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Research article

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

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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.

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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

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.

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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.

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