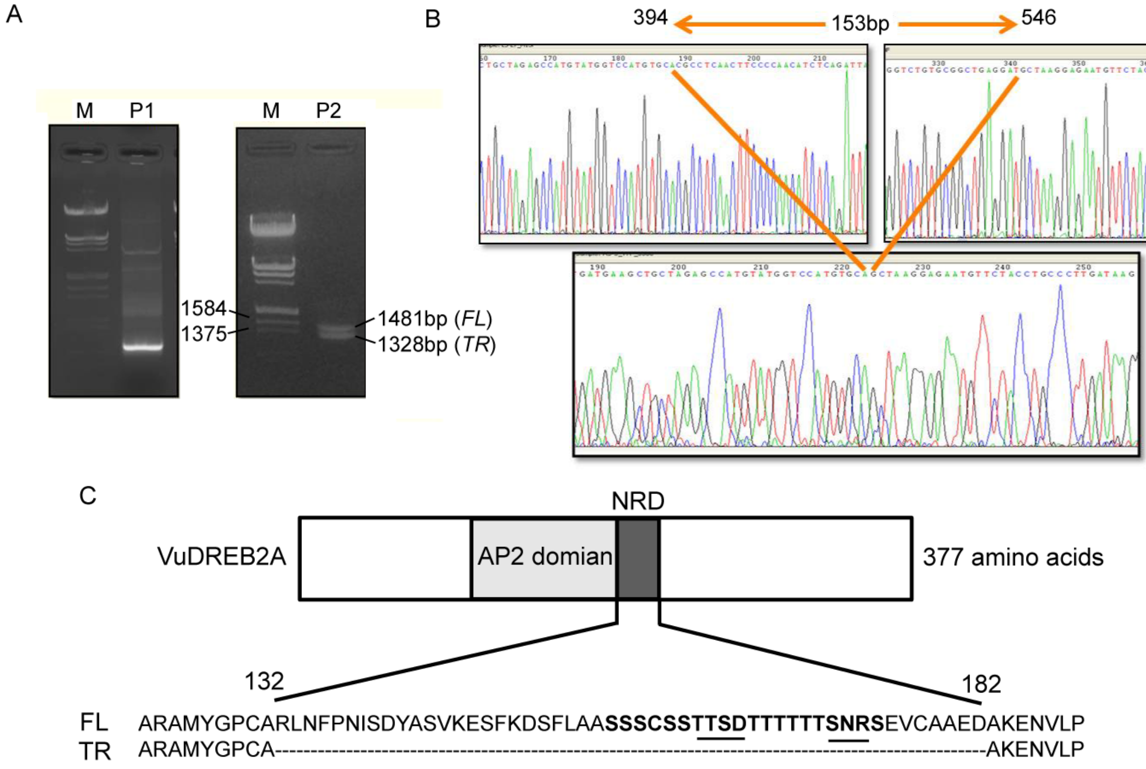


Figure 4.13 Truncation of the putative negative regulatory domain of VuDREB2A. The putative negative regulatory domain (between nucleotides 394 and 546) was deleted in the wild type full length (FL) *VuDREB2A* was removed to prepare a truncated (TR) putative active form. **A** Gel photos showing the amplification products of the 2 steps of the megaprimer PCR (see Methods). M: DNA size marker; lane P1: product of the first step of megaprimer PCR (Ta 39 °C); lane P2: product of the first step of megaprimer PCR (Ta 60 °C) showing amplification of both the 1481 bp full length and 1328 bp truncated *VuDREB2A* cDNA. **B** The sequencing analysis of the DNA in the two bands in lane P2 at the junction of the deleted region. **C** Region in the protein sequence of VuDREB2A (amino acids 132 and 182) which was deleted. This deleted region contains a poor PEST motif (PEST score -22.19) RLNFPNISDYASV and a serine/threonine-rich region SSSCSSTTSDTTTTTTSNRS containing predicted protein kinase C (TTSD) and casein kinase II (SNR) recognition sites (underlined).

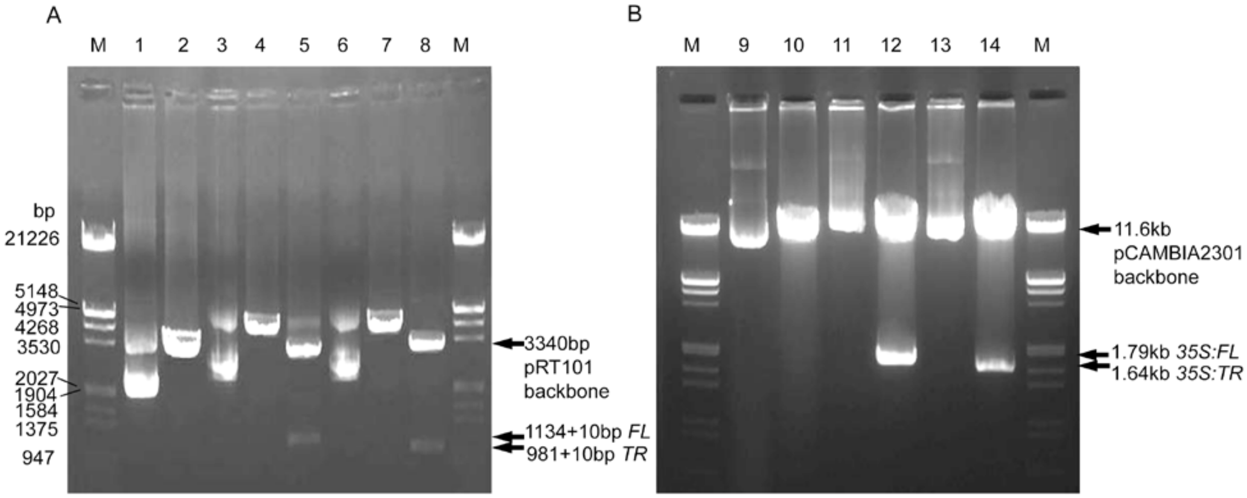


Figure 4.14 Cloning of the full length and truncated *VuDREB2A* into plant expression vectors. **A** The full length and truncated *VuDREB2A* was cloned into pRT101 between *Eco*RI & *BamH*I sites for adding the CaMV 35S promoter and polyadenylation signal (Fig. 3.6). Undigested pRT101 (lane 1) and *Eco*RI & *Bam*HI digest (lane 2) of pRT101; undigested (lane 3), *Eco*RI digest (lane 4) and *Eco*RI & *BamH*I digest (lane 5) of pRT101-*VuDREB2A full length* (*FL*) releasing the *VuDREB2A TR* of 1134 bp + 10 bp (restriction enzyme sticky ends); undigested (lane 6), *Eco*RI digest (lane 7) and *Eco*RI & *Bam*HI digest (lane 8) of pRT101-*VuDREB2A truncated* (*TR*) releasing the *VuDREB2A TR* of 981 bp + 10 bp (restriction enzyme sticky ends). **B** The 35S promoter::*VuDREB2A(FL/TR)*::polyA cassette was digested out of pRT101 by *Pst*I and cloned into plant binary vector pCAMBIA2301. Undigested (lane 9) and *Pst*I digest (lane 10) of pCAMBIA2301 (lane 9); undigested (lane 11), *Pst*I digest (lane 12) of pCAMBIA2301-35S::*VuDREB2A FL*::polyA releasing the 1.79 Kb cassette; undigested (lane 13), *Pst*I digest (lane 12) of pCAMBIA2301-35S::*VuDREB2A TR*::polyA releasing the 1.64 Kb cassette.

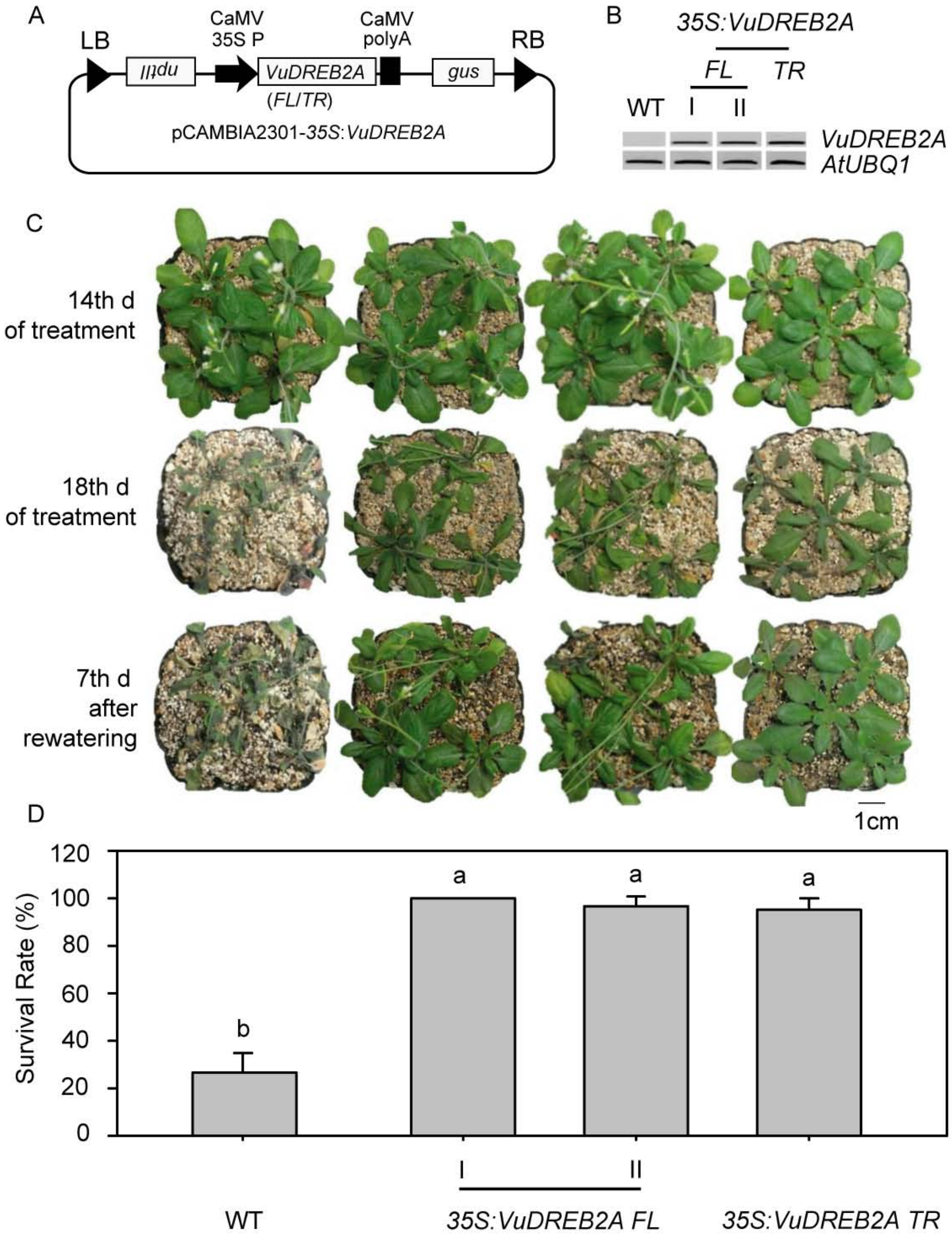


Figure 4.15 Drought-response of transgenic *Arabidopsis* carrying ectopically expressed *VuDREB2A*. Full length (*FL*) or truncated (*TR*; see Fig 4.13) *VuDREB2A* under the control of the cauliflower mosaic virus 35S promoter cloned into plant expression vector pCAMBIA2301 (**A**) were introduced into *Arabidopsis* ecotype Col-0 (WT). Transgenic lines expressing VuDREB2A, as determined by semiquantitative RT-PCR using *AtUBQ1* as an internal control (**B**), were grown in pot culture after 15 d of pre-culture on ½ MS media with 1 % sucrose. Ten plants from each line were planted in pots containing soil and were grown for a further 10 d with the bottoms of the pots immersed in nutrient solution in plastic trays. Then, the nutrient solutions were removed to impose dehydration stress, and the damage to the transgenic lines and WT on the 14th and 18th d, and the recovery from damage after 7 d of rewatering were photographed (**C**;

Bar = 1 cm). Survival rates of the wild type and transgenic plants were determined from three independent assays (**D**). Results are presented as means ± SE (n = 3 biological replicates, 10 plants per replicate). **a–b**: there are no significant differences (Tukey's test, $P \geq 0.05$) between the lines with the same characters, and significant differences (Tukey's test, $P < 0.05$) between lines with different characters. I and II represent two independent transgenic lines carrying *full length* (*FL*) *VuDREB2A*, while *TR* indicates a *truncated VuDREB2A* line. LB and RB: left and right borders of the T-DNA; *nptII*: neomycin phosphotransferase II; *gus*: ß-glucuronidase.

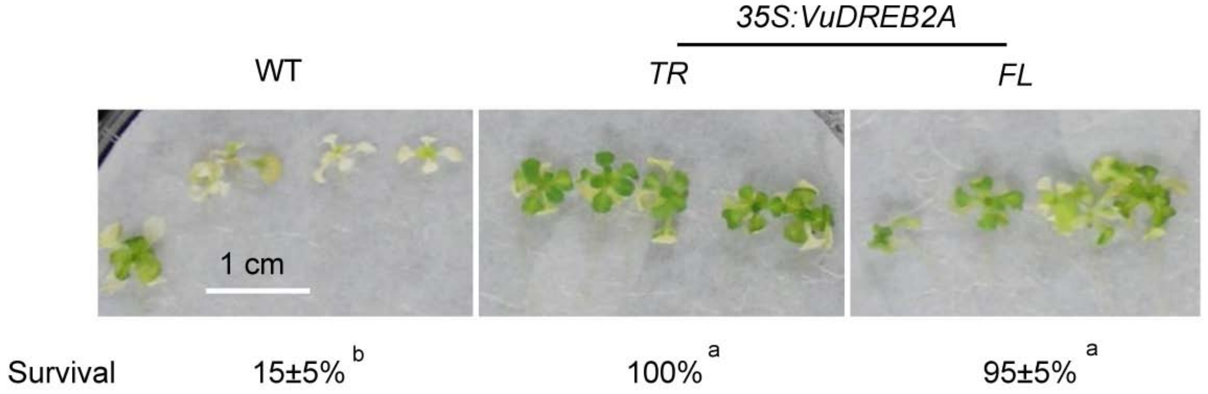


Figure 4.16 Heat shock-response of transgenic *Arabidopsis* carrying ectopically expressed *VuDREB2A*. Wild type or transgenic *Arabidopsis* overexpressing *full length* (*FL*) or *truncated* (*TR*) *VuDREB2A* were grown in ½ MS agar media for 7 d and then transferred to wet filter paper, grown at 22 °C for additional 2 d, incubated at 50 °C for 30 min, again grown at 22 °C for 7 d and photographed. Survival rates of the wild type and transgenic plants were determined from three independent assays. Results are presented as means ± SE (n = 3 biological replicates, 10 plants per replicate). **a–b**: there are no significant differences (Tukey's test, $P \geq 0.05$) between the lines with the same characters, and significant differences (Tukey's test, $P < 0.05$) between lines with different characters.

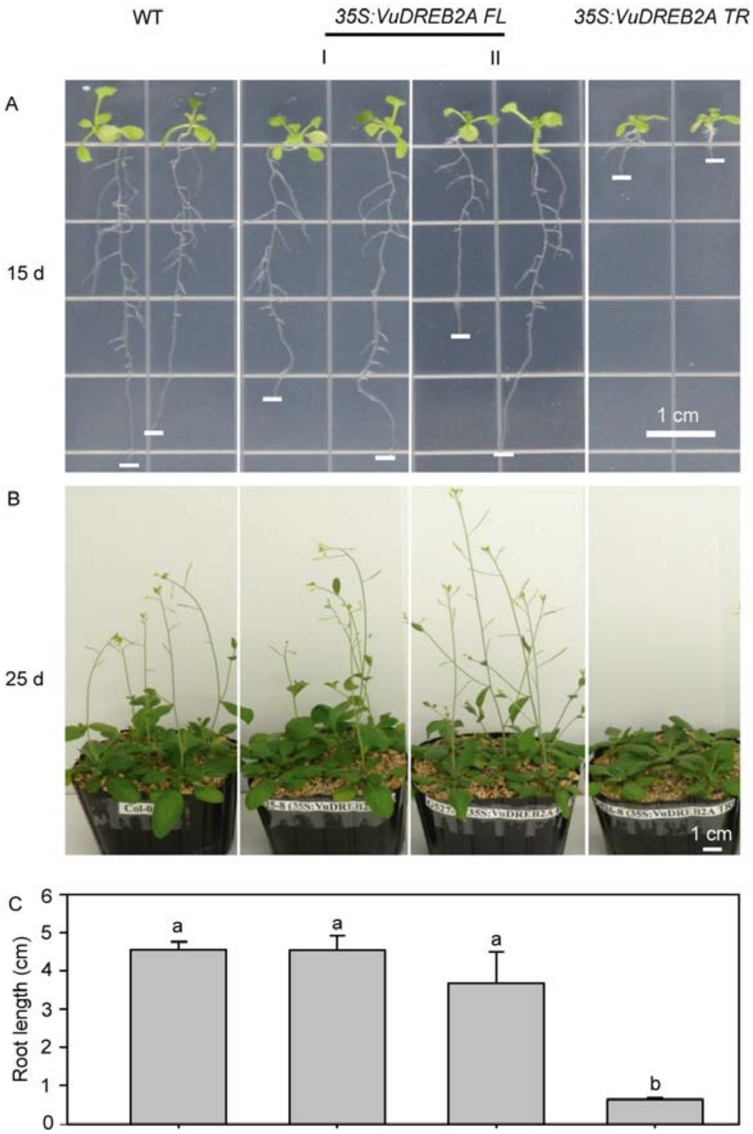


Figure 4.17 Growth of transgenic *Arabidopsis* overexpressing *VuDREB2A* under control conditions. WT (Col-0) and transgenic lines carrying *VuDREB2A* in *full length* (*FL*) form or *truncated* (*TR*; see Fig. 3) were grown in ½ strength MS medium with 1 % sucrose for 15 d (**A**), and in soil for another 10 d (**B**). I and II represent two independent transgenic lines carrying *full length* (*FL*) *VuDREB2A*, while *TR* indicates a *truncated VuDREB2A* line. Root lengths of the transgenic plants grown in media for 15 d (**C**) are presented as means ± SE (n = 10 plants per line). **a–b**: there are no significant differences (Tukey's test, $P \geq 0.05$) between the lines with the same characters, and significant differences (Tukey's test, $P < 0.05$) between lines with different characters.

4.4.3 DRE-dependent activation of transcription in the *35:VuDREB2A* transgenic plants

To understand the effects of *VuDREB2A* overexpression in *Arabidopsis*, the gene expression profiles of the transgenic plants were analyzed using an *Arabidopsis* microarray. The overexpression of *FL* and *TR VuDREB2A* caused up-regulation (fold change ≥ 3) of 15 and 6 genes, respectively, under unstressed conditions (signal intensity > 200, *t*-test *P*-value < 0.05) with 2 genes in common (Table 4.2). Several stress-responsive genes which were up-regulated in the *35S:VuDREB2A* plants (Table 4.2), were also up-regulated in *Arabidopsis* overexpressing full length or constitutively active forms of *AtDREB2A* and *GmDREB2A;2* (Sakuma *et al*. 2002, Mizoi *et al*. 2013). The results from the *FL* and *TR* plants were almost correlated (Fig. 4.18) suggesting similar target recognition by both forms of the *VuDREB2A* gene.

The up-regulated genes were found to be responsive to various stress conditions (drought, salinity, osmotic stress, oxidative stress, heat and ABA, in particular) in publicly available microarray data (Table 4.2; fold change > 3 in public array). This trend was common among the top 200 up-regulated genes (Appendix Table A9) in the transgenic lines in the meta-profile analysis (Fig. 4.19). The stress-responsive nature of the target genes could account for the improvement of drought-tolerance in transgenic *Arabidopsis*. Particular prevalence of heat shock proteins (HSPs) and heat shock transcription factors (HSFs) among the up-regulated genes (Table 4.2 and Appendix Table A9) could account for the tolerance of the transgenic plants to heat-shock (Fig. 4.16). The expression of genes found to be up-regulated in the *VuDREB2A* overexpressors from our microarray results were further confirmed by qRT-PCR (Fig. 4.20). Certain genes like PDF1.3 and HSP70 had significantly higher expression in the *35S:VuDREB2A TR* plants than *35S:VuDREB2A FL* plants (Fig. 4.20). This was a similar phenomenon as that associated with the constitutive overexpression of active forms of *Arabidopsis* DREB2A (Sakuma *et al*. 2006) and soybean *GmDREB2A;2* (Mizoi *et al*. 2013). This observation together with the dwarf phenotype (Fig. 4.17) led to the conclusion that the truncated VuDREB2A functioned as an active form of the protein in *Arabidopsis*.

The promoters of the genes up-regulated by VuDREB2A were enriched for DRE and ABRE (Table 4.3), as computed by the octamer-based method (Yamamoto *et al*. 2011), further supporting similar DNA target recognition to *Arabidopsis DREB2A.* Additional enrichment of ABREs in the promoters of VuDREB2A, as found earlier in the case of active forms of *GmDREB2A;2* (Mizoi *et al.* 2013), would be the result of secondary activation of ABA-regulated genes and/or co-localization of both DRE and ABRE in the same promoter. We could not detect complexes of VuDREB2A with ABRE core sequence ACGT in EMSA (data not shown). Also, from previous studies, no DREB ortholog was found to interact with ABRE sequences directly. In *Arabidopsis*, DREB2A was shown to interact physically and cooperate with ABA-responsive element binding factor 2 (ABF2), a transcription factor involved in ABA-responsive gene expression (Lee *et al.* 2010). Such interactions could be responsible for indirect activation of genes having ABRE in their promoters.

These findings suggest that VuDREB2A is regulated in a similar manner as *Arabidopsis* DREB2A and that it plays an important role in the dehydration and heat stress response in cowpea as a canonical DREB2 ortholog.

Table 4.2 Microarray analysis of *35S:VuDREB2A Arabidopsis*

Locus	Gene symbol	TAIR10 Short Description	Fold Change[a]	Stress Response		*Arabidopsis*/Soybean[d]	DRE/ABRE[e]
				Gene Ontology[b]	Public Microarray[c]		
Genes whose expression increased ≥3-fold in the *35S:VuDREB2A (full length)* plants compared to wild type plants							
AT1G27020		Hypothetical protein	5.6			*GmFL*	A1
AT4G12490		Bifunctional inhibitor/lipid-transfer protein/ seed storage 2S albumin-like protein	5.5		C, SA, Wo		
AT4G23600	COR13	Cystine lyase	5.2	Os, S, Wo	D, H, Os, S, Wo, MeJA, ABA	*GmΔ2*	A1, A3, D1
AT2G26010	PDF1.3	Defensin-like protein 14	4.5				D1
AT3G50480	HR4	RPW8-like protein 4	4.3		Os, Ox, SA		A1, A4
AT1G77120	ADH1	Alcohol dehydrogenase class-P	3.9	D, Os, S	D, C, Os, S, ABA, SA		A1, A2, A3, A4, A5
AT3G45140	LOX2	lipoxygenase 2	3.9	H, Os, S, W, C, Wo	D, Wo, MeJA, SA	*GmΔ2*	A1
AT1G43910		P-loop containing nucleoside triphosphate hydrolase-like protein	3.9		D, S, SA		A1
AT1G07400		Class I heat shock protein	3.9	H, Ox	D, H, Os, S, H, ABA, SA		
AT1G02930	GSTF6	Glutathione S-transferase 1	3.8	Os, Ox, S, W	D, Os, S, C, Ox, Wo		D1, A1, A3, A4
AT5G22460		Esterase/lipase/thioesterase family protein	3.7		D, Os, S, ABA		
AT1G09240	NAS3	Nicotianamine synthase 3	3.5		D, C, ABA		
AT5G51440		Heat shock protein 23.5	3.4	H, Os, Ox	D, H, S, Ox, SA		D1, A1
AT5G52640	HSP90.1	Heat shock protein 81-1	3.1	H, Ox,	D, H, Os, ABA, SA	*GmΔ2*	A1
AT5G13170	SAG29	Senescence-associated protein 29	3.0	Os	D, H, Os, ABA, SA		A1, A3
Genes whose expression increased ≥3-fold in the *35S:VuDREB2A (truncated)* plants compared to wild type plants							
AT2G26010	PDF1.3	Defensin-like protein 14	22.5				D2
AT5G44420	PDF1.2	Defensin-like protein 16	17.6	Wo	Os, MeJA		D1, A1, A3
AT1G07400		Class I heat shock protein	3.9	H, Ox			
AT5G52640	HSP90.1	Heat shock protein 81-1	3.7	H, Ox,	D, H, Os, Ox, ABA	*GmΔ2*	A1
AT5G48570	*ROF2*	Peptidylprolyl isomerase	3.2	Ox, H	D, H, Os, S, Ox, ABA	*At FL*	
AT3G12580	*HSP70*	Heat shock protein 70-4	3.1	Ox, H	D, H, Os, S, Ox, ABA, SA	*AtCA, GmFL&Δ2*	D1

[a] Ratio of fluorescence intensity of each cRNA of *35S:VuDREB2A(FL/TR)* /fluorescence intensity of each cRNA of wild type. Average data of biological replicates (*t*-test $P < 0.05$, signal intensity > 200).

[b] Gene Ontology based annotations for genes responsive to stress.

[c] Genes upregulated more than 3 fold in public microarray experiments with stress/phytohormones (data mined with Genevestigator).
Os, osmotic stress; S, salt stress; D, drought stress; W, water deprivation; H, heat; C, cold; Wo, wound; Ox, oxidative stress; ABA, abscissic acid; MeJA, methyl jasmonate; SA, salicylic acid.

[d] Genes that were also up-regulated in transgenic *Arabidopsis* overexpressing the *Arabidopsis* and soybean homologs of *DREB2A. AtFL, 35S:AtDREB2A FL; AtCA, 35S:AtDREB2A CA; Gm FL, 35S:GmDREB2A;2 FL and GmΔ2, 35S:GmDREB2A;2 Δ2* (Mizoi *et al.* 2013).

[e] ABRE/DRE motifs within 1Kb upstream of transcription start site of the genes. ABRE consensus sequences from PLACE database: A1, ACGTG; A2, CACGTGGC; A3, ACGTSSSC; A4, MACGYGB; A5, CCACGTGG. DRE consensus sequences: D1, RCCGAC; D2, ACCGAGA.

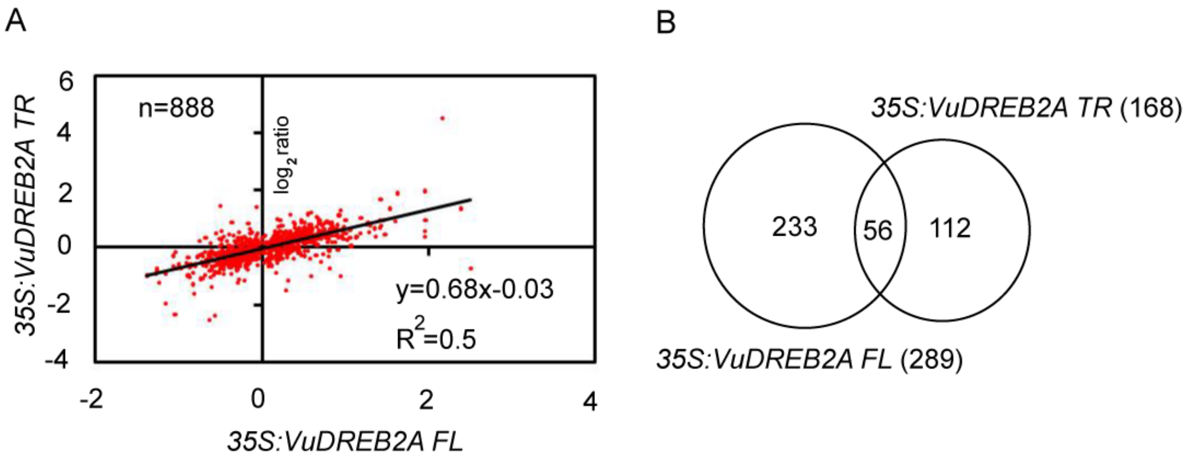


Figure 4.18 Comparison between the target genes of *full length* (*FL*) and *truncated* (*TR*) *VuDREB2A* in transgenic *Arabidopsis* A Correlation analysis between significantly (signal intensity > 200, *t*-test *P* < 0.05) up- and down-regulated genes of full length (*FL*) and truncated (*TR*) *VuDREB2A* overexpressing plants. Scatter plot with regression equation and correlation coefficient shown. **B** Overlapping genes between *35S:VuDREB2A FL* and *TR* which were up-regulated more than 1.5 fold, are shown as Venn diagram.

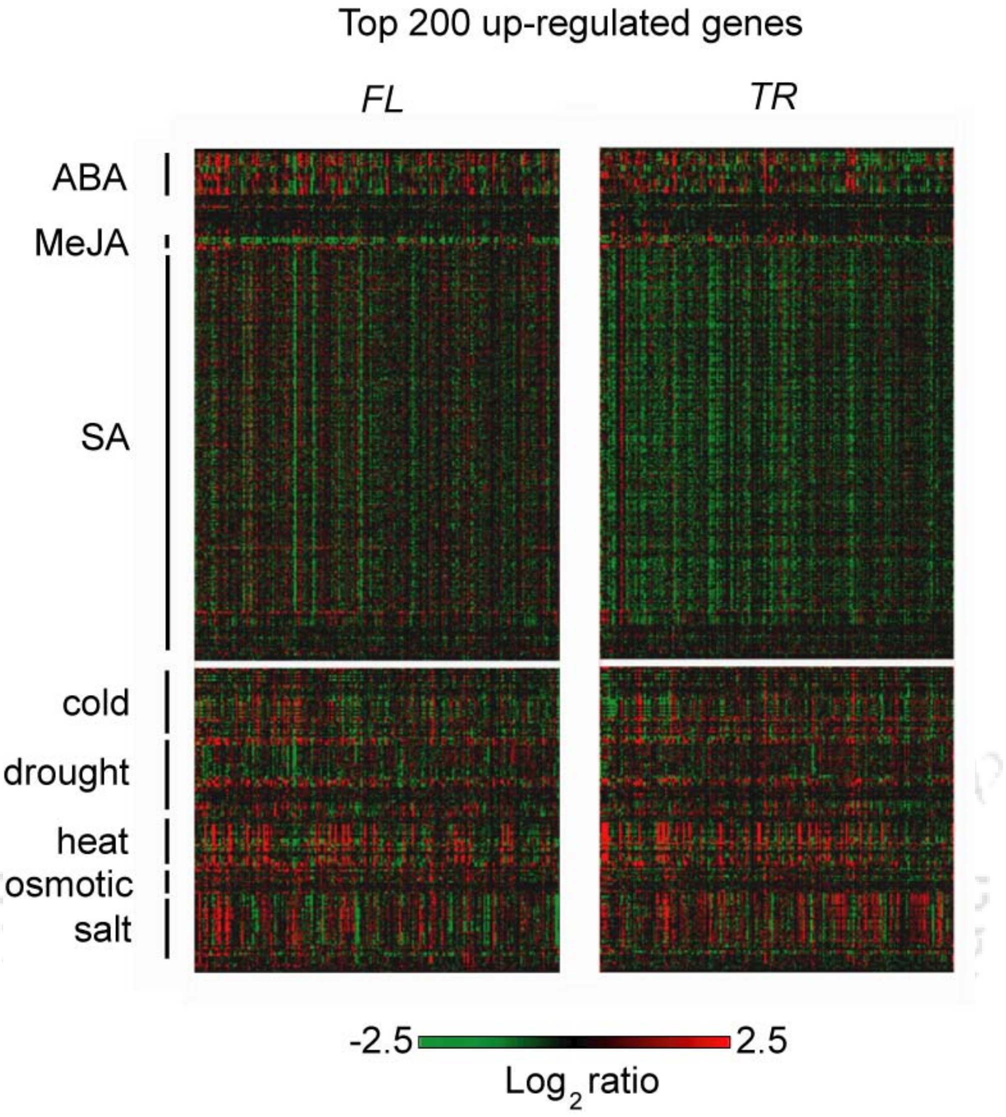


Figure 4.19 Meta-profile analyses of up-regulated genes in *Arabidopsis* carrying *VuDREB2A*. Induction ($\log_2$) ratios of the top 200 up-regulated genes in *35S:VuDREB2A full length* (*FL*) and *truncated* (*TR*) plants in control conditions were compared with public microarray experiments with cold, heat and osmotic stressed conditions and phytohormones; the results are shown with a heat map. The experiments with hormones/stress are in the *y*-axis and genes in the *x*-axis (in order of decreasing fold change in *VuDREB2A* overexpressing plants from left to right).

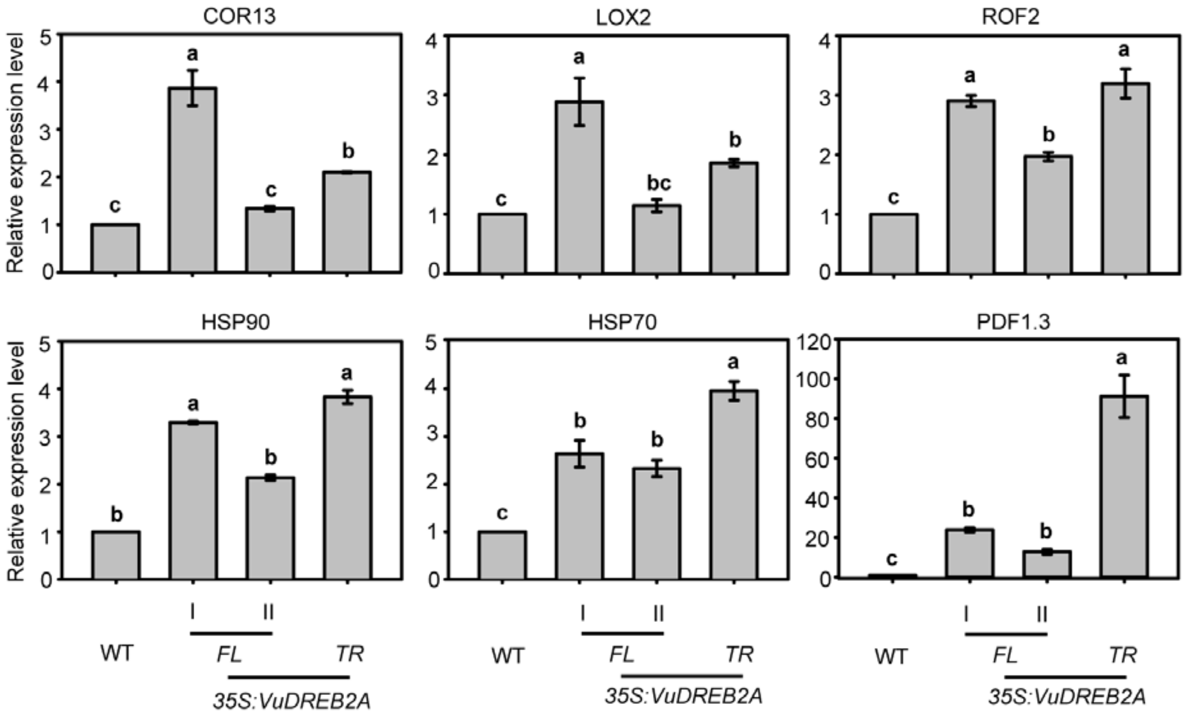


Figure 4.20 Quantification of the expression levels of *VuDREB2A* target genes in *Arabidopsis*. The expression levels of up-regulated genes in *35S:VuDREB2A FL* and *TR* plants in the microarray experiment were quantified by real-time qRT-PCR. Columns indicate relative expression levels normalized against the levels of *AtUBQ1* (means ± SE of $n = 3$ biological replicates). I and II represent two independent transgenic lines carrying *full length* (*FL*) *VuDREB2A*, while *TR* indicates a *truncated VuDREB2A* line. Results are presented as means ± SE ($n = 3$ biological replicates, 10 plants per replicate). **a–c**: there are no significant differences (Tukey's test, $P \geq 0.05$) between the lines with the same characters, and significant differences (Tukey's test, $P < 0.05$) between lines with different characters.

Table 4.3 Overrepresentation analysis of octamer sequences in the promoters of top 200 up-regulated genes in *35S:VuDREB2A* transgenic plants

Octamer[a]	Motif	Occurance in 24956 genes	Occurance in 200 genes[b]	RARf [c]	P-Value (Two Sided Fisher's ExactTest)
VuDREB2A FL transgenic *Arabidopsis*					
CGGTCGGC	DRE	24	2	10.4	0.01834
GCGGTCGG	DRE	30	2	8.3	0.02711
GCGTCGGG	DRE	32	2	7.8	0.03034
CGTCGGTC	DRE	76	3	4.9	0.02595
CTGTCGGA	DRE	78	3	4.8	0.02767
CGGTCGGT	DRE	79	3	4.7	0.02855
TGACCGAC	DRE	120	4	4.2	0.01823
CTACCGAC	DRE	90	3	4.2	0.03923
TAGTCGGA	DRE	127	4	3.9	0.0218
GTCGGTCA	DRE	143	4	3.5	0.03143
GACGTCTG	ABRE	62	3	6.0	0.01557
CTGTACGT	ABRE	84	4	5.9	0.00569
ACGTCTCG	ABRE	69	3	5.4	0.0204
CTACGTGC	ABRE	74	3	5.1	0.02429
CTGAACGT	ABRE	74	3	5.1	0.02429
GAACGTTC	ABRE	124	5	5.0	0.00408
GCACGTAG	ABRE	78	3	4.8	0.02767
CGTACGTT	ABRE	193	7	4.5	0.00132
TAGACGTC	ABRE	113	4	4.4	0.01506
ACGTCGCT	ABRE	85	3	4.4	0.03416
CCGTACGT	ABRE	85	3	4.4	0.03416
TACGTGCT	ABRE	85	3	4.4	0.03416
TCTGACGT	ABRE	181	6	4.1	0.00436
ACGTACGG	ABRE	91	3	4.1	0.04028
AGCACGTA	ABRE	92	3	4.1	0.04136
GCACGTAT	ABRE	96	3	3.9	0.04578
GCTACGTG	ABRE	96	3	3.9	0.04578
GTACGTTT	ABRE	303	9	3.7	0.00115
TGAACGTA	ABRE	174	5	3.6	0.01542
ACGTGTGA	ABRE	315	9	3.6	0.00149
GACGTTTC	ABRE	247	7	3.5	0.00495
AATACGTA	ABRE	468	13	3.5	0.00021
CCACGTAA	ABRE	290	8	3.4	0.00325
TCGTACGT	ABRE	183	5	3.4	0.01864
CACGTAAA	ABRE	415	11	3.3	0.00087
CTACGTAC	ABRE	190	5	3.3	0.02142
ACGTGGCC	ABRE	154	4	3.2	0.03929
ACGTTTCA	ABRE	394	10	3.2	0.00197
CTACGTTA	ABRE	158	4	3.2	0.0424
ACGTCAAG	ABRE	198	5	3.2	0.02491
CACGTCTC	ABRE	238	6	3.1	0.01489
ACACGTCT	ABRE	239	6	3.1	0.01516
ACGTCAAC	ABRE	241	6	3.1	0.01571
TACAACGT	ABRE	241	6	3.1	0.01571
TACGTACT	ABRE	241	6	3.1	0.01571
TTCTACGT	ABRE	283	7	3.1	0.00985

Octamer[a]	Motif	Occurance in 24956 genes	Occurance in 200 genes[b]	RARf [c]	P-Value (Two Sided Fisher's ExactTest)
VuDREB2A TR transgenic *Arabidopsis*					
AGTCGGCC	DRE	47	3	8.0	0.00764
CCCGTCGG	DRE	33	2	7.6	0.03201
CGTCGGTA	DRE	65	3	5.8	0.01755
CCGACATG	DRE	134	6	5.6	0.00103
CTACCGAC	DRE	90	4	5.5	0.00716
TAGTCGGC	DRE	68	3	5.5	0.01966
TACCGACG	DRE	77	3	4.9	0.0268
GCCGACAC	DRE	78	3	4.8	0.02767
GGACCGAC	DRE	119	4	4.2	0.01776
GGTCGGAA	DRE	120	4	4.2	0.01823
ATTGTCGG	DRE	171	5	3.6	0.01444
CTCCGACT	DRE	137	4	3.6	0.02757
TTCCGACG	DRE	149	4	3.3	0.03559
TACCGACA	DRE	154	4	3.2	0.03929
TTGCCGAC	DRE	161	4	3.1	0.04481
CGGACGTT	ABRE	59	4	8.5	0.00171
TAGACGTC	ABRE	113	6	6.6	0.00044
ACGTTGCG	ABRE	57	3	6.6	0.01257
ACGTGGCC	ABRE	154	8	6.5	0.00006
GAACGTCC	ABRE	62	3	6.0	0.01557
CACTACGT	ABRE	125	6	6.0	0.00073
GACGTCTA	ABRE	106	5	5.9	0.00214
AGACGTTC	ABRE	110	5	5.7	0.0025
TGAACGTC	ABRE	118	5	5.3	0.00333
AGACGTCT	ABRE	125	5	5.0	0.00421
CCACGTAC	ABRE	101	4	4.9	0.01047
CGACGTTA	ABRE	130	5	4.8	0.00494
GCACGTAG	ABRE	78	3	4.8	0.02767
CGTACGTC	ABRE	79	3	4.7	0.02855
ATAGACGT	ABRE	188	7	4.6	0.00115
CACGTCCT	ABRE	108	4	4.6	0.01302
TCGACGTG	ABRE	108	4	4.6	0.01302
GTGCACGT	ABRE	83	3	4.5	0.03223
TACGTGGG	ABRE	87	3	4.3	0.03614
TCACGTCC	ABRE	120	4	4.2	0.01823
ATGAACGT	ABRE	253	8	3.9	0.00145
CGACGTGT	ABRE	127	4	3.9	0.0218
GTACGTCA	ABRE	98	3	3.8	0.04808
ACGTAAAC	ABRE	458	14	3.8	0.00005
CCCACGTA	ABRE	99	3	3.8	0.04925
TCCACGTA	ABRE	268	8	3.7	0.00204
AACGTAAA	ABRE	804	24	3.7	0
TACGTCAC	ABRE	172	5	3.6	0.01476
ATTACGTC	ABRE	208	6	3.6	0.00823
CACGTACG	ABRE	139	4	3.6	0.02882
TGAACGTA	ABRE	174	5	3.6	0.01542
GTAGACGT	ABRE	141	4	3.5	0.03011
CGCCACGT	ABRE	286	8	3.5	0.003
AGACGTGG	ABRE	179	5	3.5	0.01716
ATCGACGT	ABRE	149	4	3.3	0.03559
CACAACGT	ABRE	187	5	3.3	0.02019
ACGTCACG	ABRE	150	4	3.3	0.03631
GGAGACGT	ABRE	150	4	3.3	0.03631
TCACGTGT	ABRE	340	9	3.3	0.00245
CAAACGTC	ABRE	228	6	3.3	0.01236
ACGTGTCC	ABRE	229	6	3.3	0.0126
CGTACGTT	ABRE	193	5	3.2	0.02269

[a] Overrepresented octamers containing DRE core motif (CCGAC/ its reverse complement) and ABRE motif (ACGT). [b] Top 200 genes up-regulated in *35S:VuDREB2A* plants. [c] Relative appearance ratio (RAR) of each octamer = (count of the octamer in promoters of top 200 up-regulated genes/ 200)/ (count of the octamer in total 24956 promoters/ 24956). RARf = filtered RAR ($P < 0.05$ and RAR > 3.0).

CHAPTER 5

CONCLUDING REMARKS

5.1 Significance of the study and its key findings

DREB2 genes are well-known for their potential roles in drought, salinity and heat tolerance and are conserved across a wide range of higher plants. Keeping in view the ever increasing problem of drought in agriculture, the use of valuable homolog of tolerance genes from natural drought-tolerant species cowpea would provide immense promise for sustainable agriculture in the future. Particularly, cowpea has been predicted to play a big role in ‘drought-proofing’ global food and fodder supplies for the future (Avery 2011). Unlike other legumes like common bean and soybean, studies on the molecular mechanisms for drought tolerance are scarce in cowpea, which is poorly characterized at the genomic level. Extensive breeding efforts for genetic improvement of cowpea for drought tolerance are being perused at the International Institute for Tropical Agriculture (IITA) and the Texas A&M University. Genetic enhancement of cowpea for drought tolerance by incorporating tolerance genes into early maturity cowpea lines has been proposed to be the most cost-effective method for stable crop yield in changing climates (Agbicodo *et al*. 2009). However there are scanty reports on cowpea genes conferring drought tolerance. One approach in such gene discovery is to identify candidate genes known to be relevant to drought tolerance from previous studies in other crops and test its functionality in cowpea.

Here an attempt has been made to isolate a functional *DREB2A* ortholog from cowpea, engineer its active form by deletion of a negative regulatory domain and assess its functionality in *Arabidopsis* by constitutive overexpression. The key conclusions drawn from this work are:

1. *VuDREB2A* exists as a canonical *DREB2A* ortholog in cowpea.
2. *VuDREB2A* is highly induced in desiccation, salinity and heat stress, slightly by exogenous ABA, MeJA and SA, and in prolonged low temperature treatment.
3. The *VuDREB2A* promoter carries *cis*-elements responsive to stress and phytohormones viz. ABRE, CE3, HSE, CGTCA motif, LTE, etc.
4. VuDREB2A exhibit *in vitro* binding to DRE from *Arabidopsis* promoters.

5. *VuDREB2A* can enhance drought and heat resistance in transgenic *Arabidopsis* by induction of many stress-responsive genes having DRE and/or ABRE in their promoters

6. VuDREB2A carries a functional negative regulatory domain similar to *Arabidopsis* DREB2A, removal of which leads to a dwarf phenotype and enhanced induction of some downstream target genes.

5.2 Future Perspectives

This cloned *VuDREB2A* could find use in molecular breeding to improve drought tolerance in cowpea and other related legume crops. Direct association of drought tolerance with a single nucleotide polymorphism (SNP) was identified in foxtail millet (*Setaria italica* L., *SiDREB2*) (Lata *et al.* 2011a). Sequencing the CDS and/or the regulatory elements of the *VuDREB2A* gene in cowpea varieties contrasting in drought tolerance, will allow us to examine whether the VuDREB2A locus determines drought tolerance in cowpea varieties. Identification of functional markers for such well characterized genes like *VuDREB2A* will open ways for marker assisted selection of drought resistance in cowpea. Drought tolerance has been described to be a potent target for comparative plant genomics, since this approach has the potential to unravel key genetic determinants of an inherently complex physiological process (Bennetzen 2000). Recent progress in genome-sequencing of cowpea (e.g. Timko *et al.* 2008) will soon make possible genome-wide analysis of DREB homologs in cowpea, as well as comparison with other drought-susceptible legume species. However, our lab has developed efficient genetic transformation methods in cowpea (Solleti *et al*. 2008a; Bakshi *et al.* 2011; Bakshi *et al*. 2012) and developed transgenic cowpea for storage pest resistance (Solleti *et al.* 2008b) and salt tolerance (Mishra *et al.* 2014). Therefore, implementing the *cis*-genic approach by overexpressing *VuDREB2A* in cowpea is expected to enhance drought tolerance in this crop.

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APPENDIX

General reagents: Di-sodium hydrogen phosphate, glycerol, hydrochloric acid, isopropanol, methanol, potassium acetate, ethanol, sodium chloride, sucrose and all salts for plant growth media (all of AR grade) – from SRL, India and Merck, India and Merck, Japan.

Molecular biology grade reagents/ chemicals: Acrylamide, APS, bis-acrylamide, tris base, EDTA, IPTG, TEMED, DAB, CTAB, phenol, chloroform, hydrogen peroxide, tween 20, triton X 100, (+)-*cis, trans*-ABA, methyl jasmonate, salicylic acid – from Sigma Aldrich, USA or SRL, India. Magnesium chloride, boric acid, SDS – from Merck, India. Ampicillin and kanamycin from Duchefa Biochemie, The Netherlands.

Components for bacterial culture media: Bactoagar, tryptone and yeast extract from Himedia, India.

Enzymes: ExTaq and Primestar Max DNA polymerase, SYBR Green PCR master mix II from Takara-Bio, Japan. *Bam*HI, *Xho*I, *Eco*RI, *Pst*I, *Hind*III from Fermentas, USA. T4 DNA ligase and *Nde*I from NEB, UK. ReverTraAce reverse transcriptase from Toyobo, Japan.

Blotting membranes: Hybond™-P hydrophobic polyvinylidene difluoride (PVDF) membrane from GE Healthcare Bio-sciences Corp., USA.

DNA and protein markers: DNA ladders from Sigma, USA, Axygen, USA, Fermentas, USA and Bangalore GeneI, India. Protein molecular weight marker (14-97 KDa) from Bangalore GeneI, India.

Plastic and Glasswares: PCR tubes – from Axygen, USA; micro pipette tips, micro centrifuge tubes, petridishes from Tarsons Products Pvt. Ltd., India and Sanplatech, Japan. Glasswares from Borosil International, India and Japan.

Table A1 List of commercial kits

Name	Use	Supplier
RNAqueous Kit	RNA isolation	Ambion, USA
GenElute™ Plasmid Miniprep Kit	Plasmid isolation	Sigma, USA
HiPurA™ Quick Gel Purification Kit	Purification of DNA from agarose gels	Himedia, India
First strand cDNA synthesis kit	cDNA synthesis from RNA	Fermentas, USA
5’ and 3’ RACE systems	5’ and 3’ RACE	Invitrogen, USA

Table A2 List of Plasmid Vectors

Name	Use	Promoter	Selection marker(s)	Cloning Site
pTZ57R/T(Fermentas)	T/A cloning	-	Amp^R	T/A
pET28a (Novagen)	Bacterial expression	T7	Kan^R	*VuDREB2A* cloned between *Bam*HI and *Xho*I
pRT101	Plant expression	CaMV35S	Amp^R	*VuDREB2A* cloned between *Eco*RI and *Bam*HI
pCAMBIA2301(CAMBIA)	Plant expression	CaMV35S (from pRT101)	Kan^R	*35S:VuDREB2A(FL/TR)* cloned at *Pst*I site

Table A3 List of Bacterial Strains

Strain	Description
Escherichia coli DH5α	F’Φ80d*lac*ZΔM15Δ(*lac*ZYA-*arg*F) U169*end*A1recA1*hsd*R17($\mathrm{r_k^-m_k^+}$) *deo*R*thi*-1*pho*A*sup*E44λ*gyr*A96*rel*A1
Escherichia coli BL21(DE3) (Novagen)	F$^-$ *omp*T *gal dcm lon hsd*S$_B$($\mathrm{r_B^-\ m_B^-}$) λ(DE3 [*lac*I *lac*UV5-T7 *gene* 1 *ind*1 *sam*7 *nin*5])
Agrobacterium tumefaciens GV3101	Resistance to Rif (10 mg/L) (in genome)

Table A4 Culture media for Bacteria

Media	Constituents	Concentration	pH
Luria Broth (LB)	Bactotryptone	1.0 %	7.2
	Yeast extract	0.5 %	
	NaCl	1.0 %	
Luria Agar (LA)	LB media		7.2
	Agar	1.5 %	

Table A5 Culture media for plant

Media	Constituents	Concentration	pH
Modified MGRL medium (Fujiwara *et al.* 1992)	$CaCl_2$	200 µM	5.5
	$MgSO_4.7H_2O$	1.5 mM	
	$Ca(NO_3)_2$	40 µM	
	KNO_3	3 mM	
	Na_2EDTA	67 µM	
	$NaNO_3$	4 mM	
	$FeSO_4$	8.6 µM	
	$MnSO_4$	10.3 µM	
	H_3BO_3	30 µM	
	ZnSO4	1 µM	
	$(NH_4)_6Mo_7O_{24}$	24 nM	
	$CoCl_2$	130 nM	
	$CuSO_4$	1 µM	
Murashige and Skoog medium (Murashige and Skoog 1962)	NH_4NO_3	825 mg/L	5.8
	KNO_3	950 mg/L	
	$CaCl_2$ $7H_2O$	220 mg/L	
	$MgSO_4$ 7H2O	185 mg/L	
	KH_2PO_4	85 mg/L	
	KI	0.415 mg/L	
	H_3BO_3	3.1 mg/L	
	$MnSO_47H_2O$	11.15 mg/L	
	$ZnSO_4$	4.3 mg/L	
	$NaMoO_4$	0.125 mg/L	
	$CuSO_4$	0.0125 mg/L	
	$CoCl_2$	0.0125 mg/L	
	$FeSO_4$ $7H_2O$	13.9 mg/L	
	Na-EDTA $2H_2O$	18.7 mg/L	
	Sucrose	10 g/L	
	Thiamine-HCl	0.1 mg/L	
	Niacin	0.2 mg/L	
	Glycine	0.4 mg/L	
	Pyridoxine-HCl	0.1 mg/L	

Table A6 List of Buffers and Solutions (Sambrook and Russell, 2001)

Buffer	Composition
1X Tris-EDTA (TE) buffer	0.01 M Tris-HCl (pH 8), 0.001 M Na_2EDTA (pH 8)
5X Tris Borate EDTA (TBE) buffer (1 L)	54 g Tris base, 27.5 g Boric acid, 20 ml 0.5 M EDTA (pH 8)
50X TAEbuffer (1 L)	24.2 g Tris base, 5.71 ml CH_3COOH, 10 ml of 0.5 M EDTA
Phosphate buffered saline (PBS)	0.137 M NaCl, 2.68 mM KCl, 7.98 mM Na_2HPO4, 1.4 mM KH_2PO4, pH 7.2
PBST	PBS containing 0.1 % Tween-20
<u>Buffers/Solutions for SDS-PAGE</u>	
30 % Acrylamide-bisacrylamide solution (100 ml)	29.2 g Acrylamide, 0.8 g Bisacrylamide
Tris-HCl, pH 6.8, 0.5 M (100 ml)	6.06 g of Tris base, pH adjusted to 6.8 with 2 N HCl
Tris-HCl, pH 8.8, 0.5 M (100 ml)	18.18 g of Tris base, pH adjusted to 8.8 with 2 N HCl
5X Tris Glycine buffer, pH 8.3	15.1 g Tris base, 91 g Glycine, 50 ml 10 % SDS
Sample loading buffer (1X)	50 mM Tris-HCl pH 6.8, 2 % SDS, 10 % Glycerol, 1 % ß-mercaptoethanol, 0.02 % Bromophenol blue
Staining solution (100 ml)	10 ml ethanol, 100 mg Coomasie Brilliant Blue G250, 5 g Ammonium sulfate, 1 ml Ortho phosphoric acid
<u>Buffers/Solutions for Western Blot</u>	
Transfer Buffer	25 mM Tris base, 39 mM Glycine, 20 % Methanol
Blocking solution	5 % non fat milk in PBST
Washing buffer	PBST

Table A7 Sequence of the oligonucleotides used in the study

Target Gene	GenBank Accn	Primer Name	Sequence (5'→ 3')
DREB2 Degenerate Primers			
legume DREB2		*DREB2*_deg_Fw	GAAATGGGTKGSTGAGATTAGGGA
legume DREB2		*DREB2*_deg_Rv	CATCCWTWGGCTCTYGCTTCATTC
Primers for RACE			
VuDREB2A	JN629045	*VuDREB2A*_3'RACE_GSP1	AGGATGCTAAGGAGAATGTTCTACCTG
VuDREB2A	JN629045	*VuDREB2A*_3'RACE_GSP2	GCAGAATGAAGCAAGAGCCTAAGGA
VuDREB2A	JN629045	*VuDREB2A*_5'RACE_GSP1	GATGTAGTAGTAGTAGTAG
VuDREB2A	JN629045	*VuDREB2A*_5'RACE_GSP2	GCAGCCAAGAAAGAGTCCTTAAACGATTCC
VuDREB2A	JN629045	*VuDREB2A*_5'RACE_GSP3	GGCTCCCTAATCTCACCAACCC
Primers for cloning full length *VuDREB2A*			
VuDREB2A	JN629045	*VuDREB2A_FL*_1	CTGAATCCGCTATAGCCATATCAAAC
VuDREB2A	JN629045	*VuDREB2A_FL*_1	GTATAACTGTATTCGTCTAAGGCACC
Primers for inverse PCR			
VuDREB2A	JQ066264	F1	AGGATGCTAAGGAGAATGTTCTACCTG
VuDREB2A	JQ066264	R1	GGCTCCCTAATCTCACCAACCC
VuDREB2A	JQ066264	F2	GTCCACAAAACTTCACTGCTAAGT
VuDREB2A	JQ066264	R2	GCAGAATGAAGCAAGAGCCTAAGGA
Primers for deletion of nucleotides 394-546 in *VuDREB2A*			
VuDREB2A	JN629045	*VuDREB2A*_∆394-546_Fw	CTGAATCCGCTATAG
VuDREB2A	JN629045	*VuDREB2A*_∆394-546_mRv	TAGAACATTCTCCTTAGCTGCACATGGA
VuDREB2A	JN629045	*VuDREB2A*_∆394-546_Rv	GTATAACTGTATTCGTCTAAGGCACCTA
Primers for cloning *VuDREB2A* coding sequence			
VuDREB2A	JN629045	*VuDREB2A*_pRT101_Fw	CCGGAATTCATGGGTGCTTATGATCAA
VuDREB2A	JN629045	*VuDREB2A*_pRT101_Rv	CCGGGATCCTCATTCCTTGCTTGCTAC
VuDREB2A	JN629045	*VuDREB2A*_pET28a_Fw	CCGGGATCCATGGGTGCTTATGATCAA
VuDREB2A	JN629045	*VuDREB2A*_pET28a_Rv	CCGCTCGAGTTCCTTGCTTGCTACC
Primers for qRT-PCR			

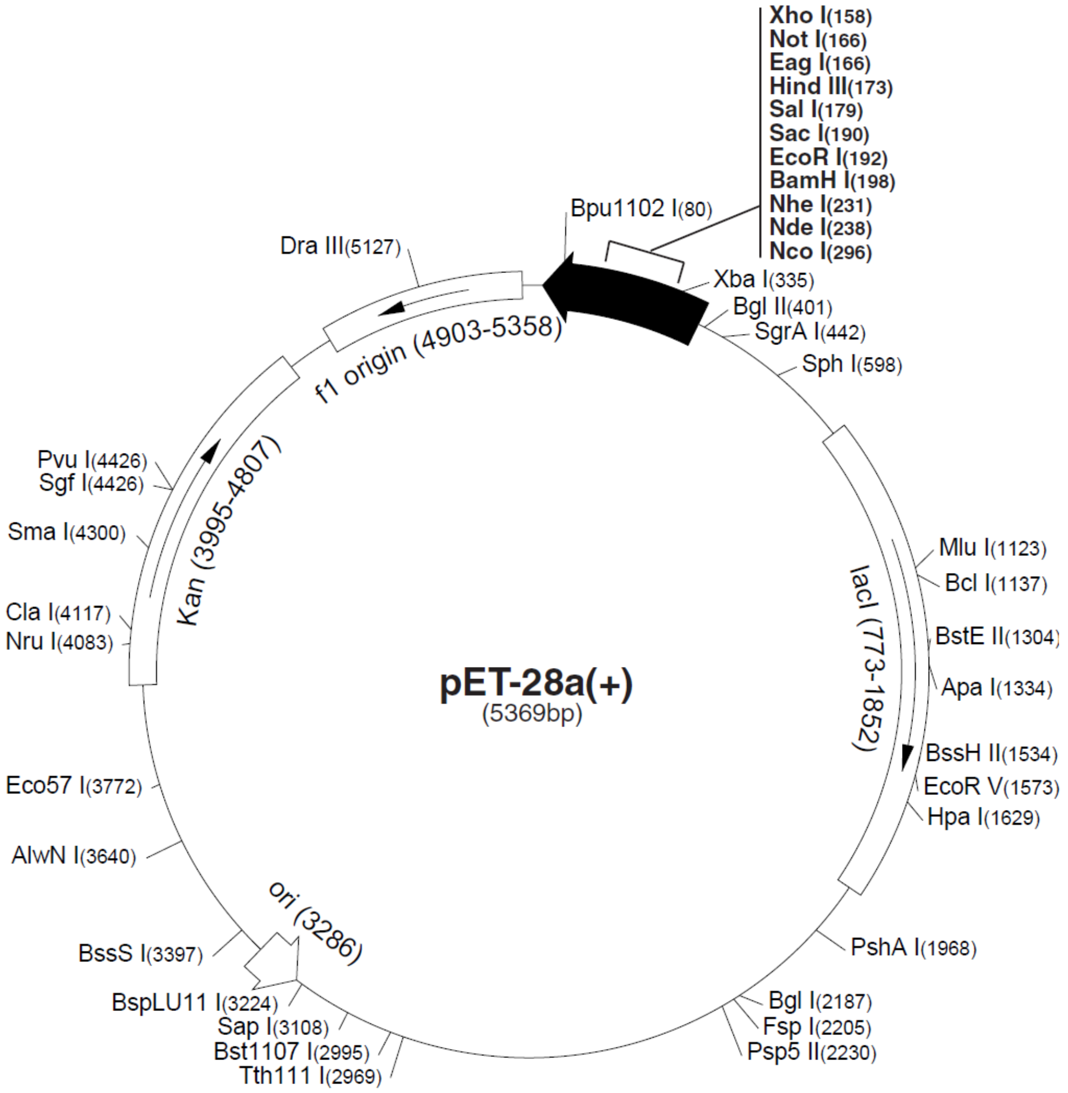
VuDREB2A	JN629045	*VuDREB2A*_qPCR_Fw	TCATGAAAACAGTTGTGCCAG
VuDREB2A	JN629045	*VuDREB2A*_qPCR_Rv	CCTTGCTTGCTACCATTTCC
VuUBQ1 EST	FG895491	*VuUbq*_qPCR_Fw	TCAGTTGAGGCCGAAGAAGA
VuUBQ1 EST	FG895491	*VuUbq*_qPCR_Rv	AAACCAGTCCCAGTCCCAAA
AtUBQ1	AY035148	*AtUBQ1*_qPCR_Fw	AGAGCTGTCAACTGCAGGAAGAA
AtUBQ1	AY035148	*AtUBQ1*_qPCR_Rv	ACAAGAAAAACAAACCCTATCAAAGG
AtPDF1.3	NM_128160	*AtPDF1.3*_qPCR_Fw	GCAATGCGTGCAAGAATCAG
AtPDF1.3	NM_128160	*AtPDF1.3*_qPCR_Rv	AATACACACCATGAAGCACCAA
AtHSP90	NM_124642	*AtHSP90*_qPCR_Fw	GACGCTGCTGAAGAGAGCAA
AtHSP90	NM_124642	*AtHSP90*_qPCR_Rv	TGACAAACCCAACCCTAGAA
AtROF2	NM_124233	*AtROF2*_qPCR_Fw	TCTGAAACAGTAACACACACCAAGA
AtROF2	NM_124233	*AtROF2*_qPCR_Rv	AAGGAAGCACAAGCGATGAG
AtCOR13	NM_118491	*AtCOR13*_qPCR_Fw	GCATTCAGTCAGAAGAACTGGTTG
AtCOR13	NM_118491	*AtCOR13*_qPCR_Rv	GACCCTTTACTTAACACCATTGACG
AtHSP70	NM_112093	*AtHSP70*_qPCR_Fw	GCATCAAATGATCACACACCA
AtHSP70	NM_112093	*AtHSP70*_qPCR_Rv	GCAGGAGGAATGGATGACGA
AtLOX2	NM_114383	*AtLOX2*_qPCR_Fw	CATTGGAGAACAACAAGAAGCA
AtLOX2	NM_114383	*AtLOX2*_qPCR_Rv	GAACACCCATTCCGGTAACA

Probes for EMSA

		ACC_DRE_Fw	TAAAAGATATACTACCGACATGAGTTCCAAAAAGC
		ACC_DRE_Rv	GCTTTTTGGAACTCATGTCGGTAGTATATCTTTTA
		GCC_DRE_Fw	TAAAAGATATACTGCCGACATGAGTTCCAAAAAGC
		GCC_DRE_Rv	GCTTTTTGGAACTCATGTCGGCAGTATATCTTTTA
		Mut1_DRE_Fw	TAAAAGATATACTAAAGACATGAGTTCCAAAAAGC
		Mut1_DRE_Rv	GCTTTTTGGAACTCATGTCTTTAGTATATCTTTTA
		Mut2_DRE_Fw	TAAAAGATATACTGAAGACATGAGTTCCAAAAAGC
		Mut2_DRE_Rv	GCTTTTTGGAACTCATGTCTTCAGTATATCTTTTA
		Mut3_DRE_Fw	TAAAAGATATACTTTTTTTATGAGTTCCAAAAAGC
		Mut3_DRE_Rv	GCTTTTTGGAACTCATAAAAAAAGTATATCTTTTA
		Mut4_DRE_Fw	TAAAAGATATACTAAATTTATGAGTTCCAAAAAGC
		Mut4_DRE_Rv	GCTTTTTGGAACTCATAAATTTAGTATATCTTTTA

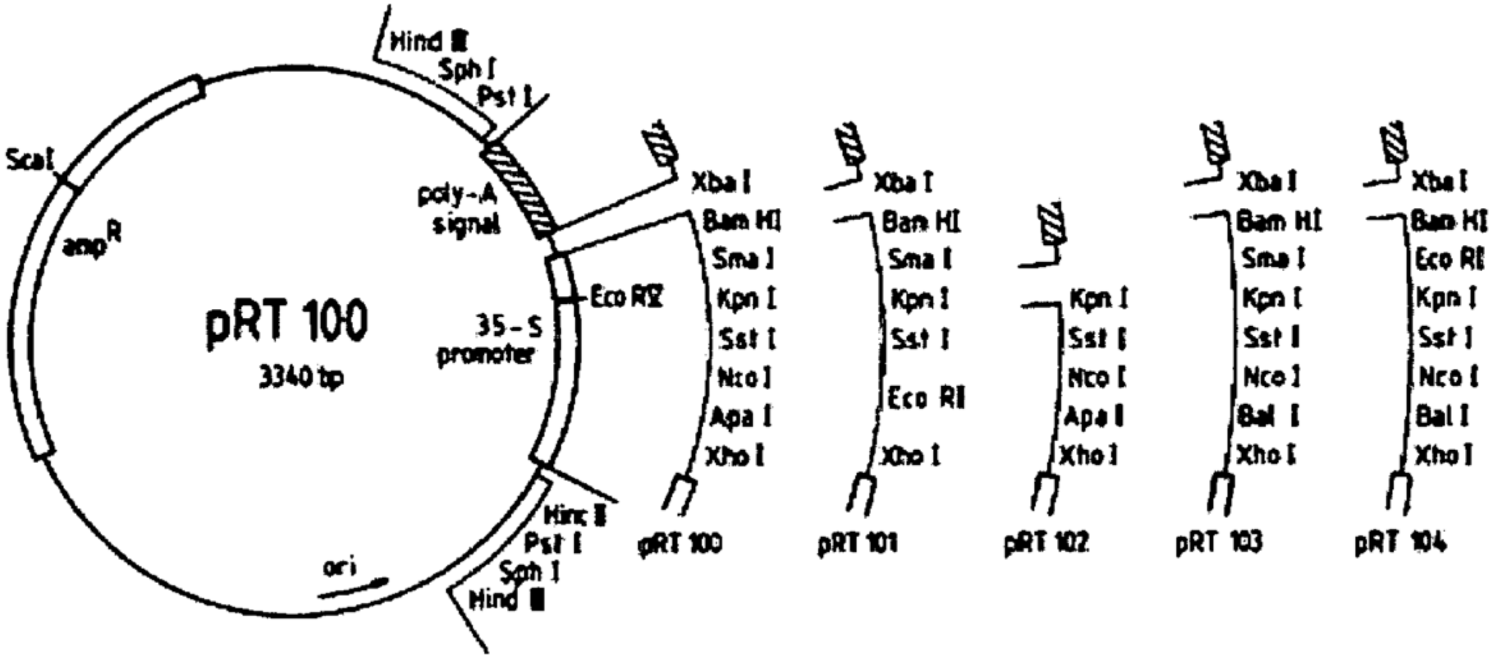
Vector Map 1: pET28a

(Source: http://biochem.web.utah.edu/hill/links/pET28.pdf)



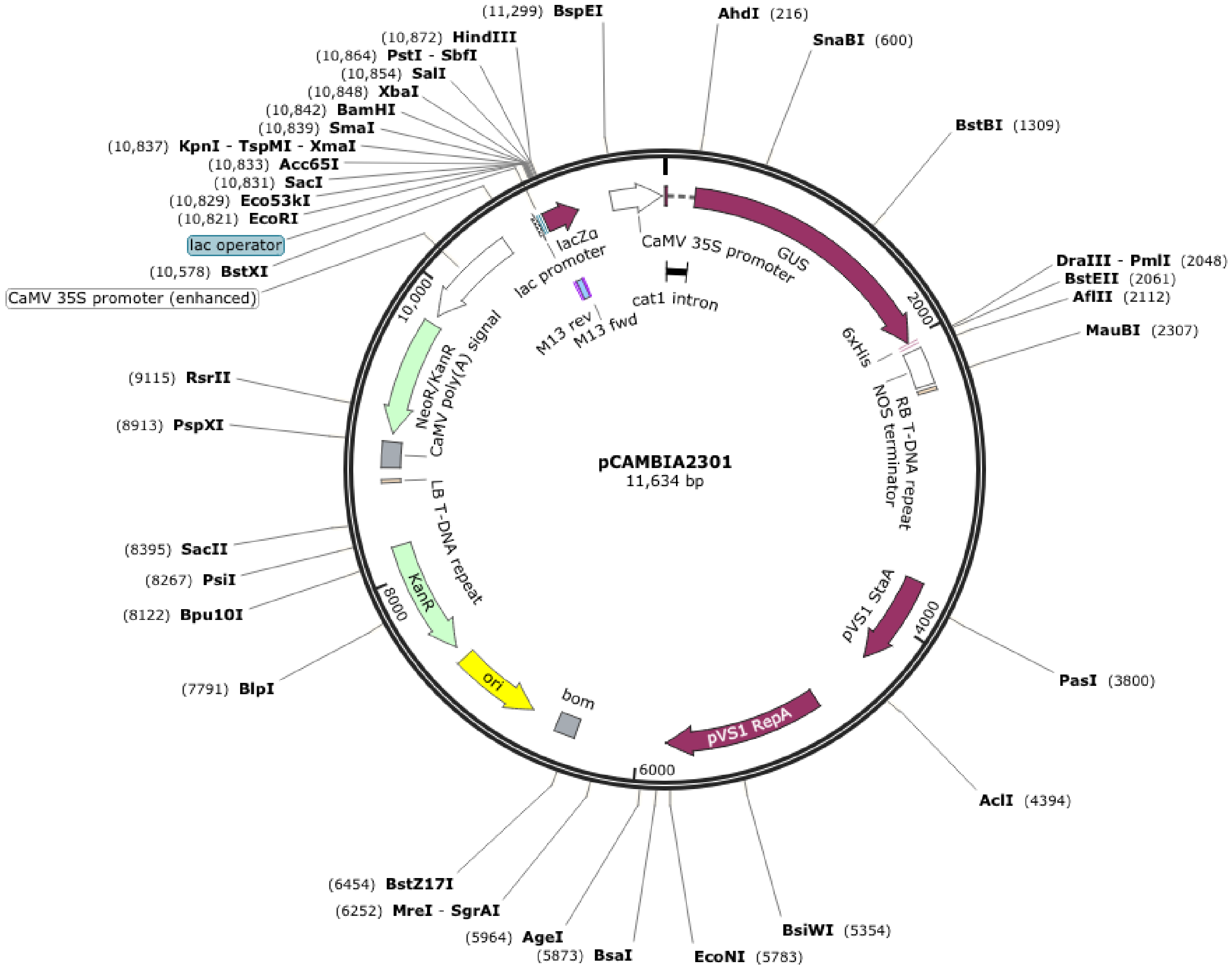
Vector Map 2: pRT100 series

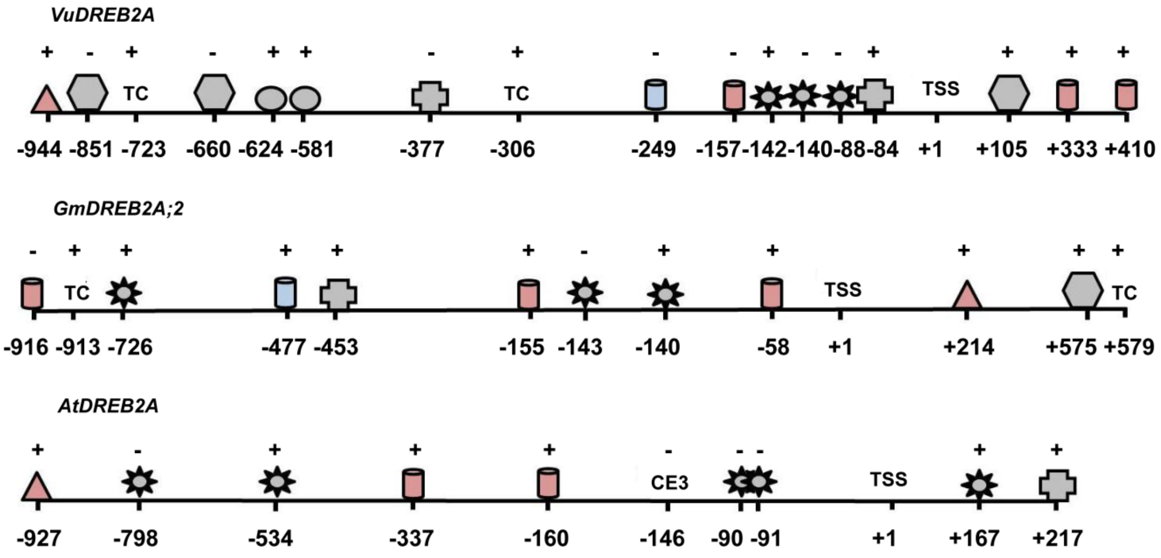
(Source: http://patentimages.storage.googleapis.com/US20090307801A1/US20090307801A1-20091210-D00000.png)



Vector Map 3: pCAMBIA2301

(Source: http://www.snapgene.com/resources/plasmid_files/plant_vectors/pCAMBIA2301/pCAMBIA2301_1x.png)





	Cis element	Conserved Motifs
✚	MeJA-responsive element	CGTCA
⬭	WRKY binding motif	TGAC
✹	ABA-responsive element	CACCTG, ACCTGCC, ACTACG, TACGTACACC
▮	Heat stress-responsive element	CGGGAAGGTTCGAC, AGAAAATCG, AGAAGATTCG, GGAAGGTTCGAGCGC
▯	Low temperature-responsive element	CCGAAA
▲	MYB recognition site	AAACCA, TAACTG
TC	TC-rich repeats/ defense and stress-responsive element	ATTTTATCCA, ATTTTATTCA, ATTCTCTAAC, GCTTCTTAC
CE3	Coupling element	GACGAGTGTC
⬢	SA-responsive element	CAGAAAAGGA, CCATCTTTTT
TSS	Transcription start site	

Figure A1 Comparative models of the promoters and 5’ UTRs of cowpea, soybean and *Arabidopsis DREB2A* genes (predicted by TSSP, PLACE and PlantCARE; see Methods). Transcription start sites in *Arabidopsis* and soybean *DREB2A* are based on cDNA sequences in Phytozome v9.1 database. +/- indicate coding/complementary genomic DNA strands.

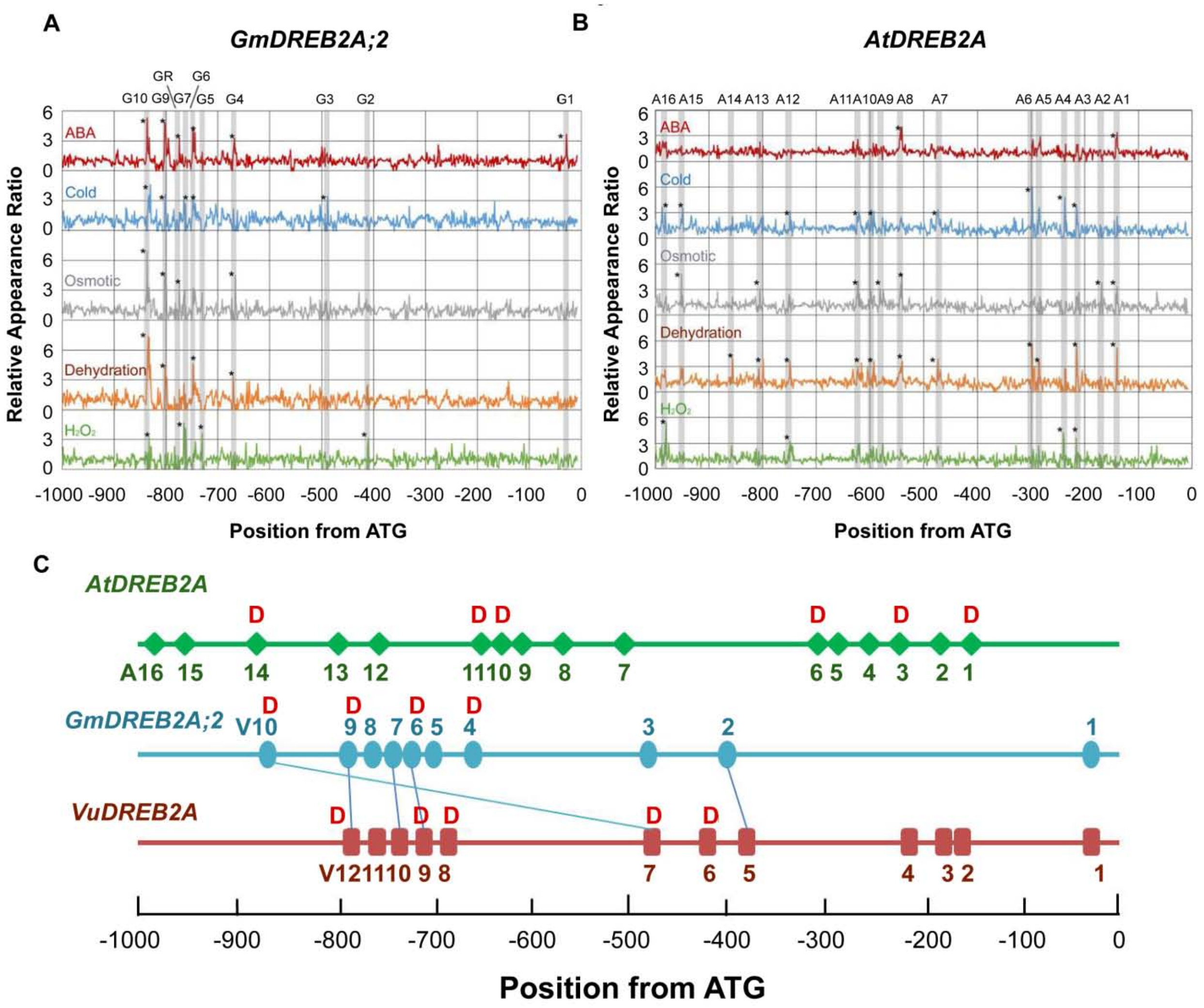


Figure A2 Comparative models of the promoters and 5’ UTRs of cowpea (*VuDREB2A*), soybean (*GmDREB2A;2*) and *Arabidopsis* (*AtDREB2A)* genes (based on predicted *cis*-elements in the overrepresentation analysis; see Methods). The graphs show scanning results of the *GmDREB2A;2* (**A**) and *AtDREB2A* (**B**) promoters based on microarray data of the ABA response (red), the cold response (blue), the osmotic stress response (gray), the dehydration response (orange) and the H_2O_2 response (green) of *Arabidopsis*. Asterisks indicate octamers with RAR > 3. Locations of predicted *cis*-elements are indicated by gray columns (A1–16 and G1-10; Appendix Table A8). Comparison between predicted *cis*-elements in the three promoters (**C**). Blue lines indicate ≥ 10 bp match between the predicted *cis*-elements in the soybean and cowpea *DREB2A* promoters. ‘D’ indicates overrepresentated octamers (RAR > 3, two-sided Fisher’s exact test $P < 0.05$) related to the dehydration response of *Arabidopsis*.

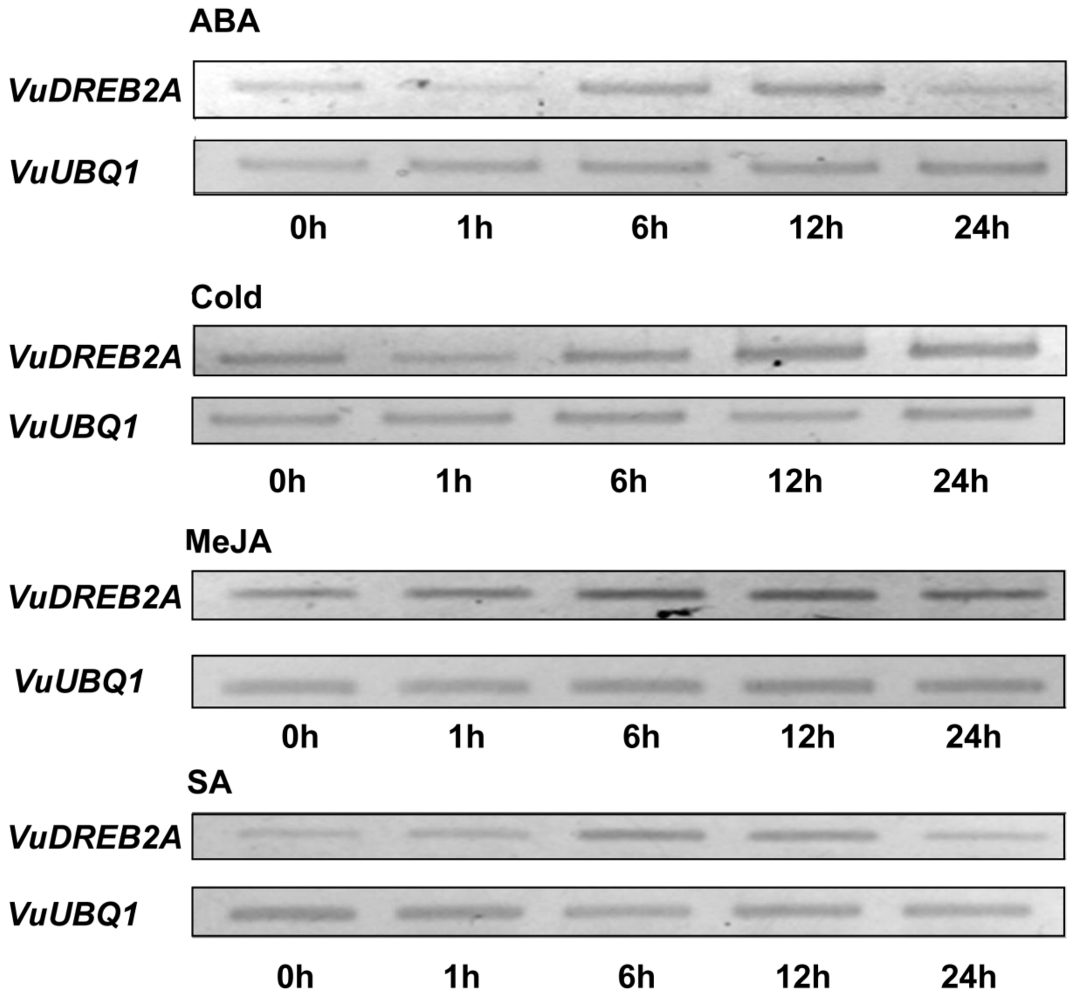
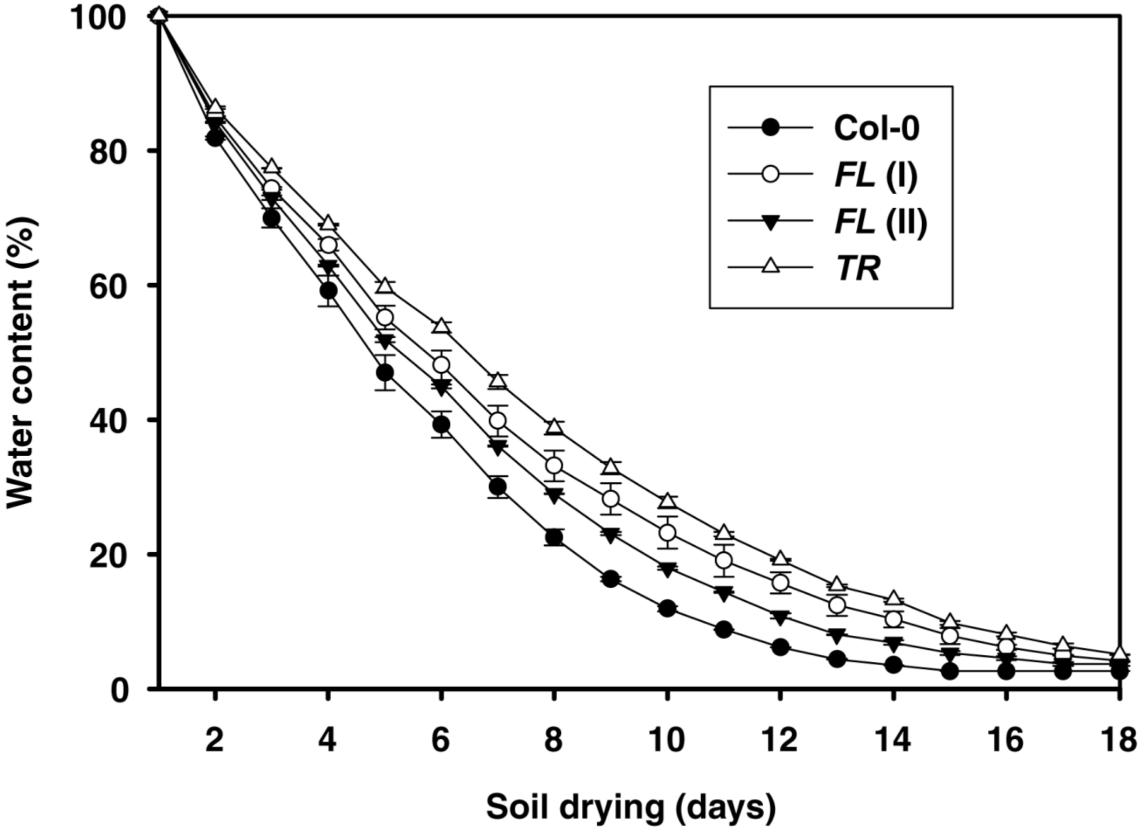


Figure A3 Expression of *VuDREB2A* in the shoots of cowpea plants grown for one week and then exposed to different treatments (ABA, cold, MeJA and SA; see Methods). Transcript abundance of *VuDREB2A* at different time points is shown together with the levels of *VuUBQ1*, the gene used for data normalization.



$$\text{Water content (\%)} = \frac{\text{Weight at day N - dry weight}}{\text{Weight at day 1 - dry weight}}$$

Figure A4 Water contents from wild type and transgenic *Arabidopsis* overexpressing *full length* (*FL*) and *truncated* (*TR*) *VuDREB2A*. Water contents were monitored by weighing plants in pots subjected to progressive drought treatment (see Fig. 4.15) each day at the same hour. Dry weights were taken after keeping the plants for a day in an 80 °C oven. Water contents were calculated according to the formula shown in the figure. I and II represent independent transgenic lines of *35S:VuDREB2A* (*FL*).

Table A8 Predicted *cis*-elements in the *AtDREB2A* and *GmDREB2A;2* promoters

Position from ATG[a]	Octamer[b]	Treatment[c]	RARf[d]	*cis*-element[e]	Sequence	Motif[f]	Reference[g]
AtDREB2A							
-987	TCGTACGT	cold	3.64	A16	TCGTACGTACACCGGAAACC	ABRE	Kim *et al.* 2011
-982	CGTACACC	H_2O_2	3.06				
-980	TACACCGG	H_2O_2	5.50				
-975	CGGAAACC	H_2O_2	3.02				
-951	TAACCGTG	osmotic stress	4.77	A15	TAACCGTGAT		
-949	ACCGTGAT	cold	3.51				
-856	GGCAATAA	dehydration	3.87	A14	GGCAATAA		
-805	CAAACTGG	osmotic stress	3.06	A13	CAAACTGGCAATAA		
-799	GGCAATAA	dehydration	3.87				
-749	TGTCAGGG	cold	3.17	A12	TGTCAGGG		
-749	TGTCAGGG	dehydration	3.64				
-749	TGTCAGGG	H_2O_2	3.25				
-623	ACAGCGTG	osmotic stress	3.82	A11	ACAGCGTGTTTCGAG		
-622	CAGCGTGT	osmotic stress	4.02				
-621	AGCGTGTT	cold	3.11				
-616	GTTTCGAG	dehydration	3.41				
-597	GGACACTC	cold	3.34	A10	GGACACTCGTCG	CGCG box/ CE3	Yang *et al.* 2002
-593	ACTCGTCG	cold	3.15				Kaplan *et al.* 2006
-593	ACTCGTCG	dehydration	3.61				Kim *et al.* 2011
-581	GAAAAGTC	osmotic stress	3.06	A9	GAAAAGTC		
-544	TGACACGT	ABA	3.29	A8	TGACACGTAACC	ABRE	Kim *et al.* 2011
-543	GACACGTA	ABA	3.98				
-543	GACACGTA	osmotic stress	3.59				
-541	CACGTAAC	ABA	4.00				
-541	CACGTAAC	osmotic stress	4.72				
-541	CACGTAAC	dehydration	3.50				
-540	ACGTAACC	dehydration	3.58				
-474	CTCCGTAG	cold	3.37	A7	CTCCGTAG		
-474	CTCCGTAG	dehydration	3.86				
-298	TCGTCCCC	cold	6.07	A6	TCGTCCCC		
-298	TCGTCCCC	dehydration	5.57				

-286	GGTACGTG	cold	3.28	A5	GGTACGTGG	ABRE	Kim *et al.* 2011
-286	GGTACGTG	dehydration	3.76				
-285	GTACGTGG	cold	3.61				
-285	GTACGTGG	dehydration	3.45				
-240	TAGGCCCG	H_2O_2	4.21	A4	TAGGCCCGTCA	CGTCA motif/ MeJA responsive element	Reinbothe *et al.*1994 Wang *et al.*2011
-239	AGGCCCGT	H_2O_2	3.16				
-237	GCCCGTCA	cold	4.78				
-216	GGGTTACC	dehydration	3.20	A3	GGGTTACCC		
-216	GGGTTACC	H_2O_2	3.58				
-215	GGTTACCC	cold	3.75				
-215	GGTTACCC	dehydration	5.37				
-169	TACCCTGT	osmotic stress	3.82	A2	TACCCTGT		
-141	GGTGTCCC	dehydration	3.03	A1	GGTGTCCCT		
-140	GTGTCCCT	ABA	3.35				
-140	GTGTCCCT	osmotic stress	3.29				
-140	GTGTCCCT	dehydration	5.12				
GmDREB2A;2							
-837	CGACGTGC	dehydration	3.14	G10	CGACGTGCGCGTTTT	CGCG box/ CE3	Yang *et al.* 2002 Kaplan *et al.* 2006 Kim *et al.* 2011
-836	GACGTGCG	ABA	5.29				
-836	GACGTGCG	osmotic stress	6.94				
-836	GACGTGCG	dehydration	3.86				
-835	ACGTGCGC	dehydration	5.66				
-834	CGTGCGCG	cold	3.22				
-834	CGTGCGCG	osmotic stress	3.32				
-834	CGTGCGCG	dehydration	7.38				
-833	GTGCGCGT	cold	3.53				
-833	GTGCGCGT	dehydration	4.04				
-832	TGCGCGTT	ABA	3.30				
-832	TGCGCGTT	cold	3.15				
-832	TGCGCGTT	osmotic stress	3.25				
-832	TGCGCGTT	dehydration	7.22				
-832	TGCGCGTT	H_2O_2	3.23				
-831	GCGCGTTT	dehydration	3.14				
-830	CGCGTTTT	cold	4.61				
-830	CGCGTTTT	dehydration	4.53				
-802	GCGCCACG	ABA	4.85	G9	GCGCCACGTGCCAGC	ABRE HSE	Kim *et al.* 2011 Sun *et al.* 2006 Yoshida *et al.* 2011
-802	GCGCCACG	osmotic stress	4.77				
-801	CGCCACGT	ABA	4.34				
-801	CGCCACGT	cold	3.62				

-801	CGCCACGT	osmotic stress	3.20				
-801	CGCCACGT	dehydration	4.45				
-800	GCCACGTG	ABA	3.53				
-800	GCCACGTG	dehydration	3.12				
-799	CCACGTGC	osmotic stress	3.50				
-799	CCACGTGC	dehydration	3.12				
-798	CACGTGCC	dehydration	3.29				
-795	GTGCCAGC	ABA	3.23				
-774	CGACGCAT	ABA	3.31	G8	CGACGCAT		
-774	CGACGCAT	osmotic stress	3.72				
-765	ATAACGCG	cold	3.05	G7	ATAACGCGACTA	CGCG box/ CE3	Yang *et al*. 2002
-765	ATAACGCG	H_2O_2	4.69				Kaplan *et al*. 2006
-762	ACGCGACT	H_2O_2	4.14				Kim *et al*. 2011
-761	CGCGACTA	H_2O_2	3.45				
-748	TCGCCACG	ABA	4.02	G6	TCGCCACGTCAC	ABRE	Kim *et al*. 2011
-748	TCGCCACG	cold	3.06			CGTCA motif/ MeJA responsive element	Reinbothe *et al*.1994
-748	TCGCCACG	osmotic stress	3.16				Wang *et al*.2011
-748	TCGCCACG	dehydration	4.68				
-747	CGCCACGT	ABA	4.34				
-747	CGCCACGT	cold	3.62				
-747	CGCCACGT	osmotic stress	3.20				
-747	CGCCACGT	dehydration	4.45				
-744	CACGTCAC	ABA	3.84				
-744	CACGTCAC	osmotic stress	3.02				
-730	TCTGGGGT	H_2O_2	3.66	G5	TCTGGGGT		
-670	AGGTGCCA	dehydration	3.33	G4	AGGTGCCAGCCC		
-668	GTGCCAGC	ABA	3.23				
-666	GCCAGCCC	osmotic stress	4.49				
-488	GCATAGGT	cold	3.44	G3	GCATAGGT		
-410	TGTGCGAT	H_2O_2	3.13	G2	TGTGCGAT		
-28	GCGAAGTC	ABA	3.64	G1	GCGAAGTC		

[a] Position from start codon of *AtDREB2A*/*GmDREB2A;2*. [b] Overrepresented octamers in promoters of genes up-regulated > 3 fold in Arabidopsis under [c] different treatments (viz., ABA, cold, osmotic stress, dehydration and H_2O_2). [d] Relative Appearance Ratio (RAR) of each octamer = count of the octamer in n genes up-regulated under different treatments/n)/(count of the octamer in total 24956 promoters/24956). n = 643 (ABA)/ 337 (cold)/ 327 (osmotic stress)/ 294 (dehydration)/329 (H_2O_2). RARf = filtered RAR (RAR > 3, $P < 0.05$). [e] Predicted cis-elements that are related to ABA, cold, osmotic stress, dehydration and H_2O_2 (A1-16, G1-10; Appendix Fig. A2). [f] Motifs in the predicted cis-elements, based on functional analyses in other plant promoters. [g] Source of functional analyses.

Table A9 Upregulated genes in transgenic Arabidopsis overexpressing full length CDS (*VuDREB2A FL*) and truncated CDS (*VuDREB2A TR*) of *VuDREB2A*. Transcriptome of transgenic plants growing under unstressed conditions was compared to that of wild type Col-0 by microarray analysis. Upper 200 genes from the quality controlled genes (intensity of spot>200, $P<0.05$) are listed with gene symbols and short descriptions of TAIR10

							Average signal intensity	
Rank	Locus	Gene symbol	Gene /TAIR10 Short description	Regulation	Fold change	*P*-value	Wild type	*35S:VuDREB2A FL*
1	AT1G27020	AT1G27020	hypothetical protein	up	5.6	4.41E-02	698.7	3923.9
2	AT4G12490	AT4G12490	bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin-like protein	up	5.5	3.65E-02	246.0	1346.4
3	AT4G23600	CORI3	cystine lyase	up	5.2	1.54E-02	369.1	1906.8
4	AT2G26010	PDF1.3	defensin-like protein 14	up	4.5	3.65E-02	193.8	863.5
5	AT3G50480	HR4	RPW8-like protein 4	up	4.3	1.45E-02	2263.0	9610.0
6	AT1G77120	ADH1	alcohol dehydrogenase class-P	up	3.9	5.64E-03	415.9	1633.5
7	AT3G45140	LOX2	lipoxygenase 2	up	3.9	1.38E-02	1984.8	7671.6
8	AT1G43910	AT1G43910	P-loop containing nucleoside triphosphate hydrolase-like protein	up	3.9	6.25E-03	243.4	938.3
9	AT1G07400	AT1G07400	class I heat shock protein	up	3.9	3.55E-03	551.5	2126.5
10	AT1G02930	GSTF6	glutathione S-transferase 1	up	3.8	4.88E-02	644.1	2468.7
11	AT5G22460	AT5G22460	esterase/lipase/thioesterase family protein	up	3.7	4.82E-02	211.4	768.6
12	AT1G09240	NAS3	Nicotianamine synthase 3	up	3.5	9.58E-03	564.0	1985.8
13	AT5G51440	AT5G51440	heat shock protein 23.5	up	3.4	3.46E-02	669.9	2286.5
14	AT5G52640	HSP90.1	heat shock protein 81-1	up	3.1	2.19E-02	1695.8	5202.7
15	AT5G13170	SAG29	senescence-associated protein 29	up	3.0	1.93E-02	872.6	2626.1
16	AT2G37770	AT2G37770	NAD(P)-linked oxidoreductase-like protein	up	3.0	2.94E-02	205.3	614.4
17	AT3G49110	PRXCA	peroxidase 33	up	2.9	1.12E-02	292.9	861.8
18	AT1G65370	AT1G65370	meprin and TRAF homology domain-containing protein	up	2.9	1.13E-02	382.7	1106.3
19	AT4G15680	AT4G15680	monothiol glutaredoxin-S4	up	2.8	4.17E-02	267.1	750.2
20	AT1G17020	SRG1	protein SRG1	up	2.8	1.09E-02	469.4	1308.8
21	AT5G40395			up	2.8	1.87E-02	1603.6	4433.2
22	AT2G43510	TI1	defensin-like protein 195	up	2.7	2.37E-02	364.7	996.9
23	AT4G04223	AT3G56210	armadillo/beta-catenin-like repeat-containing protein	up	2.7	1.69E-02	493.6	1324.0
24	AT5G48570	ROF2	peptidylprolyl isomerase	up	2.7	1.61E-02	1126.5	3001.0
25	AT1G79245			up	2.7	9.59E-03	628.2	1666.1
26	AT2G07675	AT2G07675	ribosomal protein S12	up	2.7	5.90E-03	1346.4	3569.8
27	AT1G01320	AT1G01320	tetratricopeptide repeat-containing protein	up	2.6	1.92E-02	221.3	586.2
28	AT1G69870	NRT1.7	nitrate transporter 1.7	up	2.6	2.50E-02	382.8	975.7

29	AT3G28500	AT3G28500	60S acidic ribosomal protein P2-3	up	2.5	3.89E-02	200.3	511.3
30	AT3G22550	AT3G22550	hypothetical protein	up	2.5	3.60E-02	1475.6	3757.0
31	AT2G16060	HB1	non-symbiotic hemoglobin 1	up	2.5	3.22E-02	2343.0	5838.4
32	AT2G21640	AT2G21640	hypothetical protein	up	2.5	2.58E-02	398.7	975.0
33	AT4G24120	YSL1	metal-nicotianamine transporter YSL1	up	2.4	3.10E-02	430.6	1046.9
34	ATMG01390			up	2.4	4.09E-02	54456.0	131687.1
35	AT1G54575	AT1G54575	hypothetical protein	up	2.4	4.10E-02	468.0	1127.1
36	AT2G20560	AT2G20560	DNAJ heat shock protein-like protein	up	2.4	5.45E-03	751.9	1802.1
37	AT1G43800	AT1G43800	stearoyl-acyl-carrier protein desaturase-like protein	up	2.4	7.11E-03	409.2	971.9
38	AT5G09570	AT5G09570	Cox19-like CHCH family protein	up	2.4	3.98E-02	312.4	743.4
39	AT5G09440	EXL4	protein exordium like 4	up	2.3	3.65E-02	4098.6	9542.8
40	AT2G18193	AT2G18193	P-loop containing nucleoside triphosphate hydrolase-like protein	up	2.3	2.82E-02	200.2	462.6
41	AT5G53460	GLT1	glutamate synthase 1 [NADH]	up	2.3	2.12E-03	611.0	1408.1
42	AT5G17760	AT5G17760	AAA-type ATPase family protein	up	2.3	4.68E-02	323.6	732.4
43	AT3G22840	ELIP1	chlorophyll A-B binding, early light-inducible protein	up	2.3	3.75E-02	8565.5	19367.2
44	AT1G66920	AT1G66920	protein kinase-like protein	up	2.3	3.56E-03	281.1	634.8
45	AT4G36980	AT4G36980	splicing factor, arginine/serine-rich 16	up	2.3	6.80E-03	259.0	582.8
46	AT5G59720	HSP18.2	heat shock protein 18.2	up	2.2	3.41E-02	234.2	524.5
47	AT2G07725	AT2G07725	60S ribosomal protein L5	up	2.2	2.14E-02	2946.8	6520.4
48	EG518275	AT1G34315	hypothetical protein	up	2.2	4.39E-02	482.9	1055.9
49	ATMG00210	AT2G07725	60S ribosomal protein L5	up	2.2	3.34E-02	2928.1	6416.9
50	AT5G02490	AT5G02490	heat shock protein 70	up	2.2	2.39E-02	2053.9	4495.2
51	AT2G03150	emb1579	ATP/GTP-binding protein-like protein	up	2.2	3.06E-02	249.8	542.8
52	AT4G23680	AT4G23680	SRPBCC ligand-binding domain-containing protein	up	2.2	1.55E-02	2033.2	4413.8
53	AT4G12500	AT4G12500	bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin-like protein	up	2.1	3.58E-02	258.9	553.5
54	AT3G21890	AT3G21890	B-box type zinc finger-containing protein	up	2.1	4.23E-02	337.0	718.9
55	AT1G27980	DPL1	Sphingosine-1-phosphate lyase	up	2.1	4.49E-02	1294.3	2738.6
56	AT1G76960	AT1G76960	hypothetical protein	up	2.1	4.65E-02	236.9	502.5
57	AT5G28030	DES1	cysteine synthase A	up	2.1	3.06E-02	504.7	1054.3
58	AT2G32240	AT2G32240	hypothetical protein	up	2.1	6.52E-04	225.3	469.4
59	AT1G80130	AT1G80130	tetratricopeptide repeat domain-containing protein	up	2.1	1.43E-02	468.0	974.0
60	AT4G37430	CYP91A2	cytochrome P450 81F1	up	2.1	1.54E-02	233.9	484.3
61	AT2G43620	AT2G43620	chitinase-like protein	up	2.1	4.79E-02	675.2	1396.7
62	AT2G18960	HA1	H(+)-ATPase 1	up	2.1	9.12E-03	320.0	661.8
63	AT4G00040	AT4G00040	chalcone synthase 2-like protein	up	2.1	3.82E-03	527.8	1089.9
64	AT3G16530	AT3G16530	legume lectin-like protein	up	2.1	3.48E-02	1305.7	2689.6
65	AT3G63052	AT3G63052	hypothetical protein	up	2.0	4.12E-02	1691.1	3447.2
66	AT4G12470	AZI1	azelaic acid induced 1	up	2.0	3.32E-03	206.5	420.9
67	AT3G51430	YLS2	strictosidine synthase	up	2.0	4.76E-02	399.5	812.8

68	AT4G01870	AT4G01870	tolB-related protein	up	2.0	8.70E-03	2209.5	4464.1
69	AT3G03470	CYP89A9	cytochrome P450, family 87, subfamily A, polypeptide 9	up	2.0	1.50E-02	326.2	658.4
70	AT1G08115			up	2.0	1.11E-02	5706.1	11495.6
71	AT1G54570	AT1G54570	acyltransferase-like protein	up	2.0	3.76E-02	519.2	1038.3
72	AT1G60270	BGLU6	beta glucosidase 6	up	2.0	1.62E-02	341.0	679.8
73	AT4G34230	CAD5	cinnamyl alcohol dehydrogenase 5	up	2.0	2.70E-02	3936.9	7838.7
74	AT2G07695	AT2G07695	cytochrome c oxidase subunit II	up	2.0	2.97E-02	1278.8	2530.5
75	AT4G15248	AT4G15248	B-box type zinc finger-containing protein	up	2.0	3.75E-02	474.7	938.0
76	AT5G14780	FDH	Formate dehydrogenase	up	2.0	4.34E-02	5450.2	10678.9
77	AT1G65040	AT1G65040	ubiquitin-protein ligase synoviolin	up	2.0	4.03E-02	373.6	733.6
78	AT3G14310	PME3	pectinesterase 3	up	2.0	8.38E-04	612.0	1198.3
79	AT5G04950	NAS1	nicotianamine synthase 1	up	2.0	5.21E-03	4635.9	9069.2
80	AT1G79930	HSP91	heat shock protein 91	up	2.0	4.58E-02	261.8	508.8
81	AT3G13080	MRP3	ABC transporter C family member 3	up	1.9	1.28E-02	601.5	1172.5
82	AT5G17860	CAX7	calcium exchanger 7	up	1.9	1.24E-02	648.1	1261.9
83	AT5G16730	AT5G16730	hypothetical protein	up	1.9	1.88E-03	466.6	900.9
84	AT1G02220	NAC003	NAC domain-containing protein 3	up	1.9	3.23E-02	330.0	633.0
85	AT5G47560	TDT	tonoplast dicarboxylate transporter	up	1.9	2.28E-02	450.4	865.5
86	AT4G12400	AT4G12400	putative stress-inducible protein	up	1.9	4.30E-02	1080.8	2069.5
87	AT3G54500	AT3G54500	hypothetical protein	up	1.9	2.36E-03	264.2	505.0
88	AT2G41230	AT2G41230	hypothetical protein	up	1.9	1.31E-02	960.9	1835.3
89	AT4G15258			up	1.9	2.45E-02	1300.2	2482.2
90	AT3G15356	AT3G15356	lectin-like protein	up	1.9	3.24E-02	1038.4	1967.0
91	AT1G74310	HSP101	heat shock protein 101	up	1.9	2.97E-02	413.4	779.6
92	AT3G19550	AT3G19550	hypothetical protein	up	1.9	3.00E-02	274.4	516.0
93	AT2G03760	SOT12	flavonol sulfotransferase-like protein	up	1.9	2.35E-03	784.7	1473.9
94	AT1G70810	AT1G70810	calcium-dependent lipid-binding domain	up	1.9	1.38E-02	1502.7	2808.2
95	AT3G24500	MBF1C	multiprotein-bridging factor 1c	up	1.9	6.38E-03	6682.8	12460.9
96	AT2G04030	CR88	Chaperone protein htpG family protein	up	1.9	1.21E-02	2490.7	4632.6
97	AT5G07830	GUS2	Heparanase-like protein 1	up	1.9	2.19E-02	1059.2	1965.7
98	AT5G25450	AT5G25450	ubiquinol-cytochrome c reductase subunit 7	up	1.9	1.56E-02	315.8	584.2
99	AT5G56850	AT5G56850	hypothetical protein	up	1.8	2.21E-02	197.0	363.9
100	AT5G43450	AT5G43450	putative 2-oxoglutarate-dependent dioxygenase	up	1.8	1.66E-03	786.1	1447.2
101	AT1G61275			up	1.8	3.29E-03	1197.6	2202.8
102	AT1G02850	BGLU11	beta glucosidase 11	up	1.8	3.71E-02	569.2	1045.4
103	AT1G28660	AT1G28660	GDSL esterase/lipase	up	1.8	4.34E-02	1444.3	2647.6
104	AT4G16563	AT4G16563	aspartyl protease family protein	up	1.8	5.00E-03	509.2	931.9
105	AT3G21250	MRP6	multidrug resistance-associated protein 6	up	1.8	6.78E-03	1464.1	2677.8
106	AT1G73600	CPuORF32	conserved peptide upstream open reading frame 32	up	1.8	2.18E-02	5152.4	9409.1

107	AT1G15405			up	1.8	4.11E-02	28209.1	51420.4
108	AT1G65440	GTB1	transcription elongation factor SPT6	up	1.8	4.87E-02	261.2	477.4
109	AT4G36990	HSF4	heat stress transcription factor B-1	up	1.8	4.97E-03	1824.9	3323.8
110	AT2G43150	AT2G43150	Proline-rich extensin-like family protein	up	1.8	1.30E-02	7127.3	12983.8
111	AT1G70620	AT1G70620	cyclin-related protein	up	1.8	4.07E-02	529.2	961.6
112	AT1G37130	NIA2	Nitrate reductase (NADH)	up	1.8	5.50E-03	538.0	975.0
113	AT5G25560	AT5G25560	CHY and CTCHY and RING-type zinc finger protein	up	1.8	3.02E-02	780.5	1414.0
114	AT3G53230	AT3G53230	cell division control protein 48-D	up	1.8	4.17E-02	485.2	875.0
115	AT4G02510	TOC159	translocase of chloroplast 159	up	1.8	3.86E-02	226.8	408.2
116	AT5G61790	CNX1	calnexin 1	up	1.8	9.51E-03	4916.2	8823.0
117	AT2G42610	LSH10	hypothetical protein	up	1.8	1.83E-02	965.9	1732.8
118	AT4G01700	AT4G01700	class II chitinase-like protein	up	1.8	4.23E-02	725.7	1294.9
119	AT2G16586	AT2G16586	hypothetical protein	up	1.8	4.21E-02	26571.6	47408.2
120	AT1G32230	RCD1	WWE protein-protein interaction domain family protein	up	1.8	2.05E-02	511.9	913.0
121	AT2G27420	AT2G27420	cysteine proteinase-like protein	up	1.8	2.43E-02	2273.5	4042.4
122	AT1G79520	AT1G79520	Cation efflux family protein	up	1.8	1.93E-02	788.0	1400.5
123	AT2G07708	AT2G07708	hypothetical protein	up	1.8	4.86E-02	1771.4	3139.5
124	AT4G13300	TPS13	terpenoid synthase 13	up	1.8	6.96E-04	242.4	429.9
125	AT4G05631	AT4G05631	hypothetical protein	up	1.8	1.70E-02	297.8	528.6
126	AT3G58070	GIS	C2H2 and C2HC zinc finger-containing protein	up	1.8	1.78E-02	408.1	724.0
127	AT4G15690	AT4G15690	monothiol glutaredoxin-S5	up	1.8	4.97E-02	433.8	766.4
128	ATCG00180			up	1.8	1.73E-02	14472.2	25633.5
129	AT5G43310	AT5G43310	COP1-interacting protein-like protein	up	1.8	2.23E-02	281.4	495.1
130	AT2G16430	PAP10	purple acid phosphatase 10	up	1.7	6.26E-03	2368.0	4134.6
131	AT4G02520	GSTF2	glutathione S-transferase PM24	up	1.7	2.99E-02	9017.6	15698.5
132	AT2G07726			up	1.7	4.06E-02	1807.0	3151.6
133	AT3G26210	CYP71B23	cytochrome P450 71B23	up	1.7	2.74E-02	360.8	628.6
134	AT3G48990	AT3G48990	AMP-dependent synthetase and ligase-like protein	up	1.7	1.12E-03	4970.5	8636.5
135	AT2G33435	AT2G33435	RNA recognition motif-containing protein	up	1.7	2.52E-02	278.2	481.9
136	AT1G12880	NUDT12	nudix hydrolase 12	up	1.7	3.90E-02	306.1	529.6
137	AT3G43510			up	1.7	4.05E-02	5336.8	9249.9
138	AT4G32980	ATH1	homeobox protein ATH1	up	1.7	2.43E-02	765.6	1324.3
139	AT4G27657	AT4G27657	hypothetical protein	up	1.7	1.59E-02	296.8	513.4
140	ATCG00170			up	1.7	2.49E-03	5509.6	9507.8
141	AT3G44300	NIT2	nitrilase 2	up	1.7	4.81E-02	3404.6	5893.5
142	AT1G13710	CYP78A5	cytochrome P450, family 78, subfamily A, polypeptide 5	up	1.7	1.03E-02	190.7	328.9
143	AT2G05520	GRP-3	glycine-rich protein 3	up	1.7	9.89E-03	36873.0	63426.9
144	AT2G38860	YLS5	class I glutamine amidotransferase-like domain-containing protein	up	1.7	4.08E-02	773.9	1330.2
145	AT2G46430	CNGC3	cyclic nucleotide gated channel	up	1.7	8.04E-03	278.3	478.4

146	AT2G19650	AT2G19650	cysteine/histidine-rich C1 domain-containing protein	up	1.7	3.67E-02	587.3	1007.6
147	AT2G07676	AT2G07676	hypothetical protein	up	1.7	3.88E-02	218.3	374.2
148	AT5G06530	AT5G06530	ABC transporter G family member 22	up	1.7	2.61E-03	1343.2	2297.8
149	AT3G17790	PAP17	purple acid phosphatase 17	up	1.7	3.18E-02	1174.3	2004.6
150	AT4G14690	ELIP2	early light-inducible protein 2	up	1.7	3.13E-02	6614.4	11268.3
151	AT3G52140	AT3G52140	tetratricopeptide repeat (TPR)-containing protein	up	1.7	3.37E-02	281.7	479.9
152	ATCG00520			up	1.7	9.79E-03	16588.6	28185.0
153	AT2G01010			up	1.7	4.12E-02	89902.9	152653.6
154	AT5G57240	ORP4C	OSBP(oxysterol binding protein)-related protein 4C	up	1.7	5.72E-03	330.6	559.9
155	AT1G59730	TH7	thioredoxin H7	up	1.7	3.55E-02	211.0	357.2
156	AT2G37760	AT2G37760	NAD(P)-linked oxidoreductase-like protein	up	1.7	1.79E-02	1110.1	1874.4
157	AT4G23260	CRK18	cysteine-rich receptor-like protein kinase 18	up	1.7	2.97E-02	207.9	351.0
158	AT1G79830	GC5	golgin candidate 5	up	1.7	1.39E-02	430.0	724.1
159	AT5G54130	AT5G54130	calcium-binding endonuclease/exonuclease/phosphatase family protein	up	1.7	1.44E-02	599.2	1004.9
160	ATCG00270			up	1.7	4.27E-03	53079.7	88866.0
161	AT5G42020	BIP2	Luminal-binding protein 2	up	1.7	3.42E-02	3508.6	5868.9
162	AT2G36630	AT2G36630	Sulfite exporter TauE/SafE family protein	up	1.7	1.84E-02	2147.1	3593.5
163	AT1G20920	AT1G20920	DEAD-box ATP-dependent RNA helicase 42	up	1.7	3.72E-02	506.3	848.5
164	AT5G67480	BT4	BTB and TAZ domain protein 4	up	1.7	4.48E-02	525.1	877.2
165	AT5G02600	AT5G02600	heavy metal transport/detoxification domain-containing protein	up	1.7	2.58E-02	190.4	317.6
166	AT3G04070	NAC047	NAC domain containing protein 47	up	1.7	7.80E-03	198.5	329.6
167	AT3G59930	AT3G59930	defensin-like protein 206	up	1.7	1.62E-02	900.3	1493.7
168	AT2G25450	AT2G25450	putative 2-oxoacid dependent dioxygenase	up	1.7	4.04E-03	3574.0	5909.7
169	AT4G19060	AT4G19060	putative disease resistance protein	up	1.7	4.44E-02	622.4	1032.5
170	AT4G15700	AT4G15700	monothiol glutaredoxin-S3	up	1.7	1.53E-02	419.1	693.3
171	AT1G73370	SUS6	sucrose synthase 6	up	1.6	2.94E-02	255.3	421.1
172	AT1G55020	LOX1	lipoxygenase 1	up	1.6	2.84E-02	1407.3	2311.3
173	AT1G20970	AT1G20970	hypothetical protein	up	1.6	8.78E-03	339.0	556.2
174	ATCG00820			up	1.6	4.29E-02	24262.2	39699.2
175	AT3G29762			up	1.6	6.62E-03	7242.8	11850.1
176	AT4G23300	CRK22	cysteine-rich receptor-like protein kinase 22	up	1.6	4.88E-02	496.8	810.0
177	AT1G11700	AT1G11700	hypothetical protein	up	1.6	2.22E-02	3946.2	6443.4
178	AT2G21187			up	1.6	1.82E-02	428.6	697.4
179	AT2G35940	BLH1	BEL1-like homeodomain 1	up	1.6	3.74E-02	663.7	1078.4
180	AT4G01600	AT4G01600	GEM-like protein 2	up	1.6	3.67E-02	569.6	923.6
181	AT2G44670	AT2G44670	hypothetical protein	up	1.6	3.45E-02	10690.6	17356.4
182	AT2G47850	AT2G47850	zinc finger CCCH domain-containing protein 32	up	1.6	3.49E-02	427.2	691.7
183	AT2G27395			up	1.6	2.66E-02	1179.8	1910.3
184	AT4G36648			up	1.6	2.68E-02	320.0	516.1

185	AT4G02280	SUS3	sucrose synthase 3	up	1.6	2.34E-02	200.9	323.5
186	AT1G21310	EXT3	extensin 3	up	1.6	1.76E-02	14106.8	22702.0
187	AT4G05390	RFNR1	ferredoxin--NADP+ reductase	up	1.6	4.18E-02	1406.9	2261.0
188	AT2G23210	AT2G23210	UDP-glycosyltransferase-like protein	up	1.6	1.38E-02	216.7	347.1
189	AT5G54960	PDC2	pyruvate decarboxylase-2	up	1.6	2.81E-02	515.5	825.7
190	AT5G12110	AT5G12110	Elongation factor 1-beta 1	up	1.6	5.66E-03	6632.7	10625.5
191	AT4G27300	AT4G27300	S-locus lectin protein kinase-like protein	up	1.6	1.24E-02	752.3	1205.5
192	AT5G26240	CLC-D	chloride channel protein CLC-d	up	1.6	4.47E-02	626.1	1001.8
193	AT3G09350	Fes1A	protein Fes1A	up	1.6	4.49E-02	983.2	1571.5
194	AT3G25570	CPuORF11	S-adenosylmethionine decarboxylase-like protein	up	1.6	2.92E-02	456.0	730.2
195	AT2G07698	AT2G07698	F-type H+-transporting ATPase subunit alpha	up	1.6	1.21E-02	18333.4	29307.6
196	AT5G24160	SQE6	Squalene monooxygenase 1,2	up	1.6	2.76E-02	577.6	920.9
197	AT3G21150	BBX32	B-box 32 protein	up	1.6	1.46E-02	628.7	1002.6
198	AT1G11910	APA1	aspartic proteinase A1	up	1.6	4.04E-02	384.1	609.0
199	AT5G27730	AT5G27730	heparan-alpha-glucosaminide N-acetyltransferase	up	1.6	3.63E-02	647.4	1023.5
200	AT3G58350	RTM3	restricted tev movement 3	up	1.6	3.41E-02	204.0	321.5

Rank	Locus	Gene symbol	Gene /TAIR10 Short description	Regulation	Fold change	*P*-value	Average signal intensity	
							Wild type	*35S:VuDREB2A TR*
1	AT2G26010	PDF1.3	defensin-like protein 14	up	22.5	2.40E-02	203.6	4532.3
2	AT5G44420	PDF1.2	defensin-like protein 16	up	17.6	3.77E-02	382.9	6692.8
3	AT1G07400	AT1G07400	class I heat shock protein	up	3.9	1.15E-02	537.3	2091.4
4	AT5G52640	HSP90.1	heat shock protein 81-1	up	3.7	2.37E-02	1565.9	5806.5
5	AT5G48570	ROF2	peptidylprolyl isomerase	up	3.2	4.66E-03	1054.8	3338.8
6	AT3G12580	HSP70	heat shock protein 70-4	up	3.1	2.06E-02	6848.4	21019.7
7	AT5G05250	AT5G05250	hypothetical protein	up	2.7	2.50E-02	1246.9	3368.7
8	AT5G53450	ORG1	OBP3-responsive protein 1	up	2.7	6.60E-04	864.9	2320.0
9	AT4G01080	TBL26	protein trichome birefringence-like 26	up	2.6	2.63E-02	357.4	922.2
10	AT4G23600	COR13	cystine lyase	up	2.6	4.65E-02	342.4	877.3
11	AT1G65370	AT1G65370	meprin and TRAF homology domain-containing protein	up	2.6	4.34E-02	397.1	1011.9
12	AT4G33720	AT4G33720	putative pathogenesis-related protein	up	2.5	2.43E-02	304.6	768.7
13	BX819228	AT2G41640	Glycosyltransferase family 61 protein	up	2.5	1.17E-02	616.4	1527.0
14	AT4G12400	AT4G12400	putative stress-inducible protein	up	2.5	1.27E-02	1052.3	2592.3
15	AT2G23170	GH3.3	indole-3-acetic acid-amido synthetase GH3.3	up	2.4	4.88E-02	432.5	1045.5
16	AT3G19430	AT3G19430	late embryogenesis abundant protein-like protein	up	2.3	1.28E-02	280.1	655.6
17	AT4G39210	APL3	glucose-1-phosphate adenylyltransferase large subunit 3	up	2.2	8.00E-03	494.0	1102.5
18	AT1G06000	AT1G06000	UDP-glycosyltransferase-like protein	up	2.2	2.99E-02	1116.6	2484.9
19	AT1G74310	HSP101	heat shock protein 101	up	2.2	3.00E-02	371.9	807.5

20	AT3G03780	MS2	methionine synthase 2	up	2.1	3.71E-02	23977.8	50839.0
21	AT1G28610	AT1G28610	GDSL esterase/lipase	up	2.1	2.02E-02	693.4	1458.5
22	AT1G65060	4CL3	4-coumarate--CoA ligase 3	up	2.1	3.61E-02	784.8	1643.5
23	AT3G51240	F3H	Naringenin,2-oxoglutarate 3-dioxygenase	up	2.1	6.85E-03	5558.1	11532.9
24	AT1G68570	AT1G68570	putative nitrite transporter	up	2.1	3.87E-04	1138.6	2336.8
25	AT3G22840	ELIP1	chlorophyll A-B binding, early light-inducible protein	up	2.0	3.29E-02	8313.2	16907.6
26	AT1G32900	AT1G32900	granule-bound starch synthase	up	2.0	4.43E-02	2876.5	5800.4
27	AT1G59860	AT1G59860	HSP20-like chaperone	up	2.0	2.51E-02	319.5	646.3
28	AT2G23000	scpl10	serine carboxypeptidase-like 10	up	2.0	6.03E-03	597.5	1180.7
29	AT1G19670	CLH1	chlorophyllase 1	up	2.0	1.98E-02	1442.7	2836.5
30	AT3G07720	AT3G07720	galactose oxidase/kelch repeat-containing protein	up	2.0	4.10E-02	1901.2	3719.7
31	AT3G45140	LOX2	lipoxygenase 2	up	1.9	9.79E-03	1981.8	3862.7
32	AT1G01320	AT1G01320	tetratricopeptide repeat-containing protein	up	1.9	2.35E-02	195.7	378.9
33	AT1G60270	BGLU6	beta glucosidase 6	up	1.9	3.56E-02	329.7	638.3
34	AT5G08640	FLS1	flavonol synthase/flavanone 3-hydroxylase	up	1.9	3.29E-02	3546.9	6855.2
35	AT3G07770	Hsp89.1	HEAT SHOCK PROTEIN 89.1	up	1.9	4.12E-03	230.7	444.9
36	AT5G12110	AT5G12110	Elongation factor 1-beta 1	up	1.9	2.71E-02	5937.9	11413.2
37	AT4G16370	OPT3	oligopeptide transporter	up	1.9	4.56E-02	1176.8	2262.1
38	AT5G09590	MTHSC70-2	mitochondrial HSO70 2	up	1.9	3.45E-02	2457.8	4731.8
39	AT1G54050	AT1G54050	HSP20-like chaperone	up	1.9	3.25E-03	719.8	1384.3
40	AT2G43510	TI1	defensin-like protein 195	up	1.9	1.07E-02	353.9	680.2
41	AT5G37670	AT5G37670	heat shock protein 15.7	up	1.9	1.48E-02	237.5	451.1
42	AT4G03060			up	1.9	1.87E-02	236.9	449.0
43	AT4G14690	ELIP2	early light-inducible protein 2	up	1.9	9.56E-03	6351.3	12031.2
44	AT5G13170	SAG29	senescence-associated protein 29	up	1.9	2.03E-02	802.8	1501.0
45	AT1G10370	ERD9	glutathione S-transferase	up	1.9	1.04E-02	6239.3	11641.6
46	AT5G56030	HSP81-2	heat shock protein 81-2	up	1.9	2.27E-02	9197.2	17059.8
47	AT1G60590	AT1G60590	Pectin lyase-like protein	up	1.9	4.55E-02	498.1	923.7
48	ATCG00170			up	1.8	4.63E-04	5572.9	10236.5
49	AT3G52140	AT3G52140	tetratricopeptide repeat (TPR)-containing protein	up	1.8	3.21E-02	249.4	457.9
50	AT2G20560	AT2G20560	DNAJ heat shock protein-like protein	up	1.8	1.29E-02	733.0	1339.5
51	AT5G07990	TT7	Flavonoid 3'-monooxygenase	up	1.8	7.06E-03	242.0	441.9
52	AT2G41800	AT2G41800	hypothetical protein	up	1.8	2.78E-02	1119.8	2034.2
53	AT3G09440	AT3G09440	protein heat shock protein 70-3	up	1.8	2.47E-02	3838.2	6965.1
54	AT5G56010	HSP81-3	heat shock protein 81-3	up	1.8	1.58E-02	11963.1	21689.8
55	AT4G27300	AT4G27300	S-locus lectin protein kinase-like protein	up	1.8	1.34E-03	749.2	1354.1
56	AT4G05631	AT4G05631	hypothetical protein	up	1.8	1.94E-04	299.2	540.1
57	AT2G36630	AT2G36630	Sulfite exporter TauE/SafE family protein	up	1.8	1.37E-02	1995.6	3595.6
58	AT5G44750	REV1	DNA repair protein REV1	up	1.8	3.64E-02	226.4	406.6

59	ATCG00180			up	1.8	4.83E-02	13814.8	24851.5
60	AT4G33030	SQD1	UDP-sulfoquinovose synthase	up	1.8	1.04E-02	2551.4	4562.7
61	AT2G22240	MIPS2	inositol-3-phosphate synthase isozyme 2	up	1.8	2.43E-02	3062.3	5439.9
62	AT1G09560	GLP5	germin-like protein subfamily 2 member 1	up	1.8	6.08E-03	2369.9	4200.7
63	AT2G07726			up	1.8	8.58E-03	1724.3	3047.9
64	AT1G29940	NRPA2	nuclear RNA polymerase A2	up	1.8	6.01E-03	1318.3	2323.4
65	AT4G23250	EMB1290	cysteine-rich receptor-like protein kinase 17	up	1.8	2.82E-02	291.9	513.7
66	AT1G06350	AT1G06350	delta-9 desaturase-like 4 protein	up	1.8	1.94E-02	275.5	484.1
67	AT4G39800	MIPS1	inositol-3-phosphate synthase isozyme 1	up	1.7	2.70E-02	1066.8	1867.4
68	AT5G48880	PKT2	3-ketoacyl-CoA thiolase 5	up	1.7	2.51E-02	1291.6	2256.5
69	AT3G03770	AT3G03770	leucine-rich repeat protein kinase-like protein	up	1.7	4.01E-02	801.0	1388.5
70	AT1G76130	AMY2	alpha-amylase-like 2	up	1.7	2.07E-02	661.6	1143.1
71	AT5G09570	AT5G09570	Cox19-like CHCH family protein	up	1.7	3.48E-02	301.4	518.5
72	AT2G04030	CR88	Chaperone protein htpG family protein	up	1.7	4.20E-03	2385.4	4100.9
73	AT5G58770	AT5G58770	dehydrodolichyl diphosphate synthase 2	up	1.7	4.18E-02	7559.3	12902.9
74	AT1G79520	AT1G79520	Cation efflux family protein	up	1.7	3.94E-02	710.8	1207.2
75	AT3G06530	AT3G06530	U3 small nucleolar RNA-associated protein 10 and NUC211 domain-containing protein	up	1.7	1.82E-02	576.8	977.6
76	AT1G52060	AT1G52060	jacalin-like lectin domain-containing protein	up	1.7	2.64E-02	573.0	967.7
77	AT1G52050	AT1G52050	jacalin-like lectin domain-containing protein	up	1.7	2.38E-02	1125.1	1899.6
78	AT2G24120	SCA3	DNA-directed RNA polymerase 3	up	1.7	4.72E-03	245.0	413.3
79	AT2G21640	AT2G21640	hypothetical protein	up	1.7	3.46E-02	410.8	692.6
80	AT4G04223	AT3G56210	armadillo/beta-catenin-like repeat-containing protein	up	1.7	3.09E-02	483.3	812.0
81	AT3G29762			up	1.7	1.81E-02	6872.4	11536.7
82	AT1G64600	AT1G64600	copper ion binding / methyltransferase	up	1.7	5.83E-03	330.4	554.4
83	AT1G52070	AT1G52070	jacalin-like lectin domain-containing protein	up	1.7	3.85E-03	2093.7	3500.8
84	AT5G20150	SPX1	SPX domain-containing protein 1	up	1.7	4.45E-03	1137.6	1897.5
85	AT1G65040	AT1G65040	ubiquitin-protein ligase synoviolin	up	1.7	1.40E-02	353.0	587.5
86	AT3G55120	TT5	chalcone--flavonone isomerase 1	up	1.7	9.29E-03	1611.2	2678.0
87	AT5G17890	DAR4	protein DA1-related 4	up	1.7	1.50E-02	348.5	578.2
88	AT4G02510	TOC159	translocase of chloroplast 159	up	1.7	1.24E-02	211.3	349.9
89	AT4G22485	AT4G22485	bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin-like protein	up	1.7	4.99E-02	1124.0	1855.6
90	AT5G44050	AT5G44050	mate efflux domain-containing protein	up	1.7	4.57E-02	261.5	430.9
91	AT4G04020	FIB	putative plastid-lipid-associated protein 1	up	1.7	1.67E-02	8172.2	13488.3
92	AT5G55570	AT5G55570	hypothetical protein	up	1.6	4.35E-02	258.8	425.8
93	AT5G61790	CNX1	calnexin 1	up	1.6	7.35E-04	4808.7	7891.2
94	AT3G25700	AT3G25700	aspartyl protease family protein	up	1.6	1.53E-02	455.8	743.8
95	AT5G01790	AT5G01790	hypothetical protein	up	1.6	1.20E-02	457.2	744.9
96	AT3G52890	KIPK	KCBP-interacting protein kinase	up	1.6	3.87E-02	314.8	511.1
97	AT1G58520	RXW8	lipase/hydrolase	up	1.6	3.73E-02	847.3	1370.5

98	AT1G51380	AT1G51380	DEAD-box ATP-dependent RNA helicase 34	up	1.6	1.21E-02	244.8	396.1
99	AT4G33770	AT4G33770	inositol-tetrakisphosphate 1-kinase 3	up	1.6	7.59E-03	318.6	515.2
100	AT2G25140	CLPB4	casein lytic proteinase B4	up	1.6	1.94E-02	349.5	563.9
101	AT3G25230	ROF1	rotamase FKBP 1	up	1.6	1.22E-02	2877.7	4641.9
102	AT1G15960	NRAMP6	metal transporter Nramp6	up	1.6	1.70E-02	224.9	362.3
103	AT1G26150	PERK10	proline-rich extensin-like receptor kinase 10	up	1.6	4.98E-02	736.4	1186.5
104	AT5G52310	LTI78	low-temperature-responsive protein 78/desiccation-responsive protein 29A	up	1.6	2.73E-02	280.1	450.8
105	AT2G07675	AT2G07675	ribosomal protein S12	up	1.6	2.82E-02	1313.0	2110.1
106	AT3G59140	MRP14	multidrug resistance-associated protein 14	up	1.6	1.03E-02	219.3	351.2
107	AT1G79245			up	1.6	2.54E-02	621.8	996.0
108	AT5G33355	AT5G33355	defensin-like protein 207	up	1.6	2.62E-02	258.9	413.6
109	AT3G52370	FLA15	fasciclin-like arabinogalactan protein 15	up	1.6	7.67E-03	232.5	371.0
110	AT5G24380	YSL2	metal-nicotianamine transporter YSL2	up	1.6	3.07E-02	485.9	773.1
111	AT4G27560	AT4G27560	UDP-glycosyltransferase-like protein	up	1.6	2.98E-02	419.5	667.7
112	AT3G22550	AT3G22550	hypothetical protein	up	1.6	2.59E-02	1416.1	2249.1
113	AT1G79920	AT1G79920	Heat shock protein 70	up	1.6	2.00E-02	7067.0	11171.9
114	AT1G15040	AT1G15040	class I glutamine amidotransferase domain-containing protein	up	1.6	2.57E-02	522.9	827.7
115	AT3G23810	SAHH2	adenosylhomocysteinase 2	up	1.6	8.19E-03	8151.0	12885.2
116	AT2G39250	SNZ	AP2-like ethylene-responsive transcription factor SNZ	up	1.6	1.73E-02	579.2	915.8
117	AT5G53420	AT5G53420	CCT motif family protein	up	1.6	3.44E-03	442.6	698.0
118	AT3G13080	MRP3	ABC transporter C family member 3	up	1.6	4.36E-02	559.3	881.1
119	AT2G07708	AT2G07708	hypothetical protein	up	1.6	1.28E-02	3471.7	5469.7
120	AT2G37040	PAL1	phenylalanine ammonia-lyase 1	up	1.6	7.16E-03	9008.4	14189.8
121	AT4G08870	AT4G08870	putative arginase	up	1.6	2.80E-02	1873.2	2943.5
122	AT3G48240	AT3G48240	octicosapeptide/Phox/Bem1p domain-containing protein	up	1.6	1.56E-02	244.4	383.3
123	ATCG00270			up	1.6	9.08E-03	49781.6	77958.3
124	AT1G77600	AT1G77600	sister chromatid cohesion protein PDS5	up	1.6	3.81E-02	242.8	380.2
125	AT5G15450	CLPB3	casein lytic proteinase B3	up	1.6	2.20E-02	1225.3	1911.4
126	AT3G23990	HSP60	chaperonin CPN60	up	1.6	4.51E-02	3552.1	5536.2
127	AT5G46730	AT5G46730	glycine-rich protein	up	1.6	4.14E-02	2130.0	3319.3
128	AT4G02520	GSTF2	glutathione S-transferase PM24	up	1.6	2.21E-02	9048.4	14097.6
129	AT1G54040	ESP	epithiospecifier protein	up	1.6	2.18E-02	774.5	1205.5
130	AT4G00040	AT4G00040	chalcone synthase 2-like protein	up	1.5	2.86E-02	500.9	775.0
131	AT3G07090	AT3G07090	PPPDE putative thiol peptidase family protein	up	1.5	4.43E-02	3044.0	4700.4
132	AT5G15740	AT5G15740	O-fucosyltransferase family protein	up	1.5	2.32E-02	830.6	1281.5
133	AT2G43620	AT2G43620	chitinase-like protein	up	1.5	8.59E-03	674.5	1041.3
134	ATCG01240			up	1.5	1.91E-02	68382.2	105380.3
135	AT3G53830	AT3G53830	Regulator of chromosome condensation (RCC1) family protein	up	1.5	3.09E-02	459.1	707.9
136	AT1G09140	ATSRP30	splicing factor, arginine/serine-rich 1/9	up	1.5	2.58E-02	3732.0	5747.8

137	AT3G50380	AT3G50380	hypothetical protein	up	1.5	4.58E-02	518.3	794.7
138	AT2G07698	AT2G07698	F-type H+-transporting ATPase subunit alpha	up	1.5	3.09E-02	18576.9	28421.6
139	AT5G22700	AT5G22700	F-box/FBD/LRR-repeat protein	up	1.5	2.46E-02	450.2	687.8
140	AT2G35860	FLA16	fasciclin-like arabinogalactan protein 16	up	1.5	2.87E-02	2680.3	4093.5
141	AT4G26600	AT4G26600	S-adenosyl-L-methionine-dependent methyltransferase-like protein	up	1.5	3.59E-02	208.3	318.4
142	AT3G33004			up	1.5	4.52E-02	2440.9	3727.0
143	AT1G29170	WAVE2	protein SCAR3	up	1.5	3.84E-03	312.4	476.1
144	AT2G07711			up	1.5	1.61E-02	3683.4	5611.6
145	AT2G20570	GPRI1	GBF's pro-rich region-interacting factor 1	up	1.5	3.20E-02	1537.2	2338.1
146	AT3G62460	AT3G62460	Putative endonuclease or glycosyl hydrolase	up	1.5	3.71E-02	1587.2	2410.8
147	AT3G56290	AT3G56290	hypothetical protein	up	1.5	4.39E-02	3648.4	5538.0
148	AT1G72440	EDA25	protein embryo sac development arrest 25	up	1.5	2.10E-02	391.8	593.2
149	AT3G59930	AT3G59930	defensin-like protein 206	up	1.5	2.25E-03	861.1	1300.3
150	AT1G63810	AT1G63810	hypothetical protein	up	1.5	3.50E-02	684.9	1032.6
151	AT3G22310	PMH1	DEAD-box ATP-dependent RNA helicase 9	up	1.5	4.16E-02	1426.5	2150.0
152	AT1G48920	NUC-L1	nucleolin	up	1.5	2.12E-02	511.9	771.7
153	AT1G70620	AT1G70620	cyclin-related protein	up	1.5	6.12E-03	495.7	744.6
154	AT1G56170	NF-YC2	nuclear transcription factor Y subunit C-2	up	1.5	1.54E-03	1161.0	1741.2
155	AT2G41870	AT2G41870	remorin-like protein	up	1.5	2.78E-02	509.5	763.8
156	AT4G26850	VTC2	GDP-L-galactose phosphorylase	up	1.5	1.07E-02	43192.9	64717.8
157	AT5G13930	TT4	chalcone synthase	up	1.5	4.57E-02	6804.3	10195.2
158	AT1G78070	AT1G78070	transducin/WD-40 repeat-containing protein	up	1.5	2.89E-02	1891.2	2829.7
159	AT2G30490	C4H	trans-cinnamate 4-monooxygenase	up	1.5	1.75E-02	14701.2	21951.3
160	AT3G23410	FAO3	long-chain-alcohol oxidase FAO3	up	1.5	3.05E-02	1476.4	2206.5
161	AT3G25760	AOC1	allene oxide cyclase 1	up	1.5	9.72E-03	839.0	1251.6
162	AT3G09350	Fes1A	protein Fes1A	up	1.5	3.47E-02	779.6	1162.6
163	AT1G55350	DEK1	calpain-type cysteine protease	up	1.5	2.08E-03	191.2	284.8
164	AT2G31150	AT2G31150	ATP binding / ATPase	up	1.5	3.31E-02	222.3	331.0
165	AT2G27840	HDT4	histone deacetylase HDT4	up	1.5	4.55E-02	3591.7	5347.9
166	AT1G02930	GSTF6	glutathione S-transferase 1	up	1.5	2.44E-02	1758.2	2615.6
167	AT2G37890	AT2G37890	Mitochondrial substrate carrier family protein	up	1.5	4.62E-02	261.8	389.3
168	AT2G46450	CNGC12	cyclic nucleotide gated channel	up	1.5	4.84E-02	512.4	761.2
169	AT1G30960	AT1G30960	GTP-binding protein ERG	up	1.5	1.03E-02	341.0	505.6
170	AT4G09750	AT4G09750	Rossmann-fold NAD(P)-binding domain-containing protein	up	1.5	4.91E-02	1193.3	1765.3
171	AT3G52340	SPP2	putative sucrose-phosphatase 3b	up	1.5	2.40E-02	1673.6	2469.7
172	AT5G40480	EMB3012	protein embryo defective 3012	up	1.5	4.86E-02	219.5	323.9
173	AT5G13000	GSL12	callose synthase	up	1.5	9.83E-03	2141.0	3157.4
174	AT5G54510	DFL1	indole-3-acetic acid-amido synthetase GH3.6	up	1.5	2.84E-02	963.5	1419.0
175	AT2G19310	AT2G19310	HSP20-like chaperone	up	1.5	2.89E-02	3513.0	5168.7

176	AT1G12750	RBL6	RHOMBOID-like protein 6	up	1.5	1.85E-02	869.5	1280.8
177	AT5G09870	CESA5	cellulose synthase A catalytic subunit 5 [UDP-forming]	up	1.5	3.81E-02	685.3	1008.9
178	AT1G45201	TLL1	triacylglycerol lipase-like 1 protein	up	1.5	2.16E-02	3017.2	4434.2
179	AT5G49910	CPHSC70-2	heat shock protein 70-2	up	1.5	7.05E-03	2743.8	4030.4
180	AT5G61030	GR-RBP3	glycine-rich RNA-binding protein 3	up	1.5	1.45E-02	1864.6	2738.3
181	AT2G35060	KUP11	Potassium transporter 11	up	1.5	4.33E-02	827.8	1214.7
182	AT3G56860	UBA2A	UBP1-associated protein 2A	up	1.5	9.93E-03	305.6	447.9
183	AT3G24005			up	1.5	3.51E-03	352.0	515.6
184	AT5G17050	UGT78D2	Flavonoid 3-O-glucosyltransferase	up	1.5	4.09E-02	2176.5	3183.8
185	AT4G28450	AT4G28450	WD repeat and SOF domain-containing protein 1	up	1.5	1.92E-02	488.2	711.3
186	AT5G35930	AT5G35930	AMP-dependent synthetase and ligase family protein	up	1.5	1.28E-02	214.3	312.1
187	AT3G26932	DRB3	dsRNA-binding protein 3	up	1.5	3.43E-02	381.5	554.6
188	AT1G26540	AT1G26540	agenet domain-containing protein	up	1.5	1.36E-03	192.4	279.5
189	AT3G48200	AT3G48200	hypothetical protein	up	1.5	2.85E-02	3117.3	4521.6
190	AT1G51680	4CL1	4-coumarate--CoA ligase 1	up	1.4	4.49E-02	8907.6	12885.2
191	AT4G38840	AT4G38840	SAUR-like auxin-responsive protein	up	1.4	2.72E-02	4206.8	6089.8
192	AT1G54380	AT1G54380	survival of motor neuron protein-interacting protein 1	up	1.4	4.49E-02	209.7	303.4
193	AT5G16930	AT5G16930	AAA-type ATPase family protein	up	1.4	3.43E-02	314.4	454.8
194	AT1G71340	AT1G71340	glycerophosphodiester phosphodiesterase domain-containing protein	up	1.4	1.16E-02	508.3	735.0
195	AT2G07786			up	1.4	4.50E-02	1841.2	2650.7
196	AT2G18900	AT2G18900	transducin/WD-40 repeat-containing protein	up	1.4	4.24E-04	669.1	963.9
197	AT4G16630	AT4G16630	DEAD-box ATP-dependent RNA helicase 28	up	1.4	1.41E-02	230.5	332.1
198	AT5G63920	TOP3A	DNA topoisomerase III	up	1.4	1.23E-02	392.8	564.9
199	AT5G48360	AT5G48360	formin-like protein 9	up	1.4	4.43E-02	609.4	875.5
200	AT2G15690	AT2G15690	pentatricopeptide repeat-containing protein	up	1.4	1.65E-02	1323.1	1901.2

LIST OF PUBLICATIONS

JOURNALS

1. **Sadhukhan A**, Kobayashi Y, Kobayashi Y, Tokizawa M, Yamamoto YY, Iuchi S, Koyama H, Panda SK, Sahoo L (2014) VuDREB2A, a novel DREB2-type transcription factor in the drought-tolerant legume cowpea mediates DRE-dependent expression of stress-responsive genes and confers enhanced drought resistance in transgenic *Arabidopsis*. **Planta** 240(3):645-64 DOI 10.1007/s00425-014-2111-5.

2. **Sadhukhan A,** Panda SK, Sahoo L (2014) The cowpea RING ubiquitin ligase VuDRIP interacts with transcription factor VuDREB2A for regulating abiotic stress responses. **Plant Physiology and Biochemistry** 83:51-56 DOI 10.1016/j.plaphy.2014.07.007.

3. Behura R, Kumar S, Saha B, Panda MK, Dey M, **Sadhukhan A**, Mishra S, Alam S, Sahoo DP, Sugla T, Panda SK, Sahoo L (2014) Cowpea (*Vigna unguiculata* L Walp) In: Wang K (ed) **Methods in Molecular Biology: *Agrobacterium* protocols**. Springer, USA1223:255-64. DOI 10.1007/978-1-4939-1695-5_20.

4. Thapa G, **Sadhukhan A**, Panda SK, Sahoo L (2012) Molecular mechanistic model of plant heavy metal tolerance. **Biometals** 25:489–505.

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6. Bakshi S, **Sadhukhan A**, Mishra S, Sahoo L (2011) Improved *Agrobacterium*-mediated transformation of cowpea via sonication and vacuum filtration. **Plant Cell Reports** 30:2281–2292.

CONFERENCES/SEMINARS

1. **Sadhukhan A,** Kobayashi Y, Lipka A, Hoenkga O, Gore M, Iuchi S, Kobayashi M, Koyama H (2013) Genome-wide association study in *Arabidopsis* for understanding the molecular regulation of salt tolerance. Japanese society of soil science and plant nutrition. Nagoya University, Japan. Sept 11–13.

2. **Sadhukhan A**, Mishra S, Panda SK, Sahoo L (2012) Isolation and functional characterization of DREB2A from cowpea. International Conference on Plant Biotechnology for Food Security, National Agricultural Science Center, Pusa, New Delhi, India. Feb 21–24.

NUCLEOTIDE SEQUENCE RECORDS

1. *Vigna unguiculata* dehydration responsive element binding protein 2A (DREB2A) mRNA, promoter region and complete cds (GenBank: JN629045 and JQ066264) **Sadhukhan A**, Mishra S, Panda SK, Sahoo L

2. *Vigna unguiculata* cultivar Pusa Komal E3 Ubiquitin protein ligase DREB2A interacting protein (DRIP) complete cds (GenBank: JQ066265)

   Sadhukhan A, Mishra S, Bakshi S, Panda SK, Sahoo L

3. *Vigna radiata* vacuolar sodium proton antiporter (nhx1) mRNA, complete cds (GenBank: JN656211)

   Mishra S, **Sadhukhan A**, Panda SK, Sahoo L

4. *Eichhornia crassipes* phytochelatin synthase mRNA, partial cds (GenBank: KJ776787)

   Sadhukhan A, Sahoo L

5. *Vigna unguiculata* cultivar Pusa Komal ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (rbcS) mRNA, partial cds (GenBank: JN692253)

   Sadhukhan A, Mishra S, Bakshi S, Panda SK, Sahoo L

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ORIGINAL ARTICLE

VuDREB2A, a novel DREB2-type transcription factor in the drought-tolerant legume cowpea, mediates DRE-dependent expression of stress-responsive genes and confers enhanced drought resistance in transgenic *Arabidopsis*

Ayan Sadhukhan · Yasufumi Kobayashi · Yuriko Kobayashi · Mutsutomo Tokizawa · Yoshiharu Y. Yamamoto · Satoshi Iuchi · Hiroyuki Koyama · Sanjib Kumar Panda · Lingaraj Sahoo

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Abstract

Main conclusion **VuDREB2A exists in cowpea as a canonical DREB2-type transcription factor, having the ability to bind dehydration-responsive elements in vitro and confer enhanced drought resistance in transgenic *Arabidopsis*.**

Cowpea (*Vigna unguiculata* L. Walp) is an important cultivated legume that can survive better in arid conditions than other crops. But the molecular mechanisms involved in the drought tolerance of this species remain elusive with very few reported candidate genes. The Dehydration-Responsive Element-Binding Protein2 (DREB2) group of transcription factors plays key roles in plant responses to drought. However, no DREB2 ortholog has been reported from cowpea so far. In this study, we isolated and characterized a gene from cowpea, namely *VuDREB2A*, encoding a protein of 377 amino acids exhibiting features of reported DREB2-type proteins. In cowpea, *VuDREB2A* transcript accumulation was highly induced by desiccation, heat and salt, but slightly by exogenous abscisic acid (ABA) treatment. We also isolated the *VuDREB2A* promoter and predicted stress-responsive *cis*-elements in it using *Arabidopsis* microarray data. The *E. coli*-expressed VuDREB2A protein showed binding to synthetic oligonucleotides with Dehydration-Responsive Elements (DREs) from *Arabidopsis*, in electrophoretic mobility shift assays. Heterologous expression of *VuDREB2A* in *Arabidopsis* significantly improved plant survival under drought. In addition, overexpression of a truncated version of *VuDREB2A*, after removal of a putative negative regulatory domain (between amino acids 132–182) led to a dwarf phenotype in the transgenic plants. Microarray and quantitative PCR analyses of *VuDREB2A* overexpressing *Arabidopsis* revealed up-regulation of stress-responsive genes having DRE overrepresented in their promoters. In summary, our results indicate that *VuDREB2A* conserves the basic functionality and mode of regulation of *DREB2A* in *Arabidopsis* and could be a potent candidate gene for the genetic improvement of drought resistance in cowpea.

Keywords *Arabidopsis* · *Cis*-elements · Cowpea · DREB2A · Drought

Abbreviations

ABA	Abscisic acid
ABRE	ABA-responsive element
DRE	Dehydration-responsive element
DREB	DRE-binding protein
EMSA	Electrophoretic mobility shift assay

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A. Sadhukhan · L. Sahoo (✉)
Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati 781039, Assam, India
e-mail: ls@iitg.ernet.in

Y. Kobayashi · Y. Kobayashi · M. Tokizawa · Y. Y. Yamamoto · H. Koyama
Laboratory of Plant Cell Technology, Faculty of Applied Biological Sciences, Gifu University, Gifu 501-1193, Japan

S. Iuchi
RIKEN BioResource Center Tsukuba, Tsukuba, Ibaraki 305-0074, Japan

S. K. Panda
Department of Life Science and Bioinformatics, Assam University, Silchar 788011, India

ERF	Ethylene-responsive element-binding factor
FL	Full length
HSE	Heat stress-responsive element
LTE	Low temperature-responsive element
MeJA	Methyl jasmonate
PLACE	Plant *cis*-acting regulatory DNA elements
RAR	Relative appearance ratio
rd29A	Responsive to dehydration 29A
SA	Salicylic acid
SMART	Simple modular architecture research tool
TAIR	The Arabidopsis information resource
TR	Truncated
TSS	Transcription start site

Introduction

Abiotic stresses are major limiting factors for sustainable food production as they reduce yields by more than 50 % in crop plants (Bray et al. 2000). Drought is the most serious factor among various stresses limiting crop productivity in arid regions in African (Li et al. 2009) and South Asian (Li et al. 2011) countries. Understanding and improving plant tolerance to drought stress are, therefore, of prime importance for sustenance of agriculture in these regions. Some crop plants historically cultivated in these regions show relatively enhanced tolerance to drought stress and such crop plants could be useful models to study the molecular mechanisms of drought tolerance. For example, cowpea (*Vigna unguiculata* L. Walp.) is a legume crop grown in different parts of the world, viz. Africa, India, USA and Australia, under a wide range of climatic conditions such as semiarid to sub-humid and is well adapted to heat and drought (Hall 2012). The ability of cowpea to withstand drought and its small nuclear genome (~620 Mb) make it an ideal crop model to study the molecular mechanisms of drought tolerance (Agbicodo et al. 2009).

In earlier studies, a number of genes were identified that could explain the greater drought tolerance of cowpea than other legumes. For example, various genes encoding stress-responsive molecules were isolated by differential screening of mRNA or other methods. Genes for an old yellow enzyme, group 2 LEA proteins, dihydroflavonol-4-reductase (DFR) and vestitone reductase (VR) were identified first, by differential screening (Iuchi et al. 1996), followed by other genes such as genes encoding putative phosphoinositide-specific phospholipases (El-Maarouf et al. 2001), cystatins (Diop et al. 2004), ascorbate peroxidase (D'Arcy-Lameta et al. 2006), glutathione reductase (Contour-Ansel et al. 2006) and glutathione-S-transferase (Gazendam and Oelofse 2007). In all cases, the genes reported were up-regulated under water-stressed conditions, while overexpression of one of the cowpea genes up-regulated by water-stress, *VuNCED1*, a key gene involved in ABA biosynthesis encoding 9-*cis*-epoxycarotenoid dioxygenase, improved drought tolerance in *Arabidopsis* (Iuchi et al. 2001). This suggests that transcriptional regulation plays a critical role in the drought tolerance of cowpea.

Numerous stress-induced genes thought to promote stress tolerance are regulated by transcription activation factors that interact with *cis*-acting elements in the promoters of such genes. The Dehydration-Responsive Element-Binding Proteins (DREBs) are members of the APETALA2/ethylene-responsive element-binding factor (AP2/ERF) family of transcription factors, which follow the ABA-independent signal transduction pathway and recognize the core sequence motif A/GCCGAC, termed the Dehydration-Responsive Element (DRE), in the promoters of stress-inducible genes (Yamaguchi-Shinozaki and Shinozaki 1994). Among the *DREB* gene family, the A-2 subgroup including *DREB2A* is specifically involved in the response to drought, salinity and heat stress in *Arabidopsis* (Liu et al. 1998; Sakuma et al. 2006). This signal transduction process includes the up-regulation of DREB2A itself and activation of various genes involved in stress tolerance mechanisms in various plant species (Lata and Prasad 2011b; Mizoi et al. 2012). However, this signal transduction process involving DREB2A has not been reported in cowpea.

In the present study, we report the cloning and characterization of the *DREB2A* ortholog of cowpea. First, we isolated the cDNA and promoter region of the cowpea ortholog by degenerate-oligonucleotide primed PCR (Telenius et al. 1992) and inverse PCR (Ochman et al. 1988), respectively. Bioinformatic analyses of these sequences were carried out to compare them with previously characterized *DREB2A* orthologs [e.g., *Arabidopsis* (Liu et al. 1998) and soybean (Mizoi et al. 2013)]. Second, *VuDREB2A* expression in cowpea was profiled under various stress conditions that were used in previous studies for *DREB2A* orthologs (Liu et al. 1998; Gupta et al. 2010; Lata et al. 2011a) and the results were compared with our predicted *cis*-elements in the *VuDREB2A* promoter. Third, *E. coli*-expressed VuDREB2A was tested for its ability to bind the DRE core motif in vitro. Last, we characterized the isolated gene using heterologous expression in *Arabidopsis*, which is frequently used in functional biology to characterize orthologs (e.g., Ohyama et al. 2013; STOP1 transcription factor). In summary, we demonstrate the isolation and characterization of a drought-responsive *DREB2A* ortholog from cowpea, which conserves the basic functionality and mode of regulation of *DREB2A* in *Arabidopsis*.

Materials and methods

Plant materials

Cowpea (cultivar Pusa Komal) seeds were procured from National Seeds Corporation, Pusa, New Delhi, India. *Arabidopsis thaliana* (Columbia background) seeds were procured from RIKEN BioResource Center, Tsukuba, Japan.

Growth conditions

Cowpea plants were grown hydroponically in modified MGRL medium (Fujiwara et al. 1992; 1/50 strength with inorganic phosphate eliminated and calcium concentration adjusted to 200 µM, pH 5.5) at 25 °C under a 12-h day (with photon flux density of 40 µmol m^{-2} s^{-1}) and 12-h night cycle. Wild-type or transgenic *Arabidopsis* seeds were sown on ½ MS (Murashige and Skoog 1962) agar plates or soil containing perlite and plants were grown under similar temperature and light conditions to cowpea.

Isolation of a *DREB2A* ortholog by degenerate PCR and RACE

RNA isolation from cowpea was performed as described earlier (Suzuki et al. 2008) and cDNA was prepared from the RNA using ReverTraAce® reverse transcriptase (Toyobo, Osaka, Japan) following the manufacturer's instructions. A partial cDNA was obtained by PCR using degenerate primers designed from conserved regions of reported *DREB2* sequences (Supplementary Table S1) and subcloned into pTZ57R/T (Fermentas, Hanover, MD, USA) by TA-cloning. Then, 3′ and 5′ fragments were obtained by the RACE (rapid amplification of cDNA ends) PCR system (version 2; Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Genomic DNA from cowpea was isolated by HiPura Plant Genomic DNA extraction kit (Himedia Laboratories, Mumbai, India) and amplified using primers designed from the cDNA sequence. All DNA-sequencing analyses were carried out by a commercially available DNA-sequencing service of Delhi University South Campus, New Delhi, India.

Stress treatments and expression analysis in cowpea

One-week-old cowpea plants grown hydroponically in modified MGRL medium (Fujiwara et al. 1992; 1/50 strength with inorganic phosphate eliminated and calcium concentration adjusted to 200 µM, pH 5.5) were given the following treatments: for salinity stress, plants were dipped in 250 mM NaCl in modified MGRL medium and kept at 25 °C with a 12-h photoperiod; for desiccation stress, plant roots were wrapped dry in tissue paper and kept at 25 °C with a 12-h photoperiod; for heat stress, plants were kept in an incubator maintained at 50 °C with a 12-h photoperiod; for cold stress, plants were kept at 4 °C in a refrigerator under dim light; ABA, methyl jasmonate (MeJA) and salicylic acid (SA) treatments were given both by dipping roots in and spraying shoots with 100 µM (+)- *cis*, *trans*-ABA (Sigma, St. Louis, MO, USA), 100 µM (in 0.1 % v/v ethanol) MeJA (Sigma) and 500 µM SA (Sigma), respectively. For the SA treatment, plants were kept at 25 °C in the dark. Other phytohormone treatments were carried out at 25 °C with a 12-h photoperiod. MeJA treatment was carried out in a separate growth chamber with identical conditions to avoid the effects of volatile MeJA on plants subjected to other treatments. At different time points, stressed cowpea tissues were immediately frozen in liquid nitrogen. RNA isolation from cowpea was performed as described by Suzuki et al. (2008) and cDNA was prepared from the RNA using ReverTraAce reverse transcriptase (Toyobo) following the manufacturer's instructions. Real-time PCR was carried out using Takara SYBR Green PCR master mix II and a Takara Thermal Cycler Dice Real time system II (Takara-Bio, Otsu, Japan). Gene-specific primers for real-time PCR analyses were designed using the Primer3, version 4.0 software (Koressaar and Remm 2007). All qPCR quantifications were performed by the standard curve method using a cDNA dilution series (Bustin et al. 2009), and transcript levels of particular genes were relatively quantified using an internal standard, the homolog of the *UBQ1* gene in cowpea (GenBank Accession No. FG859491).

Isolation and analyses of the *VuDREB2A* promoter

The *VuDREB2A* promoter was isolated by inverse PCR. Five micrograms of genomic DNA from cowpea was digested with *Nde*I (New England Biolabs, Beverly, MA, USA) and 0.5 µg of purified digested DNA was ligated in a total volume of 500 µL for 12 h with 5 µL T4 DNA ligase (New England Biolabs) at 15 °C. The ligation reaction was used for amplification using primers F1 and R1 and nested primers F2 and R2 (Supplementary Table S1). The resultant 1.9 kb product was sequenced and screened by the plant promoter prediction program, TSSP (http://linux1.softberry.com/berry.phtml?topic=tssp&group=programs&subgroup=promoter; Shahmuradov et al. 2003). Promoter scanning analysis using overrepresented octamers in the promoters of *Arabidopsis* genes, which were >3-fold up-regulated under the following treatments: 3 h ABA (643 genes); 3 h cold (327 genes); 3 h osmotic stress (327 genes); 1 h dehydration stress (294 genes) and 1 h H_2O_2 (329 genes), was carried out as described previously (Yamamoto et al. 2011). The PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) web-programs were also used to locate *cis*-acting motifs within the promoter.

DNA/protein sequence and phylogenetic analyses

Nucleotide and protein sequences of *DREB2A* orthologs were retrieved from the NCBI Entrez (http://www.ncbi.nlm.nih.gov/) and Phytozome v9.1 (http://www.phytozome.net/) databases. A homology search of DREB2A was carried out by the Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov/BLAST) and the Simple Modular Architecture Research Tool (SMART) program (http://smart.embl-heidelberg.de) was used for identifying potential protein domains. Multiple amino acid sequence alignment was performed using the GENETYX ver. 11 (Genetyx, Tokyo, Japan) software with the CLUSTALW program (Larkin et al. 2007). Protein structure and potential modification sites were predicted by web-based programs as follows; secondary structures, PSSpred (http://zhanglab.ccmb.med.umich.edu/PSSpred/); phosphorylation sites, NetPhosK1.0 (http://www.cbs.dtu.dk/services/NetPhosK/); PEST sequences (Rechsteiner and Rogers 1996), ePestfind (http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind). A phylogenetic tree was constructed using Molecular Evolutionary Genetic Analysis (MEGA) v 6.0.5 (Tamura et al. 2013). The distances between branches were calculated by the neighbor-joining method based on the Jones–Taylor–Thornton model (Jones et al. 1992) with 1,000 bootstrap replicates.

Bacterial expression and electrophoretic mobility shift assay (EMSA)

The CDS of *VuDREB2A* (without the stop codon for introducing Hisx6 to C-terminus) was cloned into the pET28a expression vector (Novagen, Madison, WI, USA) and transformed into *E. coli* BL21 (DE3) cells. Recombinant protein was induced with 1 mM isopropyl-ß-D-1-thiogalactoside (Sigma) at 28 °C for 12 h and purified under native conditions using a Ni–NTA purification column (GE Healthcare Bio-sciences Corp., Piscataway, NJ, USA). The purity of the proteins was evaluated by SDS-PAGE following Laemmli (1970). Western-blotting analysis was performed following Towbin et al. (1979) using anti-His×6 mouse monoclonal antibody and horse radish peroxidase-conjugated goat anti-mouse IgG (Abexome Biosciences, Bangalore, India), a Hybond^{TM}-P hydrophobic polyvinylidene difluoride (PVDF) membrane (GE Healthcare Bio-sciences Corp.) and a TE77 semi-dry transfer unit (GE Healthcare Bio-sciences Corp.) following the manufacturer's instructions. For electrophoretic mobility shift assay (EMSA), native DRE probes were designed from the *Arabidopsis* responsive to desiccation *29A* (*RD29A*) promoter as reported earlier (Agarwal et al. 2007) with mutations introduced at various regions in the core motif CCGAC for testing specificity of binding (cf. Fig. 4b). The EMSA was carried out using an EMSA kit (Invitrogen) with designed primers (Supplementary Table S1) following the manufacturer's instructions with modified buffer (Agarwal et al. 2007) in a reaction volume of 20 µL. Protein–DNA complexes were fractionated by Native-PAGE on a 12 % polyacrylamide gel with 0.5 × TBE buffer containing 5 % glycerol using the MINIVE COMPLETE electrophoresis system (GE Healthcare Bio-sciences Corp.) and an EPS 301 electrophoresis power supply (Amersham Biosciences Corp., Piscataway, NJ, USA). Gels were visualized using the Gel Doc^{TM} XR + Imaging system (Bio-Rad Laboratories, Hercules, CA, USA). Densitometric analysis from gel images was performed by the ImageJ software (Schneider et al. 2012). Graphs were generated by Sigmaplot 10 (Systat Software, San Jose, CA, USA).

Generation of *VuDREB2A*-overexpressing *Arabidopsis* and drought-response test

The CDSs of *VuDREB2A* and a modified *VuDREB2A* (designated as truncated, TR), in which the putative negative regulatory domain (between nucleotides 394–546) was removed by megaprimer PCR (Ke and Madison 1997), were first cloned into the vector pRT101 (Töpfer et al. 1987) between the *Eco*RI and *Bam*HI sites to add the CaMV 35S promoter and the CaMV strain Cabb B-D polyadenylation signal. The promoter–gene–terminator cassette was then cloned into the *Pst*I site of the T-DNA of the binary vector pCAMBIA2301 (cf. Fig. 6a). All primers used for cloning are listed in Supplementary Table S1. The constructs were mobilized to *Agrobacterium* and *Arabidopsis* transformation was carried out by the floral dip method (Clough and Bent 1998).

Drought-response testing of transgenic *Arabidopsis* was performed as described previously (Iuchi et al. 2001). Specifically, wild-type and transgenic plants were grown in ½ MS media with 1 % sucrose for 15 d and then in soil for another 10 d in uniformly sized pots, perforated at the bottom, placed on a tray with nutrient solution. The solution was removed, and the pots kept dry for 18 d. Survival rates were determined after 7 d of recovery following re-watering. Two independent lines of *35S:VuDREB2A full-length (FL)* and the dwarf *35S:VuDREB2A truncated* (*TR*) line with 10 plants per line (5 in each pot) were used in the test. Wild-type Columbia (Col-0) was used as a control. Plants in pots subjected to progressive drought stress were weighed regularly at the same hour for calculation of water contents according to Vartanian et al. (1994). Then, pots were left to dry for a day in an 80 °C oven for calculation of dry weights. Water contents were calculated according to the formula (weight at day *N* – dry weight)/(weight at day 1 – dry weight) × 100. Water loss was due to evapotranspiration and it was assumed that the increase in plant biomass was negligible compared with the water loss.

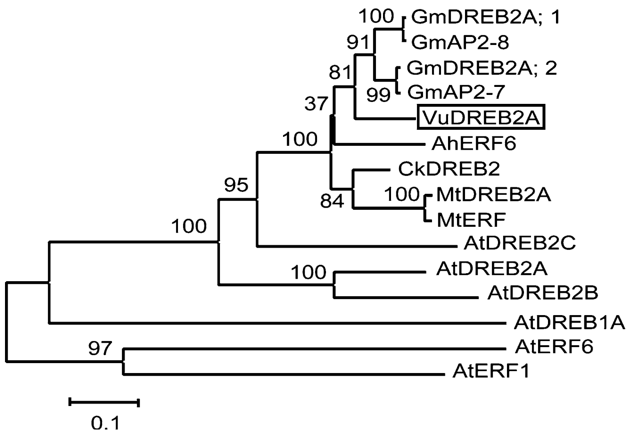

Fig. 1 Phylogenetic tree of proteins having ≥60 % sequence identity with VuDREB2A. *Arabidopsis* DREB and ethylene-responsive element-binding factor (ERF) homologs were used as out groups for the analysis. The tree was generated by MEGA 6.0.5 using the Neighbor-Joining method with 1000-replicate bootstrap testing. The numbers against each branch indicate the bootstrap values and the scale bar indicates substitution rate per site. The tree was constructed from a sequence alignment of the following proteins: *Arabidopsis thaliana* AtDREB1A [GenBank: BAA33434], AtDREB2A [GenBank: AAU93685], AtDREB2B [GenBank: BAA36706], AtDREB2C [GenBank: Q8LFR2], AtERF1 [GenBank: AAD03544], AtERF6 [GenBank: NP_567529] *Glycine max* GmDREB2A;1 [GenBank: AFU35562], GmDREB2A;2 [GenBank: AFU35563], GmAP2-7 [GenBank: NP_001240942], GmAP2-8 [GenBank: NP_001240005]; *Vigna unguiculata* VuDREB2A [GenBank: AEY75222]; *Medicago truncatula* MtDREB2A [GenBank: ABJ88942], MtERF [GenBank: XP_003616701]; *Arachis hypogaea* AhERF6 [GenBank: AFU07643]; *Caragana korshinskii* CkDREB2 [GenBank: AGI78251]

Microarray and data processing

RNA was isolated from 3-week-old transgenic *Arabidopsis* seedlings as described previously (Suzuki et al. 2004). The microarray experiment was performed by the competitive hybridization method using an Agilent Microarray system following the manufacturer's instructions. Two comparisons including one dye swap were made between biologically independent samples. The arrays were scanned with a G2505C scanner (software version 6.5) and processed using the Feature extraction software (version 10.7.3.1). Data were analyzed with GeneSpring 12.6 (Agilent Technologies, Tokyo, Japan). Our microarray raw data are available at the NCBI Gene Expression Omnibus with accession code GSE53113. A meta-profile analysis of the top 200 up-regulated genes in the transgenic plants was performed in a public microarray database (Genevestigator plant biology version; Hruz et al. 2008). The overrepresented sequences (octamers), which contained DRE core motifs (CCGAC) or ABRE core motif (ACGT) in the promoters of the top 200 up-regulated genes, in transgenic plants were computed as described previously (Yamamoto et al. 2011). The promoter sequence from −1,000 to −1 relative to the major transcription start site of each gene (determined by large scale TSS tag sequencing; Yamamoto

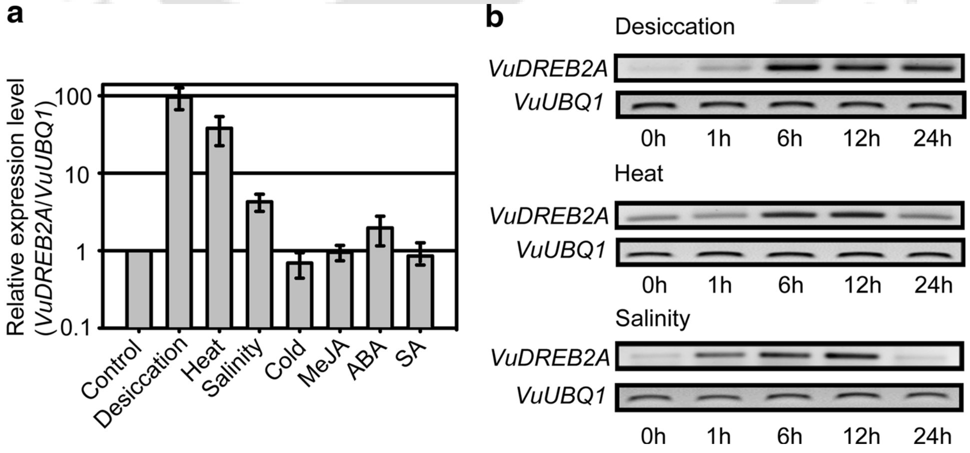

Fig. 2 Stress-inducible expression of *VuDREB2A*. Transcript levels of *VuDREB2A* in the shoots of cowpea plants grown for 1 week and then exposed to different stress treatments (desiccation, heat, cold, salinity, ABA, MeJA and SA) for 6 h (**a**). Columns indicate relative expression levels of *VuDREB2A* normalized against levels of *VuUBQ1* as calculated by real-time qRT-PCR (mean ± SE of three biological replicates). Gel photos indicate expression levels in a single experiment. Transcript abundance of *VuDREB2A* at different time points with major inducing stressors viz. desiccation, heat and salinity (**b**)

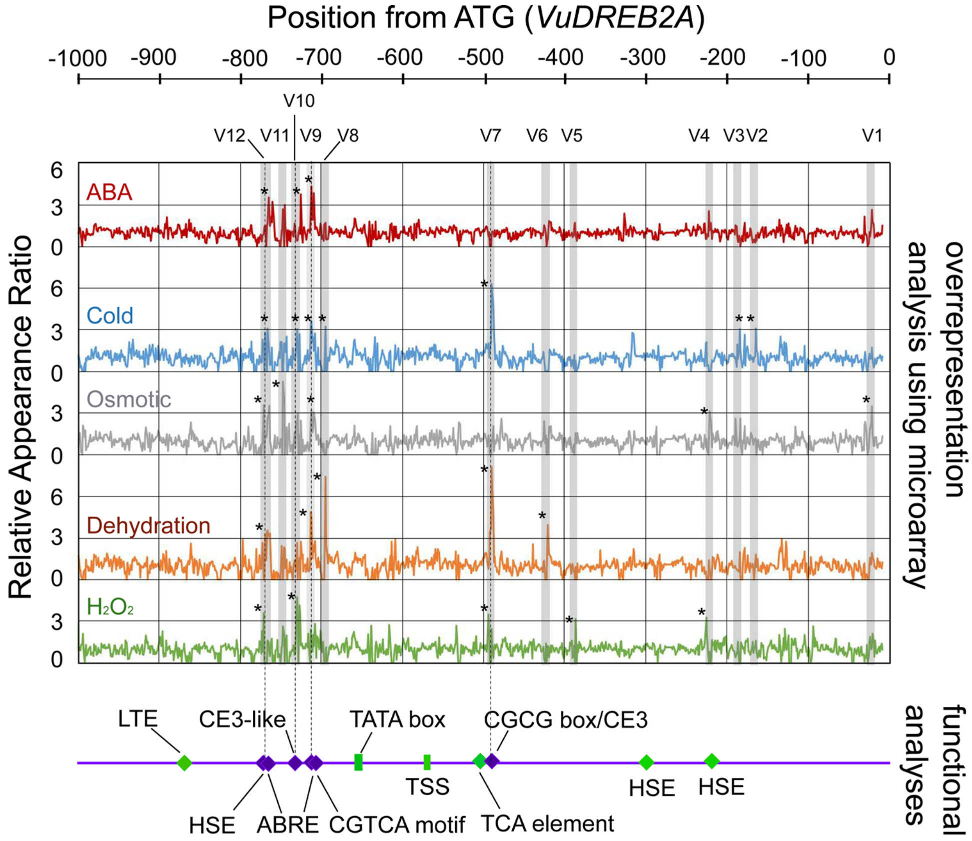

Fig. 3 Analysis of the *VuDREB2A* promoter. The *VuDREB2A* promoter and 5′-UTR (1,000 bp upstream of ATG) were scanned for *cis*-elements by evaluation of octamers in 1 bp steps based on a Relative Appearance Ratio (RAR) table as described previously (Yamamoto et al. 2011). RAR (ratio of octamer in the promoter of stress-inducible genes to genome-wide promoters) was calculated from microarray data of *Arabidopsis* under different treatments (ABA, cold, osmotic stress, dehydration and H_2O_2). *The graphs* show scanning results based on microarray data of the ABA response (*red*), the cold response (*blue*), the osmotic response (*gray*), the dehydration response (*orange*) and the H_2O_2 response (*green*) of *Arabidopsis*. Asterisks indicate octamers with RAR > 3 and $P < 0.05$. Locations of predicted *cis*-elements are indicated by *gray columns* (V1–12; Table 1). Promoter motifs determined in other plant promoters by functional analyses, are shown on the *purple line*. *Purple symbols* indicate those motifs carried by our predicted *cis*-elements, while *green symbols* indicate additional motifs detected by the PlantCARE and PLACE signal scan searches. *ABRE* ABA-responsive element, *CE3* Coupling element 3, *HSE* Heat stress-responsive element, *LTE* Low temperature-responsive element, *TSS* Transcription start site

et al. 2009) was used for the calculation and the relative appearance ratio (RAR) compared with the promoters of all genes genome-wide was computed. All data relevant to *Arabidopsis* genes were mined from The Arabidopsis Information Resource (TAIR) version 10 (http://www.arabidopsis.com/).

Statistical analyses

Statistical analyses were performed using Microsoft Excel (Microsoft Japan, Tokyo). All data were analyzed using a one-way analysis of variance (ANOVA), performed at the 95 % confidence level. A multiple comparison test (Tukey's test) was then used to compare the significant differences. The data shown are the mean values ± SE of three replicates, and the significance level relative to controls is $P < 0.05$.

Results

Isolation of the *VuDREB2A* gene by PCR-based cloning

First, we obtained a partial cDNA fragment (386 bp) by PCR using degenerate primers designed from highly conserved regions in DREB2-type proteins. Using 5′ and 3′ RACE procedures, we obtained the full-length mRNA sequence that contained a coding DNA sequence (CDS) encoding 377 amino acids with 214 bp of 5′ UTR and 157 bp of 3′ UTR. The deduced amino acid sequence was highly homologous to previously isolated DREB2 proteins, and was closest to a legume DREB2A isolated from soybean (*Glycine max*) (Fig. 1). In addition, the protein conserved the consensus amino acids in the unique APETALA 2 domain (i.e., the 14th valine and 19th glutamic acid at 79–142 amino acids), and carried a conserved nuclear

Table 1 Predicted *cis*-elements in the *VuDREB2A* promoter

Position from ATG[a]	Octamer[b]	Treatment[c]	RARf[d]	*cis*-element[e]	Sequence	Motif[f]	Reference[g]
−771	TCGAGGGC	H_2O_2	3.53	V12	TCGAGGGCC ACGTGCCAGC	HSE	Sun et al. (2006)
−771	TCGAGGGC	Osmotic stress	3.55				Yoshida et al. (2011)
−769	GAGGGCCA	Dehydration	3.20				
−767	GGGCCACG	Dehydration	3.33			ABRE	Kim et al. (2011)
−766	GGCCACGT	Cold	3.06				
−766	GGCCACGT	Dehydration	3.51				
−765	GCCACGTG	ABA	3.53				
−765	GCCACGTG	Dehydration	3.12				
−764	CCACGTGC	Dehydration	3.12				
−764	CCACGTGC	Osmotic stress	3.50				
−763	CACGTGCC	Dehydration	3.29				
−760	GTGCCAGC	ABA	3.23				
−747	CGGGAGTC	Osmotic stress	5.26	V11	CGGGAGTCC		
−746	GGGAGTCC	Osmotic stress	4.13				
−730	ATAACGCG	Cold	3.05	V10	ATAACGCG ACTAG	CGCG Box/CE3-like	Yang and Poovaiah (2002)
−730	ATAACGCG	H_2O_2	4.69				Kaplan et al. (2006)
−727	ACGCGACT	H_2O_2	4.14				
−726	CGCGACTA	H_2O_2	3.45				
−725	GCGACTAG	ABA	3.76				
−713	ACGCCACG	Cold	3.28	V9	ACGCCAC GTCAC	ABRE	Kim et al. (2011)
−713	ACGCCACG	Dehydration	4.84			CGTCA motif	
−712	CGCCACGT	ABA	4.34			(MeJA	Reinbothe et al. (1994)
−712	CGCCACGT	Cold	3.62			Responsive	Wang et al. (2011)
−712	CGCCACGT	Osmotic stress	3.20			element)	
−712	CGCCACGT	Dehydration	4.45				
−709	CACGTCAC	ABA	3.84				
−709	CACGTCAC	Osmotic stress	3.02				
−695	GGGGGGCA	Cold	3.22	V8	GGGGGGCA		
−695	GGGGGGCA	Dehydration	7.38				
−494	ATACACCG	H_2O_2	3.48	V7	ATACACCGCGTTTT	CGCG	
−492	ACACCGCG	Dehydration	3.40			Box/CE3	Kaplan et al. (2006)
−491	CACCGCGT	Dehydration	3.69				
−490	ACCGCGTT	Cold	6.30				
−490	ACCGCGTT	Dehydration	8.13				
−489	CCGCGTTT	Cold	5.62				
−489	CCGCGTTT	Dehydration	6.44				
−488	CGCGTTTT	Cold	4.61				
−488	CGCGTTTT	Dehydration	4.53				
−421	TGCACGCT	Dehydration	3.92	V6	TGCACGCT		
−386	TGTGCGAT	H_2O_2	3.13	V5	TGTGCGAT		

Table 1 continued

Position from ATG[a]	Octamer[b]	Treatment[c]	RARf[d]	*cis*-element[e]	Sequence	Motif[f]	Reference[g]
−225	CTTGGGGG	H_2O_2	3.25	V4	CTTGGGGGTTAC		
−221	GGGGTTAC	Osmotic stress	3.12				
−184	TGGCTACT	Cold	3.04	V3	TGGCTACT		
−164	AGCGTGTT	Cold	3.11	V2	AGCGTGTT		
−21	TAGCAGTG	Osmotic stress	3.47	V1	TAGCAGTG		

Octamer sequences in the *VuDREB2A* promoter which were overrepresented in the promoters of *Arabidopsis* genes responsive to ABA, cold, osmotic stress, dehydration and H_2O_2 are listed together with their relative appearance ratios (see "Materials and methods"). *Cis*-elements V1-V12 predicted on the basis of these overrepresented octamers were looked up for occurrence of functionally characterized promoter motifs from literature

[a] Position from start codon of *VuDREB2A*

[b] Overrepresented octamers in promoters of genes up-regulated >3- fold in Arabidopsis under

[c] Different treatments (viz., ABA, cold, osmotic stress, dehydration and H_2O_2)

[d] Relative Appearance Ratio (RAR) of each octamer = count of the octamer in n genes up-regulated under different treatments/n)/(count of the octamer in total 24,956 promoters/24,956). $n = 643$ (ABA)/337 (cold)/327 (osmotic stress)/294 (dehydration)/329 (H_2O_2). RARf = filtered RAR (RAR > 3, two-sided Fisher's exact test $P < 0.05$)

[e] Predicted cis-elements that are related to ABA, cold, osmotic stress, dehydration and H_2O_2 (V1–12; Fig. 3)

[f] Motifs in the predicted cis-elements, based on functional analyses in other plant species

[g] Source of functional analyses

localization signal (Supplementary Figs. S1, S2). It was thus assumed that the gene was the cowpea ortholog of *DREB2A*, and it was deposited to GenBank (as *VuDREB2A*; accession number: JN629045.3, theoretical average molecular mass of 41.76 kDa and pI of 4.76). Using primers designed at the 3′ and 5′ ends of the cDNA, we obtained amplification from genomic DNA. Comparison of the genomic amplicon [GenBank: JQ066264.2] and the full-length cDNA showed that *VuDREB2A* carried an intron of 418 bp in the 5′ UTR.

Expression profile and promoter structure of *VuDREB2A*

The expression of *VuDREB2A* was profiled under various stress treatments and with chemical signal inducers. After 6 h treatment, the *VuDREB2A* transcript was found to be highly induced by heat, salinity and desiccation stresses in cowpea shoots (Fig. 2a), similarly to its ortholog in *Arabidopsis* (Liu et al. 1998). All these stressors activated the expression very quickly, as identified by the time course analyses (Fig. 2b). Exogenous application of ABA only slightly induced *VuDREB2A*, again similar to its *Arabidopsis* ortholog (Kim et al. 2011). Similar pattern of expression was also observed in cowpea roots (data not shown).

In silico analysis of putative *cis*-elements, conducted with the *VuDREB2A* promoter sequence (GenBank Accession No. JQ066264.2), predicted the presence of a TATA box at position -32 from transcription start site (Supplementary Fig. S3). The promoter scanning analysis using the overrepresented octamers in the promoters of stress-responsive genes in *Arabidopsis* (fold change >3; NaCl, ABA, dehydration and H_2O_2) (Yamamoto et al. 2011) predicted 12 *cis*-elements (V1-12) that are related to salinity, dehydration, and oxidative stress, and ABA signaling (Fig. 3 and Table 1) in the *VuDREB2A* promoter. Of these, 4 elements contained functionally validated promoter motifs: ABA-responsive element (ABRE; core motif: ACGT; Kim et al. 2011) in V9 and V12; CGCG box (consensus: [A/C/G]CGCG[C/T/G]; Yang and Poovaiah 2002) and Coupling Element 3 (CE3; consensus: [A/C]ACGCG[C/T/G]; Kaplan et al. 2006) in V7 (complete identity) and V10 (partial identity); a heat stress-responsive element (Sun et al. 2006; Yoshida et al. 2011) in V12; and a CGTCA motif involved in MeJA-mediated response (Wang et al. 2011) in V9. Few additional *cis*-elements predicted by the PLACE and PlantCARE web-programs were: a low temperature-responsive element (LTE) (Dunn et al. 1998); TCA elements involved in SA response (Reinbothe et al. 1994); WRKY factor binding sites (Eulgem et al. 2000); a MYB binding site responsive to dehydration (Abe et al. 2003) and TC-rich repeats involved in defense and stress response (Rushton and Somssich 1998) (Supplementary Fig. S3).

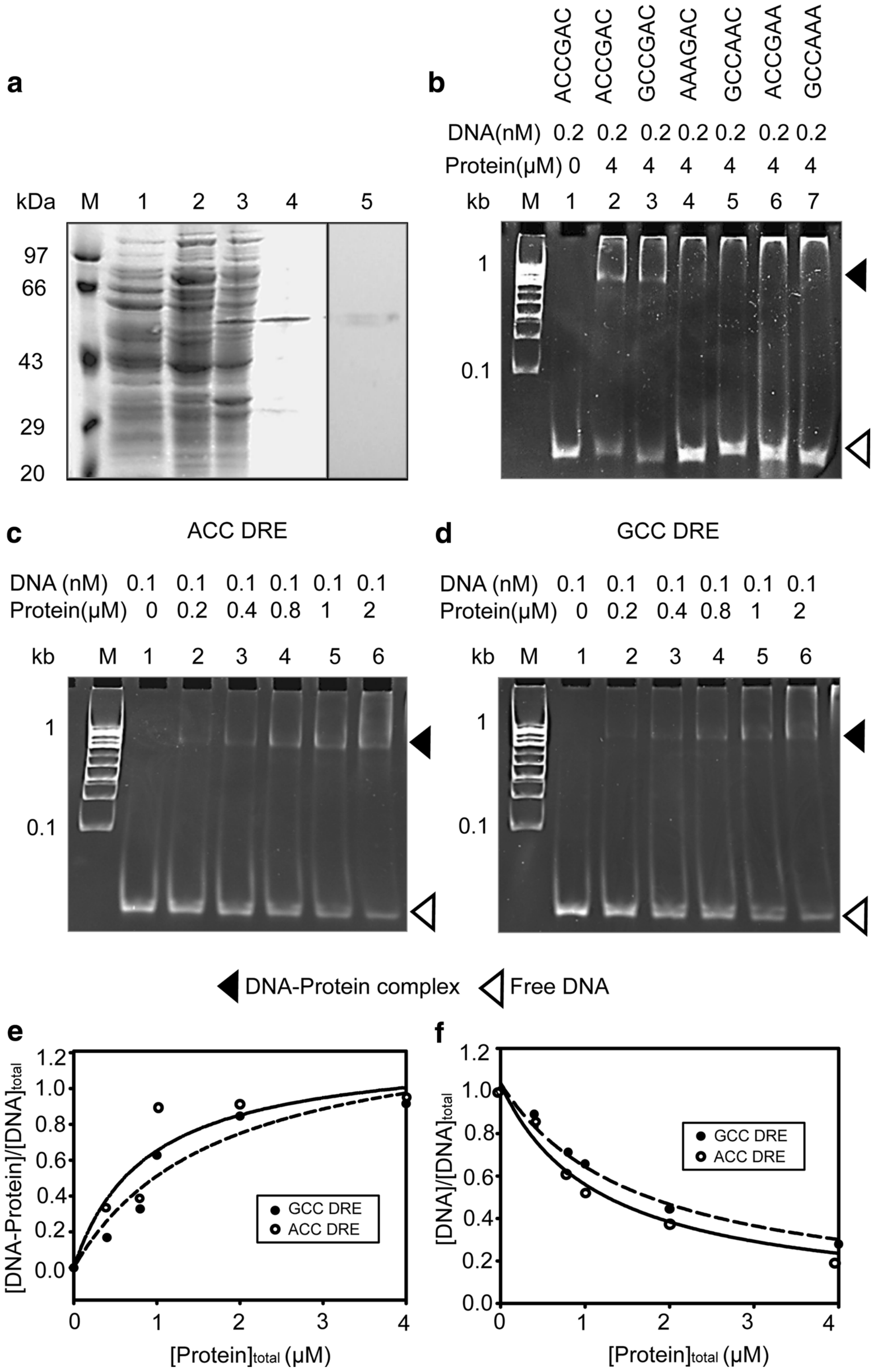

Fig. 4 **a** Electrophoretic mobility shift assay of VuDREB2A for binding ability to DRE sequences. VuDREB2A protein His×6-tagged at both the N- and C-termini was expressed in *E. coli* BL21 (DE3) cells, purified with a Ni–NTA agarose affinity column, and then checked for quality by SDS-PAGE. *Lane* M, protein size markers; *lane 1*, crude extract of BL21(DE3); *lane 2*, crude extract of BL21(DE3)pET28a; *lane 3*, crude extract of BL21(DE3) pET28a*VuDREB2A*; *lane 4*, purified *E. coli* expressing VuDREB2A (with Ni–NTA agarose affinity chromatography) and Western blotting with anti-His antibody (*lane 5*). **b** Electrophoretic Mobility Shift Assay in Native-PAGE of recombinant VuDREB2A with oligonucleotides carrying ACCGAC or GCCGAC dehydration-responsive element (DRE) sequences from the *Arabidopsis* rd29A promoter with VuDREB2A protein. Titration of fixed amounts of ACC (**c**) and GCC (**d**) DRE oligonucleotides with different amounts of recombinant VuDREB2A protein. "M" in all electrophoresis gels indicates molecular mass markers. *Black arrowheads* indicate positions of the DNA–protein complexes, while *white arrowheads* indicate positions of free DNA. Plots of fraction of bound DNA versus total protein (**e**) and fraction of free DNA versus total protein (**f**) for the ACC and GCC DREs as calculated by densitometry from (**c**) and (**d**)

Recombinant VuDREB2A interacts with dehydration-responsive elements in vitro

Using highly purified VuDREB2A protein obtained by the *E. coli* expression system [i.e., that gave single band in SDS-PAGE (Fig. 4a, lane 4) and in western blotting for the His-tag (Fig. 4a, lane 5)], we analyzed the interaction of VuDREB2A with known DRE sequences, which were previously identified in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki 1994), by electrophoretic mobility shift assay (EMSA). Protein–double-stranded oligonucleotide complexes were observed with both ACCGAC and

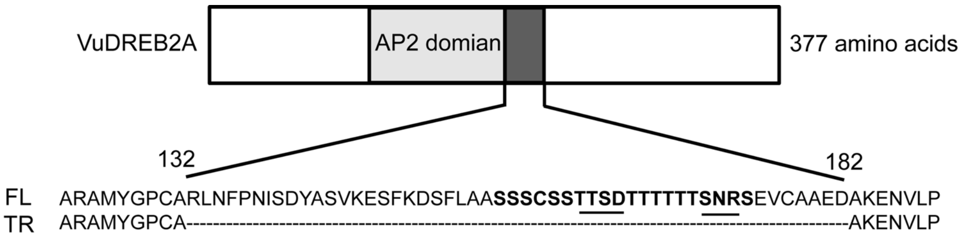

Fig. 5 Truncation of the putative negative regulatory domain of VuDREB2A. The region between amino acids 132 and 182 in the wild-type *full-length* (*FL*) VuDREB2A was removed to prepare a *truncated* (*TR*) putative active form. This deleted region contains a poor PEST motif (PEST score −22.19) RLNFPNISDYASV and a serine/threonine-rich region SSSCSSTTSDTTTTTTSNRS containing predicted protein kinase C (TTSD) and casein kinase II (SNR) recognition sites (*underlined*)

GCCGAC DRE sequences (Fig. 4b, lanes 2 and 3). The complexes disappeared when a few random mutations were introduced into the core sequence of CCGAC (Fig. 4b, lanes 4–7). At a fixed concentration (0.1 nM) of both ACCGAC and GCCGAC probes, we detected increased binding with increasing concentrations of VuDREB2A protein (0.2–4 μM) (Fig. 4c, d), which was also evident by a hyperbolic increase in the fraction of bound DNA (Fig. 4e) and a hyperbolic decay in the fraction of free DNA (Fig. 4f). These results suggested simple stoichiometric interaction with similar affinity for both DREs. In addition, we did not detect the complex when we incubated the probes with BSA (data not shown). Altogether, we concluded that VuDREB2A has the capacity to bind with the DRE consensus.

Drought resistance of transgenic *Arabidopsis* overexpressing *VuDREB2A*

To further evaluate the function of *VuDREB2A* in plant, we introduced the gene into *Arabidopsis* and characterized several phenotypes. Both the *35S:VUDREB2A full-length* (*FL*) and *35S:VuDREB2A truncated* (*TR*; the putative negative regulatory domain between amino acid residues 132 and 182 removed) (Figs. 5 and 6a) genes were introduced into the Col-0 wild type generating ten independent lines of each. Drought resistance was analyzed at the T_2 generation of the transgenic lines highly expressing *VuDREB2A* (Fig. 6b).

Two *FL* and one *TR* transgenic lines were exposed to drought stress and their survival rates were compared with the wild-type Col-0 (Fig. 6b). Both types of transgenic lines showed greater than 85 % survival rates following drought recovery, while no more than 30 % of the wild-type plants survived (Fig. 6d), indicating that *VuDREB2A* could enhance drought resistance in *Arabidopsis*. Supporting this observation, the rates of water loss from the transgenic lines were found to be slower than that from Col-0 (Supplementary Fig. S4). In the control (no stress) growth conditions, the FL transgenic lines grew comparably to Col-0, while several TR transgenic lines showed a typical dwarf phenotype (Fig. 7). The TR transgenic lines had shorter roots on gel medium (Fig. 7a, c) and delayed bolting in pot culture (Fig. 7b). This suggested that the negative regulatory domain of VuDREB2A is functional, and that the removal of this domain has a negative impact on growth, as was previously identified in *Arabidopsis DREB2A* overexpressors (Sakuma et al. 2006).

Activation of transcription in transgenic *Arabidopsis* carrying *VuDREB2A*

To understand the effects of *VuDREB2A* overexpression in *Arabidopsis*, we analyzed the gene expression profiles of the transgenic plants using an *Arabidopsis* microarray. The overexpression of FL and TR *VuDREB2A* caused up-regulation (fold change >3) of 15 and 6 genes, respectively, under unstressed conditions (signal intensity >200, *t* test P value < 0.05) with three genes in common (Table 2). These up-regulated genes were found to be responsive to various stress conditions (drought, salinity, osmotic stress, heat and ABA, in particular) in publicly available microarray data (Table 2; fold change >3 in public array). This trend was common among the top 200 up-regulated genes (Supplementary Table S2) in the transgenic lines in the meta-profile analysis (Fig. 8). The expression of genes found to be up-regulated in the *VuDREB2A* overexpressors from our microarray results was further confirmed by qRT-PCR (Fig. 9). The results indicated that VuDREB2A can activate genes for drought resistance in *Arabidopsis*. In fact, the promoter sequences of the up-regulated genes were found to contain DREs (Tables 2 and 3). Certain genes like PDF1.3 and HSP70 had significantly higher expression in the *35S:VuDREB2A TR* plants than *35S:VuDREB2A FL* plants (Fig. 9).

Discussion

cDNAs encoding DRE binding proteins responsible for activating transcription of genes for dehydration tolerance were first isolated from *Arabidopsis*; *CBF1* (Stockinger

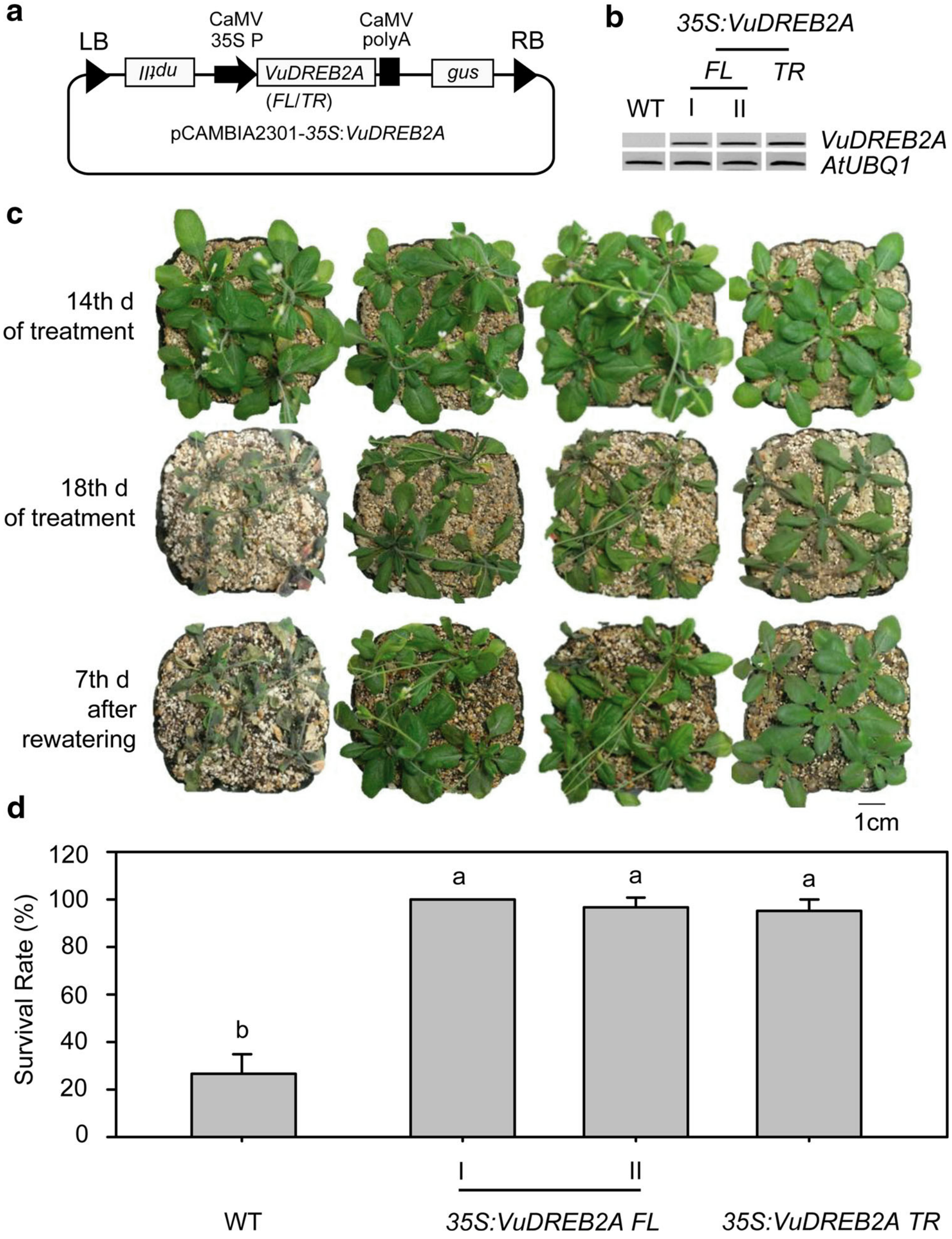

Fig. 6 Survival rates of transgenic *Arabidopsis* carrying ectopically expressed *VuDREB2A*. *Full-length* (*FL*) or *truncated* (*TR*; see Fig. 5) *VuDREB2A* under the control of the cauliflower mosaic virus 35S promoter cloned into plant expression vector pCAMBIA2301 (**a**) was introduced into *Arabidopsis* ecotype Col-0 (WT). LB and RB, left and right borders of the T-DNA; *nptII*: neomycin phosphotransferase II; *gus*: ß-glucuronidase. Transgenic lines expressing VuDREB2A, as determined by semiquantitative RT-PCR using *AtUBQ1* as an internal control (**b**), were grown in pot culture after 15 d of pre-culture on ½ MS media with 1 % sucrose. Ten plants from each line were planted in pots containing soil and were grown for a further 10 d with the bottoms of the pots immersed in nutrient solution in plastic trays. Then, the nutrient solutions were removed to impose dehydration stress, and the damage to the transgenic lines and WT on the 14th and 18th d, and the recovery from damage after 7 d of rewatering were photographed (**c**; *bar* = 1 cm). **d** Survival rates of the wild-type and transgenic plants were determined from three independent assays. Results are presented as mean ± SE ($n = 3$ biological replicates, 10 plants per replicate). There are no significant differences (Tukey's test, $P \geq 0.05$) between the lines with the same characters, and significant differences (Tukey's test, $P < 0.05$) between lines with different characters. I and II represent two independent transgenic lines carrying *full-length* (*FL*) *VuDREB2A*, while *TR* indicates a *truncated VuDREB2A* line

et al. 1997), *DREB1A* and *DREB2A* (Liu et al. 1998). Subsequently, orthologous genes have been identified from a wide variety of plants (Lata and Prasad 2011b; Kim et al. 2011), but only a few have been isolated from salt/drought-tolerant species. In the present study, we successfully isolated a functional ortholog from cowpea, which is a

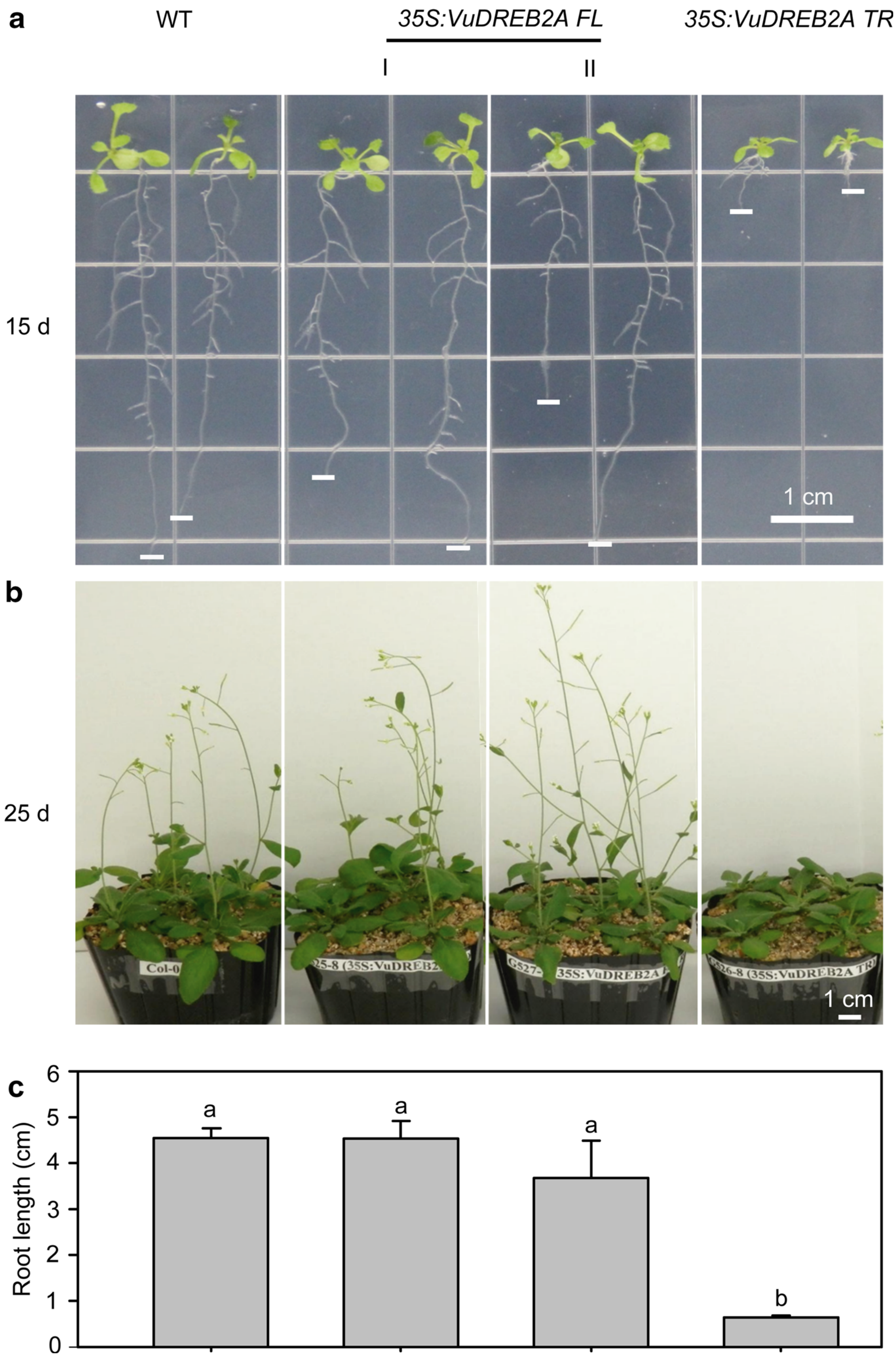

Fig. 7 Growth of transgenic *Arabidopsis* overexpressing *VuDREB2A* under control conditions. WT (Col-0) and transgenic lines carrying *VuDREB2A* in *full-length* (FL) form or *truncated* (TR; see Fig. 5) were grown in ½ strength Murashige and Skoog media with 1 % sucrose for 15 d (**a**), and in soil for another 10 d (**b**). I and II represent two independent transgenic lines carrying *full-length* (*FL*) *VuDREB2A*, while *TR* indicates a *truncated VuDREB2A* line. Root lengths of the transgenic plants grown in media for 15 d (**c**) are presented as mean ± SE ($n = 10$ plants per line). There are no significant differences (Tukey's test, $P \geq 0.05$) between the lines with the same characters, and significant differences (Tukey's test, $P < 0.05$) between lines with different characters

well-known drought-tolerant species. The isolated gene encoded a protein that was highly homologous to the functional ortholog of soybean, *GmDREB2A;2* (Kim et al. 2011) (Fig. 1). In addition, the protein shared various characteristics with DREB2A, which contains a single AP2 domain that carries valine and glutamic acid residues at the 14th and 19th positions (Supplementary Fig. S1) (Sakuma et al. 2002). The protein also had conserved motifs common to DREB2s (Nakano et al. 2006), carrying CMIV-1 and CMIV-2 in the N-terminus and CMIV-3 in the C-terminal activation domain (Supplementary Fig. S2). Thus, we finally named the gene *VuDREB2A*.

VuDREB2A was strongly induced by desiccation, salinity and heat, and slightly by application of exogenous

Table 2 Microarray analyses of *35S:VuDREB2A* (*full length* and *truncated*) *Arabidopsis* growing under unstressed conditions compared to that of wild-type Col-0

Locus	Gene symbol	TAIR10 short description	Fold change[a]	Stress response		*Arabidopsis/* soybean[d]	DRE/ABRE[e]
				Gene ontology[b]	Public microarray[c]		
Genes whose expression increased > 3-fold in the *35S:VuDREB2A (full length)* plants compared to wild-type plants							
AT1G27020		Hypothetical protein	5.6			*GmFL*	A1
AT4G12490		Bifunctional inhibitor/lipid-transfer protein/ seed storage 2S albumin-like protein	5.5		C, SA, Wo		
AT4G23600	COR13	Cystine lyase	5.2	Os, S, Wo	D, H, Os, S, Wo, MeJA, ABA	*Gm*△2	A1, A3, D1
AT2G26010	PDF1.3	Defensin-like protein 14	4.5				D1
AT3G50480	HR4	RPW8-like protein 4	4.3		Os, Ox, SA		A1, A4
AT1G77120	ADH1	Alcohol dehydrogenase class-P	3.9	D, Os, S	D, C, Os, S, ABA, SA		A1, A2, A3, A4, A5
AT3G45140	LOX2	lipoxygenase 2	3.9	H, Os, S, W, C, Wo	D, Wo, MeJA, SA	*Gm*△2	A1
AT1G43910		P-loop containing nucleoside triphosphate hydrolase-like protein	3.9		D, S, SA		A1
AT1G07400		Class I heat shock protein	3.9	H, Ox	D, H, Os, S, H, ABA, SA		
AT1G02930	GSTF6	Glutathione-S-transferase 1	3.8	Os, Ox, S, W	D, Os, S, C, Ox, Wo		D1, A1, A3, A4
AT5G22460		Esterase/lipase/thioesterase family protein	3.7		D, Os, S, ABA		
AT1G09240	NAS3	Nicotianamine synthase 3	3.5		D, C, ABA		
AT5G51440		Heat shock protein 23.5	3.4	H, Os, Ox	D, H, S, Ox, SA		D1, A1
AT5G52640	HSP90.1	Heat shock protein 81-1	3.1	H, Ox,	D, H, Os, ABA, SA	*Gm*△2	A1
AT5G13170	SAG29	Senescence-associated protein 29	3.0	Os	D, H, Os, ABA, SA		A1, A3
Genes whose expression increased > 3-fold in the *35S:VuDREB2A (truncated)* plants compared to wild-type plants							
AT2G26010	PDF1.3	Defensin-like protein 14	22.5				D2
AT5G44420	PDF1.2	Defensin-like protein 16	17.6	Wo	Os, MeJA		D1, A1, A3
AT1G07400		Class I heat shock protein	3.9	H, Ox			
AT5G52640	HSP90.1	Heat shock protein 81-1	3.7	H, Ox,	D, H, Os, Ox, ABA	*Gm*△2	A1
AT5G48570	*ROF2*	Peptidylprolyl isomerase	3.2	Ox, H	D, H, Os, S, Ox, ABA	*At FL*	
AT3G12580	*HSP70*	Heat shock protein 70-4	3.1	Ox, H	D, H, Os, S, Ox, ABA, SA	*AtCA, GmFL&*△2	D1

Up-regulated genes (fold change >3) in the transgenic plants are listed along with gene symbols, short descriptions of TAIR10, their stress responsiveness and occurrence of DRE/ABRE in their promoters

Os osmotic stress, *S* salt stress, *D* drought stress, *W* water deprivation, *H* heat, *C* cold, *Wo* wound, *Ox* oxidative stress, *ABA* abscisic acid, *MeJA* methyl jasmonate, *SA* salicylic acid

[a] Ratio of fluorescence intensity of each cRNA of *35S:VuDREB2A(FL/TR)*/fluorescence intensity of each cRNA of wild type. Average data of biological replicates (*t* test $P < 0.05$, signal intensity >200)

[b] Gene Ontology based annotations for genes responsive to stress

[c] Genes up-regulated more than threefold in public microarray experiments with stress/phytohormones (data mined with Genevestigator)

[d] Genes that were also up-regulated in transgenic *Arabidopsis* overexpressing the *Arabidopsis* and soybean homologs of *DREB2A*. *AtFL, 35S:AtDREB2A FL; AtCA, 35S:AtDREB2A CA; Gm FL, 35S:GmDREB2A;2 FL and Gm*△*2, 35S:GmDREB2A;2*△*2* (Mizoi et al. 2013)

[e] ABRE/DRE motifs within 1 kb upstream of transcription start site of the genes. ABRE consensus sequences from PLACE database: A1, ACGTG; A2, CACGTGGC; A3, ACGTSSSC; A4, MACGYGB; A5, CCACGTGG. DRE consensus sequences: D1, RCCGAC; D2, ACCGAGA

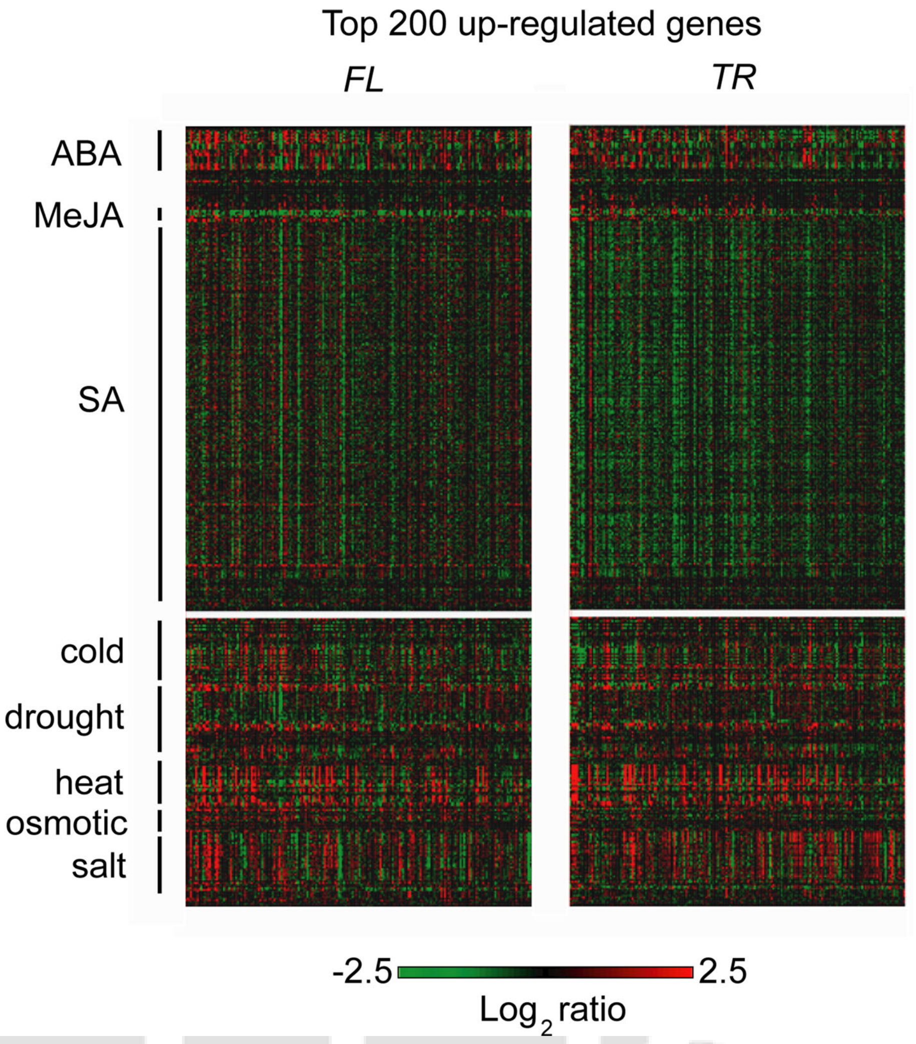

Fig. 8 Meta-profile analyses of up-regulated genes in *Arabidopsis* carrying *VuDREB2A*. Induction ($\log_2$) ratios of the top 200 up-regulated genes in *35S:VuDREB2A full-length* (FL) and *truncated* (TR) plants in control conditions were compared with public microarray experiments with cold, heat and osmotic-stressed conditions and phytohormones; the results are shown with a heat map. The experiments with hormones/stress are in the *y*-axis and genes in the *x*-axis (in order of decreasing fold change in *VuDREB2A* overexpressing plants from left to right). *ABA* abscisic acid, *MeJA* methyl jasmonate, *SA* salicylic acid

ABA (Fig. 2), which is a characteristic of *DREB2A* orthologs from *Arabidopsis* (Liu et al. 1998) and many other plant species (Lata and Prasad 2011b; Mizoi et al. 2012). This conserved mode of expression profile could be explained by conserved *cis*-elements responsive to these stressors in the promoters of *Arabidopsis*, soybean and cowpea *DREB2* genes (Supplementary Figs. S3, S5, and Supplementary Table S3). Particularly, promoters of *DREB2A* in soybean and cowpea possessed similar clusters of *cis*-elements (V5-12 and G2-10; Supplementary Fig. S5) related to the dehydration response of *Arabidopsis*, with at least 10 bp sequence identity in five cases (V5-G2, V7-G10, V9-G6, V10-G7 and V12-G9). This suggested strong conservation of the *DREB2A* promoter structure within the two legume species. The *VuDREB2A* promoter was found to conserve the ABA-responsive complex including ABRE and its Coupling Element 3 (and the similar CGCG box). Roles of the ABRE and CE3 in the dehydration responsiveness of the *AtDREB2A* promoter were demonstrated earlier (Kim et al. 2011). Additional roles of the CE3 in calcium signaling were also reported (Kaplan et al. 2006). The CGCG box is commonly observed in promoters of genes involved in ABA signaling and are specific targets of calmodulin-binding proteins in *Arabidopsis* (Yang and Poovaiah 2002). Involvement of similar pathways in the regulation of *DREB2A* in cowpea can be speculated from the localization of the ABRE-CE3 in the *VuDREB2A* promoter. The observation of the heat stress-responsive element in the *VuDREB2A* promoter could be correlated to the strong induction of *VuDREB2A* by heat stress. We could detect some additional *cis*-elements responsive to low temperature, MeJA and SA in the *VuDREB2A* promoter (Fig. 3 and Supplementary Fig. S3). Consistent with these findings, the *VuDREB2A* gene was found to be slightly induced by exogenous application of MeJA and SA. Cold had an induction effect on the gene after 12 h of treatment (Supplementary Fig. S6).

In vitro binding (i.e., EMSA, Fig. 4) and *in planta* overexpression assays revealed that *VuDREB2A* encodes a

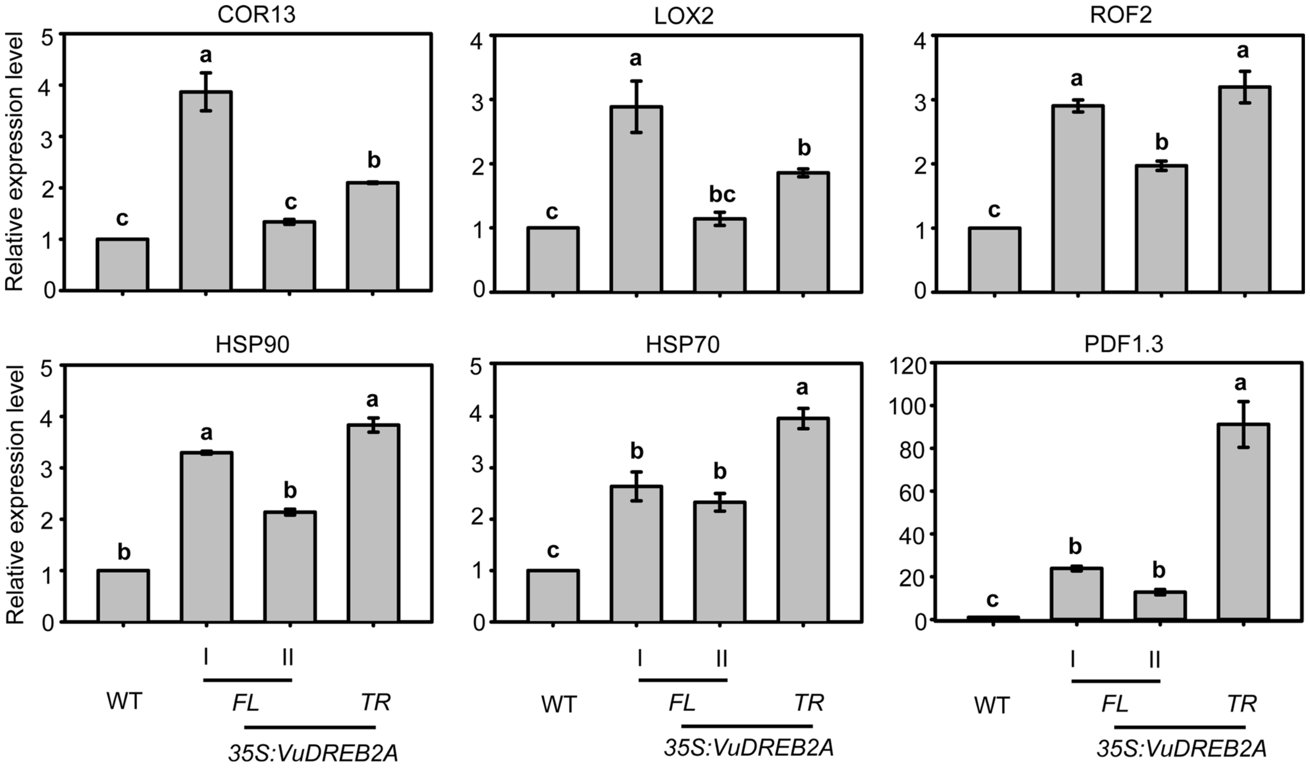

Fig. 9 Identification of *VuDREB2A* downstream targets in *Arabidopsis*. The expression levels of up-regulated genes in *35S:VuDREB2A* FL and TR plants in the microarray experiment were quantified by real-time qRT-PCR. *Columns* indicate relative expression levels normalized against the levels of *AtUBQ1* (mean ± SE of $n = 3$ biological replicates). I and II represent two independent transgenic lines carrying *full-length* (*FL*) *VuDREB2A*, while *TR* indicates a *truncated VuDREB2A* line. Results are presented as mean ± SE ($n = 3$ biological replicates, 10 plants per replicate). There are no significant differences (Tukey's test, $P \geq 0.05$) between the lines with the same characters, and significant differences (Tukey's test, $P < 0.05$) between lines with different characters

functional protein that can bind to the known target sequence of DREB2A (Sakuma et al. 2002; A/GCCGAC, Fig. 4b) and can activate the transcription of several known target genes of *AtDREB2A* and *GmDREB2A;2* in *Arabidopsis* (Table 1) (Mizoi et al. 2013). The promoters of the genes up-regulated by VuDREB2A were enriched for DRE and ABRE (Table 3), as computed by the octamer-based method (Yamamoto et al. 2011), further supporting similar DNA target recognition to *Arabidopsis DREB2A*. Additional enrichment of ABREs in the promoters of VuDREB2A, as found earlier in the case of active forms of *GmDREB2A;2* (Mizoi et al. 2013), would be the result of secondary activation of ABA-regulated genes and/or co-localization of both DRE and ABRE in the same promoter. We could not detect complexes of VuDREB2A with ABRE core sequence ACGT in EMSA (data not shown). Also, from previous studies, no DREB ortholog was found to interact with ABRE sequences directly. In *Arabidopsis* DREB2A was shown to interact physically and cooperate with ABA-responsive element-binding factor 2 (ABF2), a transcription factor involved in ABA-responsive gene expression (Lee et al. 2010). Such interactions could be responsible for indirect activation of genes having ABRE in their promoters.

Ectopic expression of *VuDREB2A* in *Arabidopsis* resulted in an increased drought-resistant phenotype (Fig. 6c), but overexpression of the truncated protein (i.e., removing the putative negative regulatory domain) induced a dwarf phenotype (Fig. 7). This was very similar to phenotypes observed with *Arabidopsis* overexpressors of *DREB2A* driven by a constitutive promoter (Sakuma et al. 2006, Chen et al. 2009; Mizoi et al. 2013). This suggests that the cowpea ortholog has an active negative regulatory domain, which remains functional in the heterologous system of *Arabidopsis*. In fact, a serine/threonine-rich region immediately downstream of the AP2 domain was conserved in VuDREB2A (Supplementary Fig. S2), which acts as a negative regulatory domain that destabilizes the DREB2A protein in *Arabidopsis* by phosphorylation (Sakuma et al. 2006; Agarwal et al. 2007) and degrades it by the ubiquitin–proteasome mediated process (Qin et al. 2008).

In summary, we succeeded in isolating a novel *DREB2* ortholog from the drought-tolerant crop cowpea, and

Table 3 Overrepresentation analysis of octamer sequences in the promoters of top 200 up-regulated genes in *35S:VuDREB2A full-length* (*FL*) and *truncated* (*TR*) transgenic plants

Octamer[a]	Motif	Occurance in 24,956 genes	Occurance in 200 genes[b]	RARf[c]	*P* Value (Two-sided fisher's Exact Test)	Octamer[a]	Motif	Occurance in 24,956 genes	Occurance in 200 genes[b]	RARf[c]	*P* Value (Two-Sided fisher's exact test)
VuDREB2A FL transgenic *Arabidopsis*						*VuDREB2A TR* transgenic *Arabidopsis*					
CGGTCGGC	DRE	24	2	10.4	0.01834	AGTCGGCC	DRE	47	3	8.0	0.00764
GCGGTCGG	DRE	30	2	8.3	0.02711	CCCGTCGG	DRE	33	2	7.6	0.03201
GCGTCGGG	DRE	32	2	7.8	0.03034	CGTCGGTA	DRE	65	3	5.8	0.01755
CGTCGGTC	DRE	76	3	4.9	0.02595	CCGACATG	DRE	134	6	5.6	0.00103
CTGTCGGA	DRE	78	3	4.8	0.02767	CTACCGAC	DRE	90	4	5.5	0.00716
CGGTCGGT	DRE	79	3	4.7	0.02855	TAGTCGGC	DRE	68	3	5.5	0.01966
TGACCGAC	DRE	120	4	4.2	0.01823	TACCGACG	DRE	77	3	4.9	0.0268
CTACCGAC	DRE	90	3	4.2	0.03923	GCCGACAC	DRE	78	3	4.8	0.02767
TAGTCGGA	DRE	127	4	3.9	0.0218	GGACCGAC	DRE	119	4	4.2	0.01776
GTCGGTCA	DRE	143	4	3.5	0.03143	GGTCGGAA	DRE	120	4	4.2	0.01823
GACGTCTG	ABRE	62	3	6.0	0.01557	ATTGTCGG	DRE	171	5	3.6	0.01444
CTGTACGT	ABRE	84	4	5.9	0.00569	CTCCGACT	DRE	137	4	3.6	0.02757
ACGTCTCG	ABRE	69	3	5.4	0.0204	TTCCGACG	DRE	149	4	3.3	0.03559
CTACGTGC	ABRE	74	3	5.1	0.02429	TACCGACA	DRE	154	4	3.2	0.03929
CTGAACGT	ABRE	74	3	5.1	0.02429	TTGCCGAC	DRE	161	4	3.1	0.04481
GAACGTTC	ABRE	124	5	5.0	0.00408	CGGACGTT	ABRE	59	4	8.5	0.00171
GCACGTAG	ABRE	78	3	4.8	0.02767	TAGACGTC	ABRE	113	6	6.6	0.00044
CGTACGTT	ABRE	193	7	4.5	0.00132	ACGTTGCG	ABRE	57	3	6.6	0.01257
TAGACGTC	ABRE	113	4	4.4	0.01506	ACGTGGCC	ABRE	154	8	6.5	0.00006
ACGTCGCT	ABRE	85	3	4.4	0.03416	GAACGTCC	ABRE	62	3	6.0	0.01557
CCGTACGT	ABRE	85	3	4.4	0.03416	CACTACGT	ABRE	125	6	6.0	0.00073
TACGTGCT	ABRE	85	3	4.4	0.03416	GACGTCTA	ABRE	106	5	5.9	0.00214
TCTGACGT	ABRE	181	6	4.1	0.00436	AGACGTTC	ABRE	110	5	5.7	0.0025
ACGTACGG	ABRE	91	3	4.1	0.04028	TGAACGTC	ABRE	118	5	5.3	0.00333
AGCACGTA	ABRE	92	3	4.1	0.04136	AGACGTCT	ABRE	125	5	5.0	0.00421
GCACGTAT	ABRE	96	3	3.9	0.04578	CCACGTAC	ABRE	101	4	4.9	0.01047
GCTACGTG	ABRE	96	3	3.9	0.04578	CGACGTTA	ABRE	130	5	4.8	0.00494
GTACGTTT	ABRE	303	9	3.7	0.00115	GCACGTAG	ABRE	78	3	4.8	0.02767
TGAACGTA	ABRE	174	5	3.6	0.01542	CGTACGTC	ABRE	79	3	4.7	0.02855
ACGTGTGA	ABRE	315	9	3.6	0.00149	ATAGACGT	ABRE	188	7	4.6	0.00115
GACGTTTC	ABRE	247	7	3.5	0.00495	CACGTCCT	ABRE	108	4	4.6	0.01302
AATACGTA	ABRE	468	13	3.5	0.00021	TCGACGTG	ABRE	108	4	4.6	0.01302
CCACGTAA	ABRE	290	8	3.4	0.00325	GTGCACGT	ABRE	83	3	4.5	0.03223
TCGTACGT	ABRE	183	5	3.4	0.01864	TACGTGGG	ABRE	87	3	4.3	0.03614

Table 3 continued

Octamer[a]	Motif	Occurance in 24,956 genes	Occurance in 200 genes[b]	RARf[c]	*P* Value (Two-sided fisher's Exact Test)	Octamer[a]	Motif	Occurance in 24,956 genes	Occurance in 200 genes[b]	RARf[c]	*P* Value (Two-Sided fisher's exact test)
CACGTAAA	ABRE	415	11	3.3	0.00087	TCACGTCC	ABRE	120	4	4.2	0.01823
CTACGTAC	ABRE	190	5	3.3	0.02142	ATGAACGT	ABRE	253	8	3.9	0.00145
ACGTGGCC	ABRE	154	4	3.2	0.03929	CGACGTGT	ABRE	127	4	3.9	0.0218
ACGTTTCA	ABRE	394	10	3.2	0.00197	GTACGTCA	ABRE	98	3	3.8	0.04808
CTACGTTA	ABRE	158	4	3.2	0.0424	ACGTAAAC	ABRE	458	14	3.8	0.00005
ACGTCAAG	ABRE	198	5	3.2	0.02491	CCCACGTA	ABRE	99	3	3.8	0.04925
CACGTCTC	ABRE	238	6	3.1	0.01489	TCCACGTA	ABRE	268	8	3.7	0.00204
ACACGTCT	ABRE	239	6	3.1	0.01516	AACGTAAA	ABRE	804	24	3.7	0
ACGTCAAC	ABRE	241	6	3.1	0.01571	TACGTCAC	ABRE	172	5	3.6	0.01476
TACAACGT	ABRE	241	6	3.1	0.01571	ATTACGTC	ABRE	208	6	3.6	0.00823
TACGTACT	ABRE	241	6	3.1	0.01571	CACGTACG	ABRE	139	4	3.6	0.02882
TTCTACGT	ABRE	283	7	3.1	0.00985	TGAACGTA	ABRE	174	5	3.6	0.01542
						GTAGACGT	ABRE	141	4	3.5	0.03011
						CGCCACGT	ABRE	286	8	3.5	0.003
						AGACGTGG	ABRE	179	5	3.5	0.01716
						ATCGACGT	ABRE	149	4	3.3	0.03559
						CACAACGT	ABRE	187	5	3.3	0.02019
						ACGTCACG	ABRE	150	4	3.3	0.03631
						GGAGACGT	ABRE	150	4	3.3	0.03631
						TCACGTGT	ABRE	340	9	3.3	0.00245
						CAAACGTC	ABRE	228	6	3.3	0.01236
						ACGTGTCC	ABRE	229	6	3.3	0.0126
						CGTACGTT	ABRE	193	5	3.2	0.02269
						GACGTGGC	ABRE	312	8	3.2	0.00495
						TACGTTGG	ABRE	156	4	3.2	0.04082
						GAACGTAA	ABRE	203	5	3.1	0.02726

Overrepresentated octamer sequences containing the DRE core motif (CCGAC/its reverse complement) and the ABRE (ACGT core motif) are listed together with their occurrence ratios (see "Materials and methods") and statistical significance of overrepresentation (two-sided Fisher's exact test *P* value)

(RAR) of each octamer = (count of the octamer in promoters of top 200 up-regulated genes/200)/(count of the octamer in total 24956 promoters/24956). RARf = filtered RAR ($P < 0.05$ and RAR > 3.0)

[a] Overrepresented octamers containing DRE core motif (CCGAC/its reverse complement) and ABRE motif (ACGT)

[b] Top 200 genes up-regulated in *35S:VuDREB2A* plants

[c] Relative appearance ratio

validated its function both in vitro using simple biochemical methods and in vivo using the well-established molecular biological platform *Arabidopsis*. The promoter of this gene was also isolated and analyzed in silico. Our results indicated conserved function and mode of regulation of the gene between *Arabidopsis* and cowpea. Our efforts in isolating *VuDREB2A* could provide a useful tool for molecular breeding to further improve drought tolerance in cowpea. Direct association of drought tolerance with a single nucleotide polymorphism (SNP) was identified in foxtail millet (*Setaria italica* L.; *SiDREB2*; Lata et al. 2011a). Similar efforts in exploration of the natural genotypic variation among cowpea varieties, contrasting in drought tolerance can lead to probable association of the VuDREB2A locus to the drought tolerance in this species. Also, recent progress in DNA-sequencing technologies (e.g., Timko et al. 2008) will soon make possible genome-wide analysis of DREB2 homologs in cowpea. However, we have already developed effective genetic transformation methods in cowpea in recent years (Solleti et al. 2008; Bakshi et al. 2011, 2012a, b). This allows us to test *cis*genic approaches (i.e., introducing *VuDREB2A* to cowpea) to enhance the drought tolerance of this crop.

Authors' Contribution LS, HK and SKP conceived of the study, designed and coordinated the experiments. AS cloned the *VuDREB2A* gene and promoter, and carried out EMSA. SI prepared plant expression constructs and transgenic *Arabidopsis* lines. Yasufumi K and Yuriko K helped AS in the microarray experiment, the drought-response test and qRT-PCR. MT and YYY performed in silico analyses of the promoters. AS and HK drafted the manuscript. All authors read and approved the final manuscript.

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Conflict of interest The authors declare that they do not have any competing interest.

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Research article

The cowpea RING ubiquitin ligase VuDRIP interacts with transcription factor VuDREB2A for regulating abiotic stress responses

Ayan Sadhukhan [a], Sanjib Kumar Panda [b], Lingaraj Sahoo [a,] *

[a] *Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati 781039, Assam, India*
[b] *Department of Life Science and Bioinformatics, Assam University, Silchar 788011, India*

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ABSTRACT

Cowpea (*Vigna unguiculata* L. Walp) is an important grain legume cultivated in drought-prone parts of the world, having higher tolerance to heat and drought than many other crops. The transcription factor, Dehydration-Responsive Element-Binding protein 2A (DREB2A), controls expression of many genes involved in osmotic and heat stress responses of plants. In *Arabidopsis*, DREB2A-interacting proteins (DRIPs), which function as E3 ubiquitin ligases (EC 6.3.2.19), regulate the stability of DREB2A by targeting it for proteasome-mediated degradation. In this study, we cloned the cowpea ortholog of *DRIP* (*VuDRIP*) using PCR based methods. The 1614 bp long *VuDRIP* mRNA encoded a protein of 433 amino acids having a C3HC4-type Really Interesting New Gene (RING) domain in the N-terminus and a C-terminal conserved region, similar to *Arabidopsis* DRIP1 and DRIP2. We found *VuDRIP* up-regulation in response to various abiotic stresses and phytohormones. Using yeast (*Saccharomyces cerevisae*) two-hybrid analysis, VuDRIP was identified as a VuDREB2A-interacting protein. The results indicate negative regulation of VuDREB2A by ubiquitin ligases in cowpea similar to *Arabidopsis* along with their other unknown roles in stress and hormone signaling pathways.

1. Introduction

Plants have developed a number of elaborate molecular mechanisms to respond and adapt to various environmental stresses, such as drought, high salinity and extreme temperatures. Transcriptional regulation is the most important of such mechanisms and a wide range of transcription factors are involved in the signal transduction network, from stress perception to the expression of stress-responsive genes leading to stress-adaptation (Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006; Mittler and Blumwald, 2010). Post-transcriptional regulation via alternative splicing, RNA processing and RNA silencing (Covarrubias and Reyes, 2010; Howell, 2013) as well as post-translational regulation of transcription factors via phosphorylation, ubiquitination and sumoylation have also been found to play vital roles in the modulation of plant stress responses (Bailey-Serres et al., 2012; Lyzenga and Stone, 2012).

Dehydration-Responsive Element-Binding Protein 2A (DREB2A), an ethylene-responsive element binding factor/APETALA2 (ERF/AP2) family transcription factor, plays a key role in the dehydration and heat stress responses of *Arabidopsis* (Morimoto et al., 2013). It regulates the expression of stress-inducible genes via the dehydration-responsive element (DRE; CCGAC core motif) in the promoter of such genes (Yamaguchi-Shinozaki and Shinozaki, 1994). The *DREB2A* gene is induced by dehydration or heat shock via ABA- and heat shock-responsive *cis*-acting elements in its promoter (Kim et al., 2011; Yoshida et al., 2011). However, the native form of *DREB2A* could not activate the transcription of target genes on overexpression, suggesting role of post-translational negative regulation on the protein (Sakuma et al., 2006; Qin et al., 2008). A 30-amino acid Ser-/Thr-rich region termed as NRD (negative regulatory domain) immediately downstream to the DNA-binding domain of DREB2A is the site for post-translational regulation (Sakuma et al., 2006). The removal of the NRD led to a constitutively active form of DREB2A (DREB2A CA) which was more

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Abbreviations: AbA, Aureobasidin A; ABA, abscisic acid; CA, constitutively active; CDS, coding sequence; DRE, dehydration-responsive element; DREB2A, DRE-binding protein 2A; DRIP, DREB2A-interacting protein; MEGA, molecular evolutionary genetic analysis; MeJA, methyl jasmonate; NRD, negative regulatory domain; RING, Really Interesting New Gene; SA, salicylic acid; SD, synthetically defined; SMART, Simple Modular Architecture Research Tool; X-α-Gal, 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside; YPDA, Yeast Peptone Dextrose Adenine.

* Corresponding author. Tel.: +91 361 2582204; fax: +91 361 2582249.
E-mail addresses: s.ayan@iitg.ernet.in (A. Sadhukhan), drskpanda@gmail.com (S.K. Panda), ls@iitg.ernet.in (L. Sahoo).

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stable than the wild-type protein (Sakuma et al., 2006; Morimoto et al., 2013). The overexpression of DREB2A CA also caused severe growth and reproductive defects in transgenic *Arabidopsis* apart from inducing target gene expression even under non-stressful conditions (Sakuma et al., 2006). The DREB2A molecule was believed to be degraded in the nucleus by the 26S proteasome pathway (Qin et al., 2008). Two C3HC4 RING-domain containing proteins, DREB2A-Interacting Protein1 (DRIP1) and DRIP2 were found to interact with DREB2A (near the NRD) and function as E3 ubiquitin ligases (EC 6.3.2.19), ubiquitinating DREB2A and targeting it for proteasome mediated degradation (Qin et al., 2008). Both disruption of DRIP (in the *drip1 drip2* double mutant) and overexpression of DREB2A in the *drip1-1* mutant caused increased expression of downstream target genes of DREB2A and a dwarf phenotype resembling the DREB2A CA overexpressor; while overexpression of DRIP resulted in delayed expression of the DREB2A target genes. Hence, DRIP was understood to negatively regulate DREB2A, lowering its abundance under non-stressful conditions, to reduce metabolic burden.

Cowpea (*Vigna unguiculata* L. Walp) represents as an ideal crop model to study the molecular mechanisms of drought tolerance owing to its enhanced tolerance to drought and relatively small genome size (Agbicodo et al., 2009). However, there are not many reports on mining drought tolerance genes from cowpea. We recently isolated a Dehydration-Responsive Element-Binding protein 2A (DREB2A) ortholog from cowpea, VuDREB2A (GenBank: JN629045.3) which was highly induced in response to desiccation, heat and salinity, and conferred enhanced drought tolerance by up-regulation of several stress-responsive genes in transgenic *Arabidopsis* (Sadhukhan et al., 2014). A Ser/Thr-rich region immediately downstream to the DNA binding domain in VuDREB2A appeared to have some role in the stability of the protein, since its removal led to a dwarf phenotype and enhanced expression of some of the downstream genes of VuDREB2A (Sadhukhan et al., 2014), similar to DREB2A CA (Sakuma et al., 2006). This provides vital clue to the possibilities of existence of similar pathways regulating VuDREB2A in cowpea.

In this paper, we report successful cloning of the DRIP ortholog from cowpea (VuDRIP) by degenerate oligonucleotide-primed PCR and Rapid Amplification of cDNA Ends (RACE). We detected the presence of highly conserved structural features of RING domain-containing ubiquitin ligases in VuDRIP. We found the up-regulation of VuDRIP in both shoots and roots of cowpea, in response to various abiotic stresses and exogenous application of phytohormones. A yeast two-hybrid analysis showed VuDRIP interacting with VuDREB2A. We predict the negative regulation of VuDREB2A by VuDRIP via ubiquitination in cowpea, in a manner similar to the *Arabidopsis* orthologs.

2. Methods

2.1. Plant materials and growth conditions

Cowpea (cultivar Pusa Komal) seeds were procured from National Seeds Corporation, Pusa, New Delhi, India. Cowpea plants were grown hydroponically in modified MGRL medium (Fujiwara et al., 1992; 1/50 strength without inorganic phosphate and calcium concentration adjusted to 200 μM, pH 5.5) at 25 °C with a 12 h photoperiod.

2.2. Cloning of VuDRIP by degenerate PCR and RACE

The VuDRIP gene was cloned by degenerate oligonucleotide-primed PCR (Telenius et al., 1992). Degenerate primers were designed from a ClustalW2 alignment of reported plant E3 ubiquitin protein ligases/DRIP sequences (Table 1). Total RNA was isolated from 6 h high salt (250 mM NaCl)-stressed cowpea seedlings by the method of Suzuki et al. (2008). cDNA was synthesized from the cowpea total RNA using ReverTraAce reverse transcriptase (Toyobo, Osaka, Japan) following supplier's instructions. A partial cDNA sequence was obtained by PCR using degenerate primers and salt stressed cowpea cDNA. Thereafter, 5′ and 3′ ends of the cDNA were cloned by 5′ RACE (Takara-Bio, Ohtsu, Japan) and 3′ RACE (Invitrogen, Carlsbad, CA, USA) systems following manufacturers'

Table 1
Sequence of oligonucleotides used for cloning and sequencing analyses of *VuDRIP*, RT-PCR and cloning into yeast vectors.

Target gene	GenBank Accn	Primer name	Sequence (5′ → 3′)	Restriction site
Degenerate primers				
legume *DRIP*		*DRIP*_deg_Fw	GGCCWGAYMACAWTTTGCAAG	
		*DRIP*_deg_Rv	GAMGCHRMWARDGARAACCAAA	
Primers for RACE				
VuDRIP	JQ066265	VuDRIP_5′RACE_Phos_GSP1	GCAATTTGTTGGGT[a]	
		VuDRIP_5′RACE_GSP2	GTCAGGTGAGCATGAACTATCTTGGCGA	
		VuDRIP_5′RACE_GSP3	AGGCTGCAAGTAGGAGCAAG	
		VuDRIP_5′RACE_GSP4	TGCTGACAACCAAAGATGAAAG	
		VuDRIP_5′RACE_GSP5	AAAGATTAGCACTGATCATGTCTCTCCA	
		VuDRIP_3′RACE_GSP1	AAAGATTAGCACTGATCATGTCTCTCCA	
		VuDRIP_3′RACE_GSP2	GGAATGACTCAATTTGGTTCTCCCTTTC	
Primers for cloning full length *VuDRIP*				
VuDRIP	JQ066265	VuDRIP_FL_1	ACAGGACACACACACACA	
		VuDRIP_FL_1	CTTTAACAGGATGATATAATGTGAATT	
Primers for cloning *VuDREB2A* coding sequence in pGBKT7				
VuDREB2A	JN629045	VuDRIP_pGBKT7_Fw	CCGGAATTCATGGGTGCTTATGATCAA	*Eco*RI
		VuDRIP_pGBKT7_Rv	GGCGGATCCTCATTCCTTGCTTGCTAC	*Bam*HI
Primers for cloning *VuDRIP* coding sequence in pGADT7				
VuDRIP	JQ066265	VuDRIP_pGADT7_Fw	CCGCCCGGGTATGACGATGAAGAGGCTT	*Sma*I
		VuDRIP_pGADT7_Rv	CCGGAGCTCTCATGATGAAGGTTGTTG	*Sac*I
Primers for qRT-PCR				
VuDRIP	JQ066265	VuDRIP_qPCR_Fw	ACCTATGGTCGGAAGGTCCA	
		VuDRIP_qPCR_Rv	GCAGCCAGTGTGCAAATAAAA	
		VuDRIP_semiqPCR_Fw	AGTCTGCAAAGAGTGCGGGACA	
		VuDRIP_semiqPCR_Rv	GCTCCTACTTGCAGCCTCTGCT	
VuUBQ1 EST	FG895491	*VuUbq*_qPCR_Fw	TCAGTTGAGGCCGAAGAAGA	
		*VuUbq*_qPCR_Rv	AAACCAGTCCCAGTCCCAAA	

[a] Phosphorylated at 5′ end.

instructions. All amplifications were carried out using ExTaq (Takara). The amplified products were cloned in pTZ57R/T vector (Fermentas) and sequenced. DNA sequencing was carried out by the commercially available DNA sequencing facility of Delhi University South Campus, New Delhi, India.

2.3. Sequence and phylogenetic analyses

Nucleotide and protein sequence homologies were analyzed using NCBI Basic Local Alignment Search Tool (BLAST). Multiple sequence alignments were carried out CLUSTALW program (Larkin et al., 2007) and visualized using GeneDoc (Nicholas et al., 1997). The phylogenetic tree was constructed using Molecular Evolutionary Genetic Analysis (MEGA) v 6.0.5 (Tamura et al., 2013). The distances between branches were calculated by the neighbor-joining method based on the Jones-Taylor-Thornton model (Jones et al., 1992) with 1000 bootstrap replicates. Characteristics of the translated protein were determined using ExPasy: SIB Bioinformatics resource portal (www.epasy.org). Potential protein domains were searched using Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de). The nuclear localization signal was predicted by WOLFPSORT (http://wolfpsort.org/) and NucPred (https://www.sbc.su.se/~maccallr/nucpred/cgi-bin/single.cgi#scorehelp).

2.4. Expression analysis of *VuDRIP*

One week-old cowpea seedlings grown in modified MGRL medium, were given the following treatments: high salinity, desiccation, heat, cold, abscisic acid (ABA), methyl jasmonate (MeJA) and salicylic acid (SA). For salinity stress, plants were dipped in 250 mM NaCl in modified MGRL medium. For desiccation stress, plant roots were wrapped dry in tissue paper. For heat stress, plants were kept in an incubator maintained at 50 °C (with same photoperiod). For cold stress, plants were kept at 4 °C in a refrigerator under dim light. ABA, MeJA and SA treatments were given both by dipping roots in and spraying shoots with 100 μM (+)-*cis*, *trans*-ABA (Sigma, St. Louis, MO, USA), 100 μM (solubilized in ethanol), MeJA (Sigma) and 500 μM SA (Sigma), respectively. For the SA treatment, plants were kept at 25 °C in the dark. MeJA treatment was carried out in a separate growth chamber to prevent effects of the volatile compound on plants subjected to other treatments. After 6 h, stressed cowpea tissues were harvested for RNA isolation (following Suzuki et al., 2008) and cDNA synthesis (by Toyobo ReverTraAce reverse transcriptase following supplier's instructions). Real-time PCR was carried out using Takara SYBR Green PCR master mix II and a Takara Thermal Cycler Dice Real time system II (Takara). Gene-specific primers for real-time PCR analyses were designed using Primer3, version 4.0 software (Koressaar and Remm, 2007). Real-time PCR quantifications were performed by the standard curve method using a cDNA dilution series (Bustin et al., 2009). The homolog of the *UBQ1* gene in cowpea (EST sequence; GenBank Accession: FG859491) was used as internal standard in relative quantifications (Supplementary Fig. 1A).

2.5. Yeast two-hybrid analysis

The interaction between VuDREB2A and VuDRIP was assessed using Matchmaker Gold Yeast Two-Hybrid System (Clonetech, Mountain View, CA, USA) according to supplier's instructions. The *VuDREB2A* ORF was cloned into bait plasmid vector pGBKT7 (containing GAL4 DNA-binding domain) between *Eco*RI and *Bam*HI sites, while the *VuDRIP* ORF was cloned into prey plasmid vector pGADT7 (containing GAL4 activation domain) between *Sma*I and *Sac*I sites. The bait and prey plasmids were mobilized into yeast strains Y2H Gold and Y187 respectively and mated together in 2X Yeast Peptone Dextrose Adenine (YPDA) media. Serial dilutions of the mated cultures were spread out on synthetically defined (SD) media, with two amino acids dropped out (double dropout media; SD/-Leu/-Trp) to select for the bait and prey plasmids. Positive interactions were confirmed by growth in double dropout as well as quadruple dropout (SD/-Ade/-Leu/-Trp/-His) media supplemented with Aureobasidin A and 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside (X-α-Gal) to select for activation of the Gal4-responsive *HIS3*, *ADE2*, *AUR1-C* and *MEL1* genes. Resultant colonies were streaked on the same respective media and photographed after growth.

3. Results

3.1. Isolation of VuDRIP by PCR-based cloning

An amplicon of 766 bp was obtained using degenerate primers and it was confirmed as partial DRIP sequence through NCBI BLAST search. The full-length mRNA sequence was obtained by 5′ and 3′ RACE. The 5′ RACE produced amplicon of 478 bp (with 285 bp unknown 5′ end cDNA region) and 3′RACE of 623 bp. The 1614 bp long *VuDRIP* mRNA sequence was submitted to GenBank with accession No. JQ066265. It encoded a protein of 433 amino acids with a theoretical average molecular mass of 48.09 KDa and a pI of 9.53 (GenBank Accession: AEY75223). The predicted protein of 433 amino acids was found to contain a RING domain of C3HC4 type in the region 20–60 (Fig. 1) typifying proteins involved in the ubiquitination pathway (Freemont, 1993; Lovering et al., 1993; Borden, 2000).

Apart from the RING domain, the VuDRIP protein sequence was also found to conserve a nuclear localization signal, and a C-terminal region without any characteristic domain, which has been implicated to interact with the N-terminal region of DREB2A in *Arabidopsis* (Qin et al., 2008).

3.2. Stress-inducible expression of VuDRIP

VuDRIP was found to be up-regulated (>2-fold) after 6 h of high salinity (250 mM NaCl), heat (50 °C), ABA (100 μM), MeJA (100 μM) and SA (500 μM) treatments in both shoots and roots of cowpea but most markedly up-regulated (>10-fold) by desiccation stress in cowpea shoots. However in cold stress, *VuDRIP* expression was unchanged in the shoots and was only slightly induced in the roots of cowpea (Fig. 2). This suggests involvement of VuDRIP in multiple abiotic stress and hormone signaling pathways, with most pronounced association with desiccation stress in cowpea. Further, primers designed from the variable region of *VuDRIP* were used for amplification of total cDNA from cowpea under both stressed and non-stressful conditions. Two partial amplicons were obtained in each case (Supplementary Fig. 1B), which were sequenced, indicating two homologs of the DRIP gene in cowpea.

3.3. Interaction of VuDRIP with VuDREB2A

To determine whether VuDRIP interacted with VuDREB2A, the full length coding sequences (CDSs) of these genes were expressed as fusions of yeast GAL4-activation domain (prey) and Gal4-DNA-binding domain (bait) respectively in vectors pGADT7 and pGBKT7. After transformation into yeast cells and yeast two-hybrid mating, all diploid cells containing either the clones or empty vectors could grow on SD/-Leu/-Trp plates (Fig. 3B). However, on the selective medium, SD/-Leu/-Trp/-His/-Ade/X-α-Gal/Aureobasidin A, only the cells harboring positive interactions between the bait and prey could grow and produce blue colonies (Fig. 3C). Known interaction

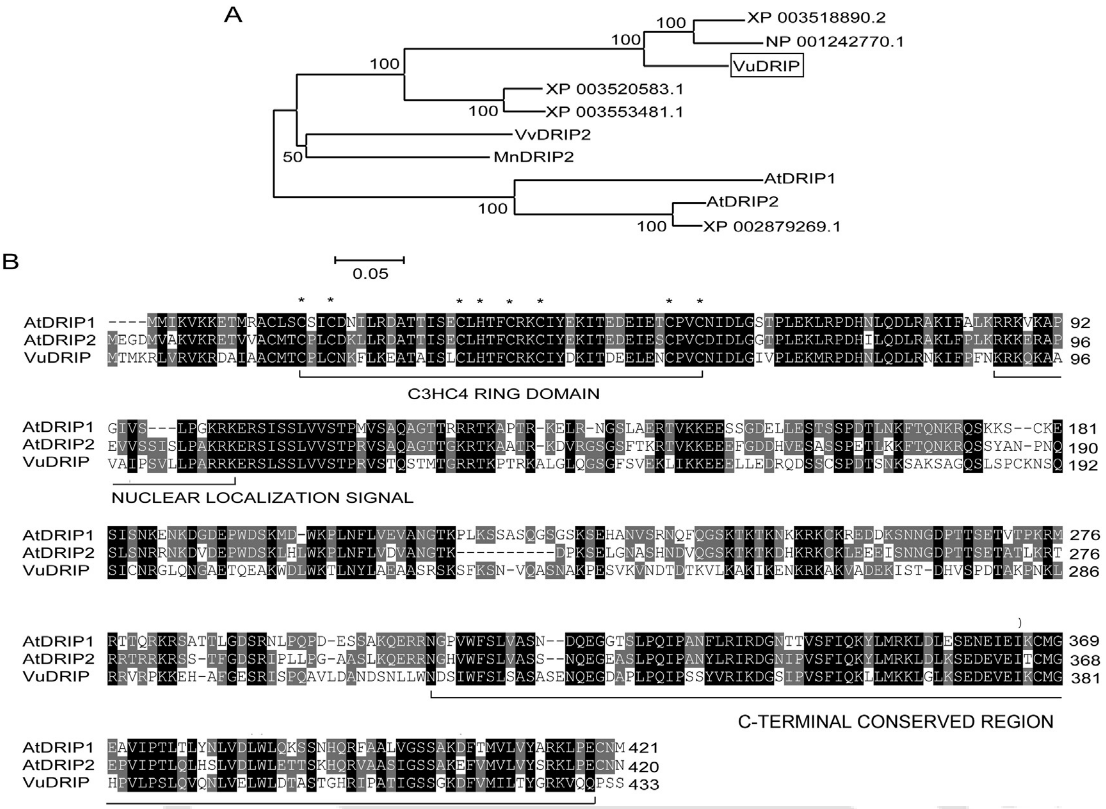

Fig. 1. Phylogeny and sequence characteristics of VuDRIP. Phylogram of DRIP orthologs (A). Proteins having >60% sequence identity with VuDRIP were used for the phylogenetic analysis. *Arabidopsis* DRIP homologs were used as out groups. The tree was generated by MEGA 6.0.5 using the Neighbor-Joining method with 1000-replicate bootstrap testing. The numbers against each branch indicate the bootstrap values and the scale bar indicates substitution rate per site. The tree was constructed from a sequence alignment of the following proteins: *Arabidopsis thaliana* AtDRIP1 [GenBank: NP_172162.3], AtDRIP2 [GenBank: NP_565702.1], *Arabidopsis lyrata* zinc finger family protein [GenBank: XP_002879269.1], *Glycine max* DRIP2-like proteins [GenBank: XP_003518890.2, XP_003520583.1, XP_003553481.1 and NP_001242770.1]; *Vigna unguiculata* VuDRIP [GenBank: AEY75223.2]; *Vitis vinifera* VvDRIP2 [GenBank: XP_002275598.1]; *Morus notabilis* MnDRIP2 [GenBank: EXB81323.1]. Alignment of the amino acid sequences of VuDRIP, AtDRIP1 and AtDRIP2 (B). Black shading indicate complete conservation while gray indicate partial conservation (with similarity groups included under same color). Amino acids 20 to 60 in VuDRIP constitute a conserved Cysteine$_3$HistidineCysteine$_4$-type RING domain with the cysteines and histidine indicated by asterisks. An additional region in the C-terminus is also conserved which had been implicated to interact with DREB2A in *Arabidopsis* (Qin et al., 2008).

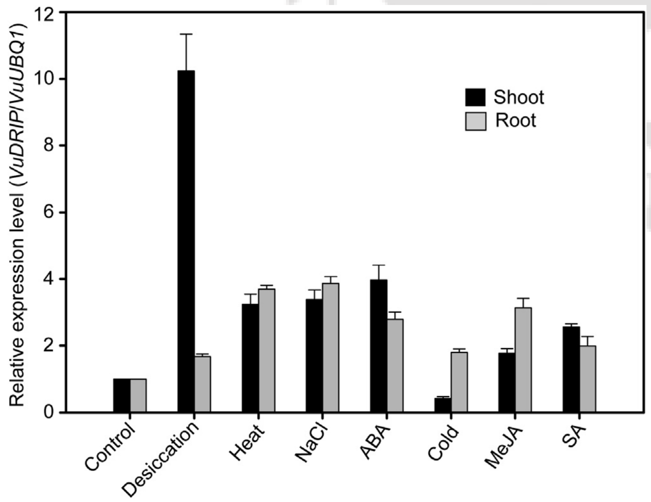

Fig. 2. Expression of VuDRIP under abiotic stress. Transcript levels of *VuDRIP* in the shoots and roots of cowpea plants grown for one week and then exposed to different stress treatments viz. desiccation, heat (50 °C), cold (4 °C), ABA (100 μM), MeJA (100 μM) and SA (500 μM) for 6 h. Columns indicate relative expression levels of *VuDRIP* normalized against levels of *VuUBQ1* (see Supplementary Fig. 1A) as calculated by real-time PCR (means ± SE of 3 biological replicates).

between the GAL4 DNA-binding domain fused with murine p53 (pGBKT7-53) and the GAL4 activation domain fused with SV40 large T-antigen (pGADT7-T) (Li and Fields, 1993; Iwabuchi et al., 1993) was used as positive control, while Gal4 DNA binding domain fused with human lamin C (pGBKT7-Lam) and pGADT7-T were mated as a negative control. We also eliminated possibility of false positives by confirming that the Gal4 DNA-binding or activation domains in the empty vectors could not interact with the bait or prey. Our results indicated that VuDRIP could interact with VuDREB2A in yeast.

4. Discussion

Post-translational regulation is a key strategy adopted by plants that determines the critical levels of regulatory proteins for maintenance of homeostasis. Covalent attachment of a 76-amino acid protein, ubiquitin on the lysine residue of a target protein leads to its recognition by the 26S protasome and subsequent degradation. Such kind of post-translational regulation is involved in a wide range of biological processes in plants (Moon et al., 2004). Typically three enzymes are involved in covalent attachment of ubiquitin protein moity to a target protein, viz. E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase). Many ubiquitin ligases are RING domain-containing proteins. The RING domain is rich in cysteines and contains eight metal ligand residues coordinating zinc ions (Borden, 2000). When the

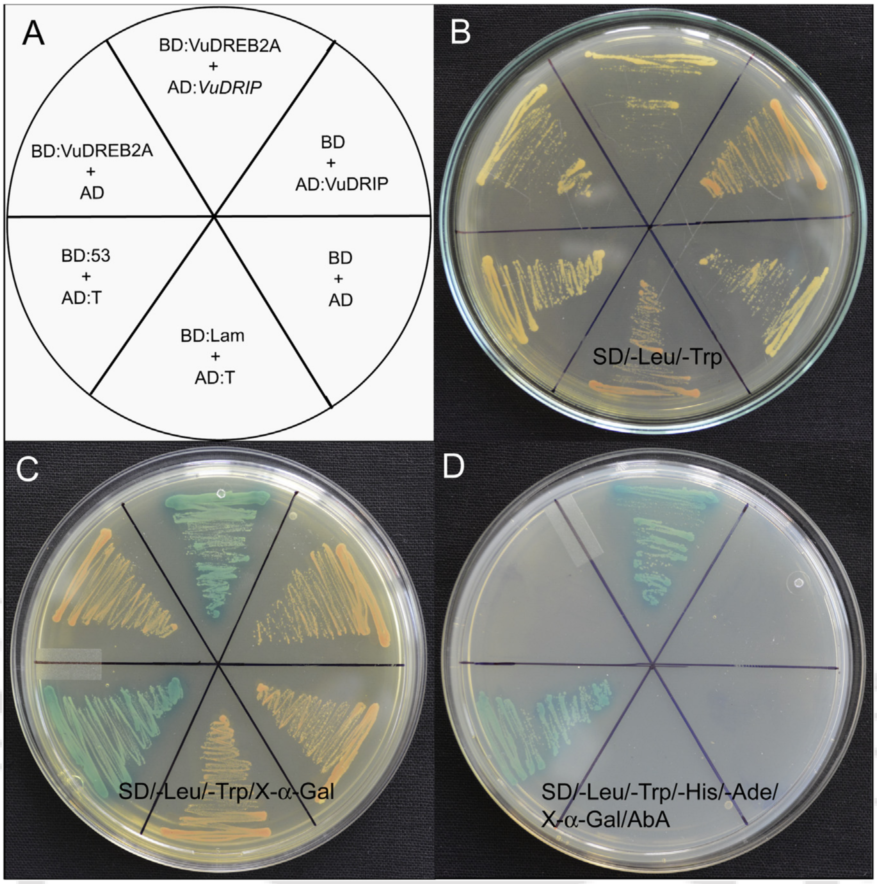

Fig. 3. Identification of VuDRIP interaction with VuDREB2A via yeast two-hybrid analysis. The VuDRIP CDS was cloned in pGADT7 vector (as fusion of the GAL4 activation domain) and the VuDREB2A CDS into pGBKT7 vector as fusion of the GAL4 DNA-binding domain). Plain vectors/clones of pGADT7 and pGBKT7 were mobilized to yeast strains Y187 and Y2H Gold respectively. These two strains were then mated to form diploid cells harboring both vectors. Map showing positions of the different diploid yeast cells in plates B, C and D (A). BD: GAL4 DNA-binding domain (pGBKT7); AD: GAL4 activation domain (pGADT7); 53: murine p53; T: SV40 large T-antigen; Lam: Human Lamin C. Interaction of BD:53 fusion and AD:T fusion was used as positive control, whereas BD:Lam and AD:T as negative control. Growth of yeast in double dropout media (SD/-Leu/-Trp) (B). All types of diploid cells can grow in this media. Growth of yeast in double dropout media supplemented with X-α-Gal (C). All cells can grow in this media, but only the cells harboring positive protein–protein interactions can produce a blue color. Growth of yeast in quadruple dropout media (SD/-Leu/-Trp/-His/-Ade) supplemented with X-α-Gal and Aureobasidin A (AbA) (D). Only the cells harboring positive protein–protein interactions can grow in this media.

metal ligand at position 5 is a His the RING domain is of the C3HC4 type (Freemont, 1993; Lovering et al., 1993). Many RING E3 proteins regulate expression of transcription factors, involved in response to environmental stress or hormone signaling in plants, by targeting them for proteasome mediated degradation. For example, in *Arabidopsis*, SINAT5 targets NAC1 for degradation to attenuate auxin signals (Xie et al., 2002); AIP2 targets ABI3 for proteolysis to negatively regulate ABA signaling (Zhang et al., 2005); HOS1 targets ICE1 for proteolysis to attenuate cold stress signals (Dong et al., 2006); and DRIP1 and 2 target DREB2A for proteolysis to reduce its levels under non-stressful conditions.

The expression of these E3 ligases SINAT5, AIP2 and HOS1 were found to be inducible under the respective phytohormone or stress treatments (auxin, ABA and cold). These regulators functioned in attenuation of the signals through degradation of their substrate transcription factors under stress, indicating the importance of antagonistic transcriptional as well as post-transcriptional mechanisms in signal transduction cascades (Xie et al., 2002; Zhang et al., 2005; Dong et al., 2006). In contrast, *Arabidopsis DRIP* homologs were constitutively expressed under both stress and non-stressed conditions with the exception of *DRIP2*, which was slightly induced under dehydration stress (Qin et al., 2008). Furthermore, through overexpression and mutant analyses, it was evident that expression of drought-responsive genes was not affected by DRIP at a later phase of the drought response. It was thus inferred that although DRIP functioned negatively in stress-responsive gene expression, it did not function to attenuate drought stress signals, rather restricted DREB2A activity under non-stressful conditions. Notably, *DREB2A* transcripts were found at low levels but the protein could not be detected under non-stressful conditions, since it underwent proteolysis due to interaction with DRIP.

In cowpea, we detected, similar to *Arabidopsis*, very low levels of *VuDREB2A* transcripts under normal growth conditions which however, increased drastically under desiccation, heat and salinity stress and slightly by exogenous ABA treatment (Sadhukhan et al., 2014). The interaction of VuDREB2A with the RING domain protein VuDRIP as emerged from the yeast two-hybrid assay and our previous observation of *VuDREB2A* up-regulation under stress suggests proteasome-mediated degradation of VuDREB2A in cowpea under non-stressful conditions, similar to AtDREB2A. However *VuDRIP* transcripts were also highly induced under the same treatments inducing *VuDREB2A* (desiccation, heat, salinity and ABA) indicating more specialized roles of VuDRIP under stress, including probable attenuation of stress signals by antagonizing VuDREB2A. Additional induction of *VuDRIP* under MeJA and SA treatments indicates the possibility of unknown roles of VuDRIP in signaling pathways relating biotic stress.

In *Arabidopsis* DRIP1 and 2, the E3 ubiquitin ligase activity and interaction with VuDREB2A were demonstrated to be mediated by the C3HC4 RING domain and another C-terminal protein–protein interactive region, respectively. Only the C-terminal region was observed to specifically interact with DREB2A and led to higher reporter gene activity in yeast than the full length DRIP. Moreover, only the N-terminal end of DREB2A up to the NRD participated in the interaction with DRIP. This provided explanation why disruption of DRIP led to phenotypes similar to that produced by removal of the NRD in DREB2A. Based on the structural similarity of the protein domains of the DRIP orthologs in *Arabidopsis* and cowpea, we designed the yeast two-hybrid assay with VuDREB2A as bait and observed positive interaction between VuDRIP and VuDREB2A. However, a detailed investigation would provide more light on the functional domains in VuDRIP and their interacting partners in VuDREB2A. *In vitro* ubiquitination assays are also necessary to confirm whether VuDRIP actually ubiquitinates VuDREB2A for subsequent proteasome-mediated degradation.

A thorough understanding of the molecular mechanisms underlying the stress responses of crop plants, especially tolerant species like cowpea is necessary for development of enhanced stress-tolerant varieties for sustainable agriculture in the future. Negative regulators of stress responses have received less attention in crops in spite of their critical roles in fine tuning of stress signals and maintenance of homeostasis in cells. Here, an attempt has been made to identify a putative negative regulator of DREB2A transcription factor from drought-tolerant crop cowpea taking cues from model plant *Arabidopsis*. Further studies through transgenic approaches, for example, knock down of such negative regulators coupled with overexpression of VuDREB2A would lead to improvement of drought tolerance as well as deeper insights into the drought stress signaling mechanism in this species.

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Author contribution

A.S. performed the experiments and analyzed the results. S.K.P. and L.S. designed and supervised the experiments. A.S. and L.S. wrote the paper.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2014.07.007.

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