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IN-SILICO STUDIES OF CBL-CIPK SIGNALING IN RICE: RESPONSE AGAINST HYPOXIA IS TRIGGERED BY INTERACTION OF CBL10 WITH CIPK15

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Abstract

In rice, *Oryza sativa,* Calcineurin B-Like (OsCBL) protein mediated activation of CBL-Interacting Protein Kinase15 (OsCIPK15) is extremely crucial for plants' ability to cope up with hypoxic stress. Ten various types of OsCBLs and OsCIPK15 have been implicated in hypoxia, but little is known about the CBL partner that activates CIPK15 signaling. *In-silico* sequence, structural and functional analyses of OsCBLs and CIPK family members were conducted to explore the OsCBL which binds with and activates OsCIPK15, thus serving as regulatory factor for plant's response to hypoxia. Sequence analysis revealed four characteristic conserved EF motifs in OsCBL family, whereas OsCIPK15 was found to contain a conserved 128 residue long NAF domain with a highly conserved 18 residue long helical fragment at its N-terminus. Structures of 10 OsCBL proteins and CIPK15 were modeled and protein-protein docking was undertaken to explore binding affinity of each of the ten OsCBL proteins for OsCIPK15. Several binding conformations of OsCBL-CIPK complexes were analyzed estimating their binding affinities on the basis of hydrogen bonding, electrostatic and hydrophobic interactions. OsCBL10 has been found to be the partner in establishing the most stable interaction with OsCIPK15's NAF domain thereby activating down the stream cascade of molecular events and configuring the plant's response to hypoxia.

Key words: Stress, Hypoxia, Anoxia, *Oriza sativa*, Rice.

Introduction

Plants being sessile organisms cannot choose their environment in which they grow and survive. To cope up with different environmental condition, they must rely on various signal transduction pathways. Among the wide repertoire of plant signaling components, calcium is a ubiquitous secondary messenger which mainly regulates key adaptation and developmental processes such as guard cell opening/closure regulation and root hair elongation in plant (Gilroy & Trewavas, 2001; Weinl & Kudla, 2009).

In plants, fluctuations in soil/root environment entail rapid fluctuations in Ca^{2+} level thereby activating calcium dependent signaling transduction pathways (Luan *et al.*, 2009). Calcium ions bind to calcium sensory proteins that transduce signals to downstream proteins to trigger stress responsive physiological processes that help plant cell in adapting to stress. Among calcium sensors, Calcineurin B-like (CBL) harbor a sensory calcium binding domain (Harper & Harmon, 2005). CBL proteins complex with specific interacting kinases (CIPKs) to form a coordinated calcium induced stress signalling network in plants where CBLs serve as calcium sensor and relay proteins as they depend on their specific CBL Interacting Kinases (CIPKs) for various stress responses (kinase activity), including hypoxia (Subbaiah *et al.*, 1994). The CBL sensory module is activated on calcium binidng, thus inducing response through CIPKs by phopshorylation and activation of diverse array of downstream signaling proteins for stress tolerance in plants (Albrecht *et al.*, 2003; Sheen, 1996).

CBL's calcium binding domain is characterized by conserved EF motifs (Shi *et al.*, 1999; Wang *et al.*, 2012), whereas CIPKs contain a c-terminal 128 residue long NAF domain, which is crucial for binding of CIPK to CBL proteins (Lee *et al.*, 2009; Mahajan *et al.*, 2006; Shi *et al.*, 1999). The domain is named after a highly conserved 24 residue long NAF motif which lies right at start of its N-terminus. While NAF domain of CIPK is crucial for interaction for CBLs, CBLs have evolved a binding pocket for binding the target NAF domain of its CIPK partner. In spite of the conservation observed in CIPKs' NAF domains and their CBL binding pockets, various CIPK's are exclusively involved in particular types of stress-response pathways. These pathways are triggered by specific CIPK's which are activated by the interaction of their NAF domain with a CBL protein which is sensitive to a unique type of stress. The rice, *Oryza sativa,* genome contains 10 CBLs (OsCBL) and 32 CIPKs (OsCIPK) which interact with each other to form specific pairs (Kolukisaoglu *et al.*, 2004). OsCBLs and OsCIPKs complexation induce responses to various stresses like flooding, drought, cold and salt (Kanwar *et al.*, 2014; Lee *et al.*, 2009; Sadiq *et al.*, 2011). OsCBL-OsCIPK interaction plays an important role in survival of rice plants under low oxygen stress or hypoxia (Lee *et al.*, 2009). This interaction leads to the activation of downstream signaling pathways, resulting in an increased production of soluble sugars and energy under flooding stress to help plants to grow under these unfavorable conditions. Lee *et al.*, (2009) identified that OsCIPK15 is responsible for increased rice tolerance to submergence, but the CBL protein(s) that interact with OsCIPK15 remains unknown. Knowledge of OsCBL-CIPK15 interaction in rice is thus of crucial importance. This study aims at investigating CBL-CIPK interaction mechanism in rice responsible for plant's response to anoxia.

Materials and Methods

Sequence analysis: Sequences of 10 OsCBL family proteins and 32 CIPKs of rice (*Oryza sativa*) were retrieved from UniProt (Wu *et al.*, 2006). Accession numbers of CBL1, CBL2, CBL3, CBL4, CBL5, CBL6, CBL7, CBL8, CBL9 and CBL10 were Q7XC27, Q3HRP5, Q75LU8, Q75KU4, Q3HRP2, Q3HRP1, Q3HRP0, Q3HRN9, Q3HRN8 and Q3HRN7 respectively. Multiple sequence alignment of retrieved protein sequences of CBL family was carried out in MEGA using ClustalW (Larkin *et al.*, 2007) to identify EF regions crucial for calcium sensing and binding. Likewise, protein sequences of 32 CIPKs were aligned to find out the conserved NAF domain residues which help CIPKs in complex formation with specific CBL proteins in *Oryza sativa*.

Structural analysis: Solved protein structures of rice CBL and CIPK15 were unavailable in Protein databank (PDB). For that matter, homology modelling was adopted for model prediction. To search appropriate templates for three-dimensional (3D) protein structure prediction, amino acid sequences of CBL1-CBL10 proteins and CIPK15 were searched individually through NCBI BLAST from Protein Databank (PDB). Based on maximum query coverage and high sequence identity, two crystal structures of calcium binding proteins of *Arabidopsis thaliana* (PDB Ids: 2ZFD_A, 2EHB_A) were selected as templates for OsCBL proteins (Akaboshi *et al.*, 2008). For modeling OsCIPK15's NAF domain, crystal structure of *Arabidopsis thaliana* CIPK23 (PDB id 4CZT) was selected as template as it showed 50% sequence identity and 98% query coverage to the OsCIPK15's NAF domain (Chaves-Sanjuan *et al.*, 2014). Models of OsCIPK15 NAF domain and CBL1-CBP10 were predicted through MODELLER program embedded in in UCSF Chimera (Huang, 1996; Pettersen *et al.*, 2004). The newly built 3D structural models were energy minimized at Yasara and validated on the basis of Ramachandran plot, free energy, steric clashes and other parameters of protein structure quality at ProCheck (Kleywegt & Jones, 1996; Krieger *et al.*, 2002). Models were refined repeatedly through energy minimization over steepest gradient to improve the quality parameters.

OsCBL and OsCIPK15 interaction: To determine the binding specificity of OsCIPK15 with OsCBL family proteins in rice, protein-protein docking was employed. Before docking, dockprep was performed on OsCIPK15 and each of the 10 OsCBL structure models in UCSF Chimera (Pettersen *et al*., 2004). Dockprep involved removal of water molecules from protein structures, followed by the addition of hydrogens and assignment of partial charges, the final structures were then used for protein-protein docking on pyDock and Cluspro (Cheng *et al.*, 2007; Kozakov *et al.*, 2017). To search the binding interface, 100 various binding conformations were generated for each of the complex. This ivolved finding binding poses through rigid body affinity followed by approximation of electrostatic and chemical affinities for each of the hundred rigid body poses or binding conformations. Out of these conformations top ranking best conformation was selected. pyDOCK results were generated in ENE file in which all the docking models were ranked according to the total energies. Top ranked structures based on the lowest energy values were analyzed further through DIMPLOT; a dedicated tool for protein protein docking interaction analysis in 2D embedded in LigPlus pakage (Laskowski & Swindells, 2011).

Results and Discussion

Sequence analysis: The sequence length of the ten OsCBL proteins range between 210–290 residues whereas length of the 32 OsCIPKs spans over 404 -540 residues whereas the length of NAF domain ranges between 102-128 residues. The OsCBL proteins shared 55-68.9% sequence identity and 83% sequence homology. Likewise, the sequence identity between NAF domains of 32 members of OsCIPK family was observed to be 48%. Multiple sequence alignment of 10 OsCBLs revealed four calcium binding EF regions; 1st EF region at 128-136 sequence position is 8 residues long, followed by 12 residues long EF2 region (160-172 sequence position). EF2 region lies at conserved distance of 22 residues from EF1. Unlike *Arabidopsis thaliana*, 11 residues long EF3 region (197-208 sequence position) lies 24 residues away towards c-terminal from EF2 region. While EF4 region (241-252) is also 11 residues long which lies 32 residue distant from EF3 (Fig. 1). Distance between the EF regions of *Oryza sativa* and *Arabidopsis thaliana* are highly conserved except EF2-EF3 distance. In case of 32 membered CIPK family, the NAF motif was identified at position 315-317, the entire NAF domain spans over 302-430 residues in case of OsCIPK15. Multiple sequence alignment of *Oryza sativa* CIPK family derived through ClustalW illustrates selection pressure along NAF domain (Fig. 2). The CIPK15 NAF domain residues are identical to CIPK14 while some positions show variation of amino acid from other CIPKs.

Structural analysis: OsCIPK15 NAF domain comprises of 128 residues. The structure revealed 5 β strands and 5α-helices connected through loops (Fig. 3). OsCIPK15 structure was validated before and after energy minimization. Before refinement, 96.1% and 2.3% residues were in favored and allowed regions respectively while 0.9 % outliers were observed as detailed in Table 1. Before minimization of OsCIPK15 model, free energy of the structure was estimated as -109609.21 kJ/mol (26222.29 kcal/mole) and Yasara score was -2.76. After energy minimization, more refined structure was obtained with energy value -277563.3 kJ/mol (-66402.70 kcal/mole) and Yasara score of -0.27. While RMSD value of refined model as compared to initial model was 0.443 Å. After energy minimization, overall structure quality got improved as Ramachandran analysis showed 96.8% and 2.3% residues in favored and allowed regions respectively while 0.9% of the residue was reported as outlier.

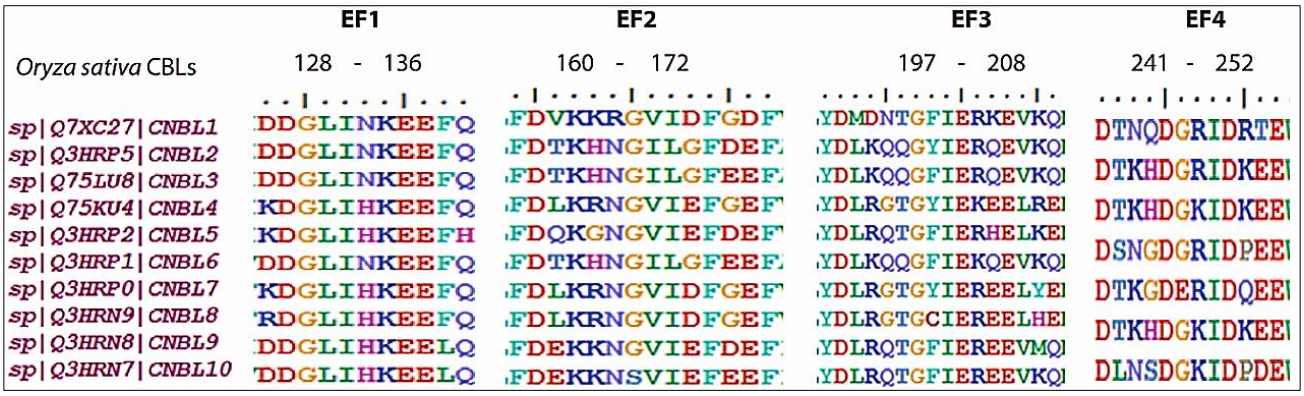

Fig. 1. Four EF regions in *Oryza sativa* CBLs as illustrated by multiple sequence alignment in ClustalW. Uniprot Ids of each CBL protein is given on left side. EF regions are numbered at the top (in bold), Position of occurrence of EF motif in the sequence is depicted on the top of the figure. In each EF region.

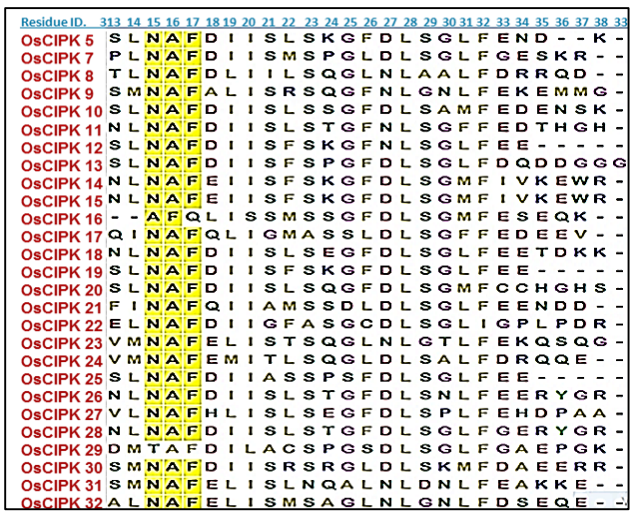

Fig. 2. Multiple Sequence Analysis of 32 *Oryza sativa* CIPKs through ClustalW. Left: CIPK names are given in red, Top: Residue ID in numeration is given, the numbering is according to CIPK15. Highly conserved Arginine (N), Alanine (A) and phenylalanine (F) residues that characterize NAF domain are highlighted in yellow.

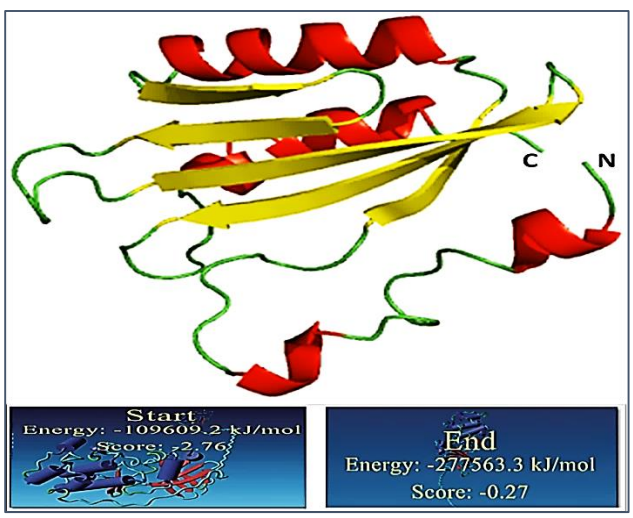

Fig. 3. Homology Model of *Oryza Sativa* (Os) CIPK15. α helices are shown in red, β strands in yellow and loops are highlighted in green N and C termini have been also indicated, the N-terminal helix-loop-helix fragment is highly conserved in OsCIPKs' NAF domains. Energy value and score of initial model is -109609.3 KJ/mol (26222.29 kcal/mole) and -2.76 respectively. After minimization stable and refined model of CIPK15 has energy value -277563.3Kj/mol (-66402.70 kcal/mole) and score (-0.27) shown in yellow.

In case of CBLs, all predicted models showed 11 helices connected through loops (Fig. 4). Model validation and refinement parameters of OsCBL1-CBL10 are given in Table 1. All the models of OsCBLs and CIPK15 were validated as 99 % of the reisdues in the models were found to be either in the most favorable or acceptable region. Except CBL7 all the models including that of CIPK15 were observed to follow the desired criteria of back bone structure with more than 95% of the residues lying in the most favorable region, (Table 1.)

OsCBL and OsCIPK15 interaction: Figure 5 shows 10 top complex conformations from each of the OsCBL proteins with CIPK15. These poses were selected from a cluster of 100 predicted binding poses, generated by PyDock for each of the complex on the basis of three variables, electrostatic energy, desolvation rnergy and Van Der Waals which cumulatively yielded an overall binding energy value. Among the top ranked docked complexes shown in Figure 5, OsCBL10-OsCIPK15 was found as the best binding pose as it showed the least binding free energy of -53.20 kcal/mole as compared to the rest of the OsCBL-CIPK15 complexes, Table 2. The OsCBL10-OsCIPK15 complex showed 12 hydrogen bonds and multiple Van Der Waal interactions, (Table 3.) In OsCBL10-OsCIPK15 complex, the N-trminal heical fragment of OsCIPK15 (helix-loop-helix) which spans over 18 residues (Leu314-Phe332) has been structurally evolved to bind well in the binding pocket of OsCBL10, (Fig. 6a 6c.) OsCIPK15 residues Asn 315, Glu318, Leu314, Glu393, Ser321, Ile320, Ser323, Lys324, Gly330 and Ile333 showed strong hydrogen bond intearction with OsCBL10 binding pocket residues Lys211, His236, Lys241, Lys242, Thr192, Glu195 and Arg119; detail of hydrogen bond network is given in Table 3. These residues build a perfect binding pocket to accomodate the N-terminal helical fragment of OsCIPK15 to interact with strong hydrongen bond network, (Fig. 6b & 6d.) Similary a number of residues were found to be involved in hydrophobic interactions including Phe 317, Phe332, Leu 328 and Phe 322 of NAF helical fragment of OsCIPK. The conserved NAF motif of the NAF domain of OsCIPK15 was found to be involved in strong interaction with OsCBL10 both in hydrogen bonding and hydrophobic interactions, Fig. 7. OsCBL10 is thus of critical importance in triggering and determining the hypoxic response in rice plant.

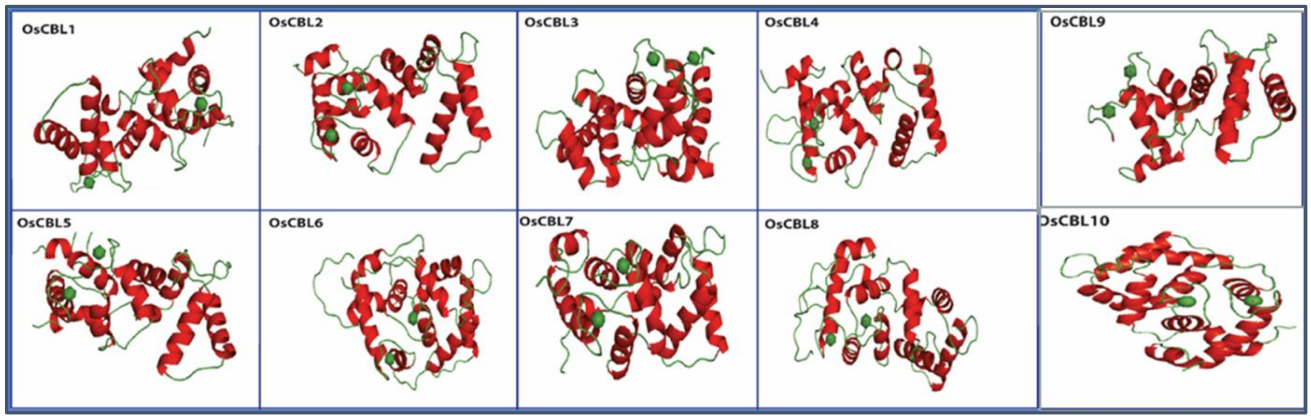

Fig. 4. Homology Models of ten *Oryza sativa* (Os) CBL proteins. Alpha helices are shown in red and loops are highlighted in green color.

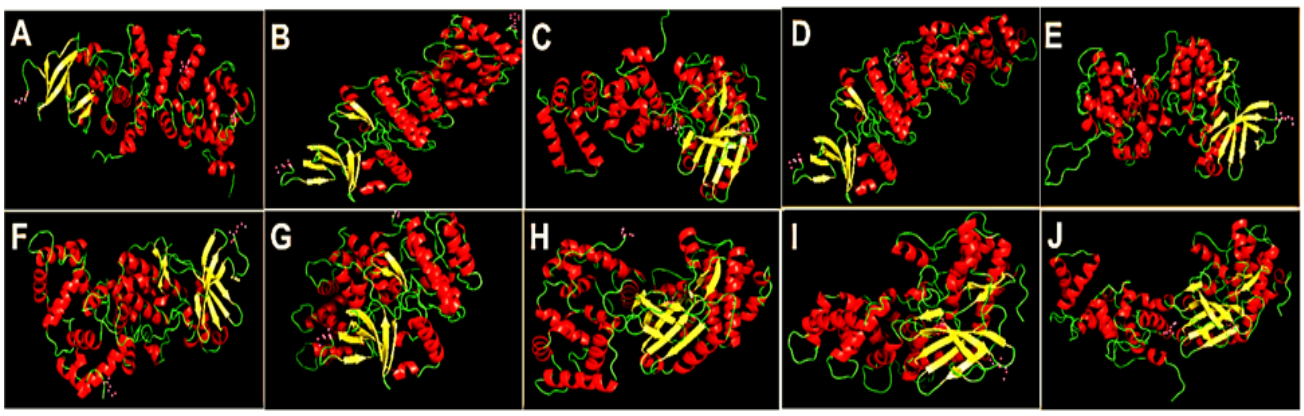

Fig. 5. Complexes of *Oryza sativa* CIPK15 with ten CBLs as generated through multiple recursive docking at pyDock.

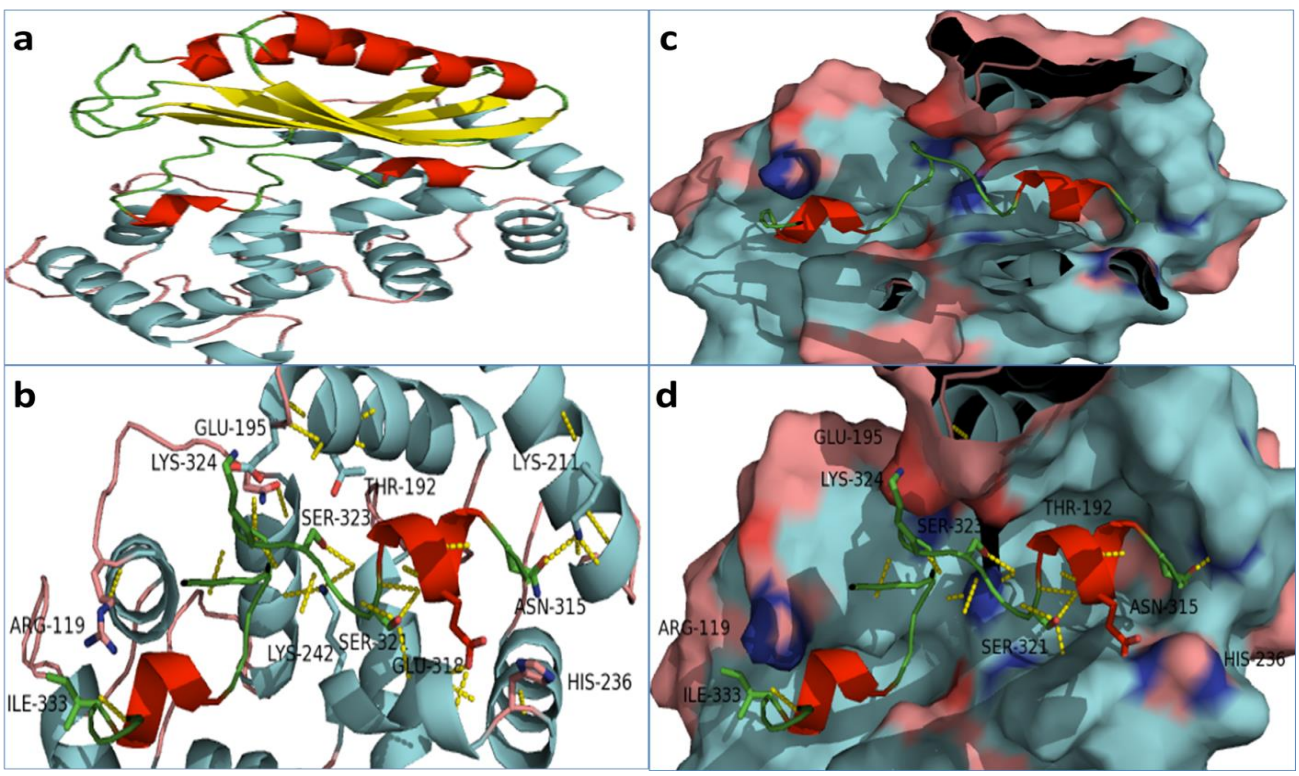

Fig. 6. Various poses of OsCBL10 binding OsCIPK15. a. OsCIPK15 - in red (α-helix), yellow (β-strand) and green (loop) - binding with OsCBL10 - in cyan (α-helix) and tint (loop) b. N-terminal helical segment of OsCIPK15 along with OSCBL10 binding pocket zoomed in to show the hotspot residues forming hydrogen bonds shown in yellow dotted lines. Side chains of some of the residues involved in hydrogen bonding have been also shown. c. OsCIPK15 binding pocket in OsCBL10 to accommodate the N-terminal helical fragment is shown in surface format. d. Same as c, zoomed in showing sidechains of hotspot residues and hydrogen bonds in yellow.

Table 1. Ramachandran evaluation of refined *Oryza sativa* (Os) CIPK15 and CBLs (1-10) models, all the models validated more than 99% of residues lied either in favored or allowed regions.

Proteins	Residues in favoured region (%)	Residues in allowed region (%)	Residues in outlier region (%)
***Os*CBL Family proteins**			
CBL1	96.2	3.3	0.5
CBL2	98.7	1.3	0.0
CBL3	97.3	2.2	0.4
CBL4	97.6	2.4	0.0
CBL5	95.4	4.2	0.5
CBL6	98.2	1.8	0.0
CBL7	93.4	6.2	0.5
CBL8	95.3	4.7	0.0
CBL9	97.2	2.8	0.0
CBL10	94.5	5.5	0.0
***Os*CIPK Family protein**			
CIPK15	96.8	2.3	0.9

Table 3. List of 14 Hydrogen bonding interactions identified between CBL10-CIPK15 complexes.

No.	Length of hydrogen bond Å	Residues (atoms) involved in hydrogen bonding	
		CIPK15	CBL10
1.	2.61	Asn315(OD1)	Lys211(N2)
2.	2.68	Glu318(OE2)	His236(ND1)
3.	2.71	Leu314(O)	Lys211(N2)
4.	2.80	Glu393(OE3)	Lys241 (NZ)
5.	2.51	Ser321(O)	Lys242(NZ)
6.	2.58	Ile320(O)	Lys242(NZ)
7.	2.85	Ser323(O)	Thr192(OG1)
8.	2.61	Lys324(NZ)	Glu195(O)
9.	2.78	Lys324(NZ)	Glu195(OE2)
10.	2.58	Gly330(O)	Arg119(NH1)
11.	2.56	Ile333(O)	Arg119(NH1)
12.	2.53	Ile333(O)	Arg119(NH2)

Table 2. Binding energies of various CBL proteins complexed with CIPK15, ranked in ascending order, the binding energy comprises of three subsets of electrostatic, desolvation and Van Der Waals energies. CBL10 showed the highest affinity for CIPK15 with minimum free energy.

CBL-CIPK Complex	Electrostatic energy kcal/mole	Desolvation energy kcal/mole	Van Der Waals Energy kcal/mole	Cumulative binding energy kcal/mole
CBL10-CIPK	-24.31	-35.61	67.19	-53.21
CBL6-CIPK	-42.83	-10.43	50.67	-48.19
CBL4-CIPK	-14.02	-39.35	68.18	-46.55
CBL8-CIPK	-30.81	-23.16	81.63	-45.81
CBL2-CIPK	-60.05	12.72	86.11	-38.71
CBL3-CIPK	-32.71	-11.78	71.84	-37.31
CBL9-CIPK	-30.83	-4.18	-10.77	-36.09
CBL1-CIPK	-34.36	0.04	-2.48	-34.57
CBL7-CIPK	-25.86	-7.77	16.02	-32.02
CBL5-CIPK	-25.12	-6.82	20.28	-29.91

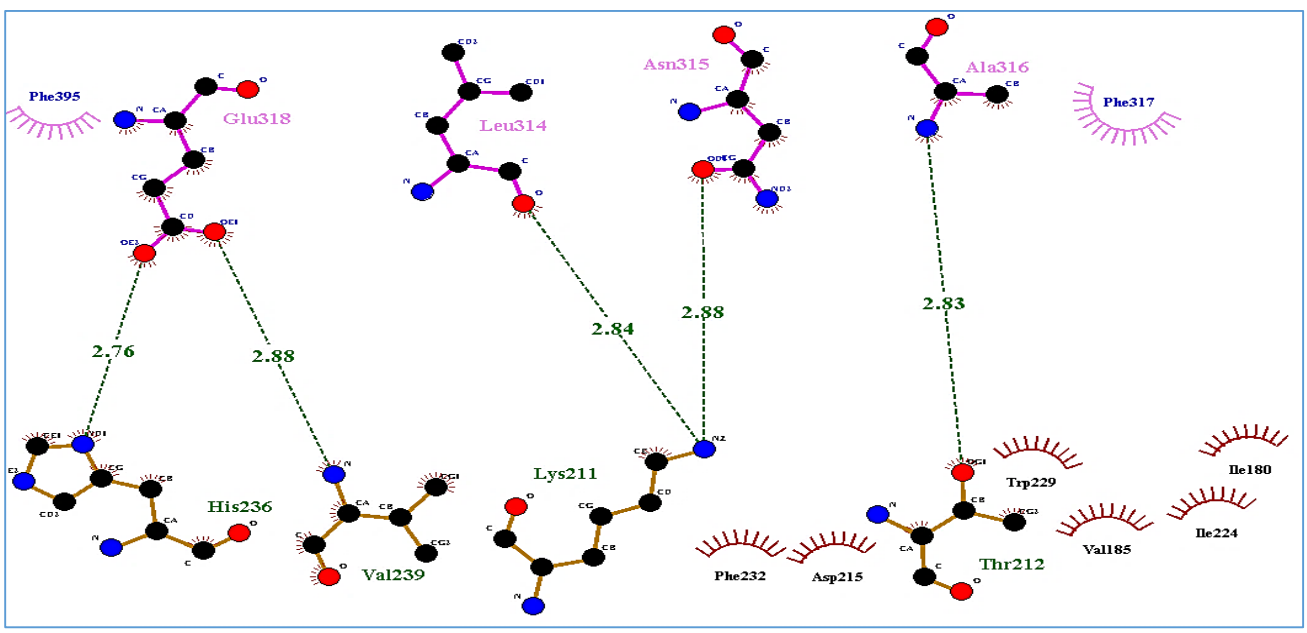

Fig. 7. A section of Dimplot analysis of OsCIPK15 with OsCBL10, OsCIPK15 residues has been shown at top whereas those of OsCBL10 are shown at bottom. Hydrogen bonds have been shown in green dotted lines, the bond length is given in Å whereas hydrophobic interactions have been shown by spiked semi circles. Ligplot+ analysis shows hydrogen bonds between side chain atoms of Leu314, Asn-315, Ala316 and Glu 318 of OsCIPK15 and Lys211, Thr212, His236, Val 239 and Lys241 of CBL10. Strong hydrophobic interaction is also observed between Phe317 of OsCIPK15 and Trp229, Val185, Ile224 and Ile180 of OsCBL10. Asn315, Ala316 and Ph317 constitute the NAF domain of OsCIPK15.

In this study, we have investigated potential of ten OsCBL proteins to bind with and activate OsCIPK15 which is implicated in plant's response to hypoxia. The OsCBL10 has been found to strongly interact and build the most stable association with OsCIPK15 with the highest binding affinity. OsCBL10 has evolved a unique binding pocket for interaction with OsCIPK15's NAF domain; in particular, OsCBL10's residues Lys211, His236, Lys241 and Lys242 have been uniquely evolved to configure its binding pocket to complex and communicate with NAF residues of OsCIPK15. Our findings suggest that the OsCBL10 is involved in activation of rice response to hypoxia through its interaction with OsCIPK15.

Functional studies of CBL and CIPK signaling pathway have implicated CBL-CIPK interaction in various types of abiotic stresses which needs to be investigated in case of rice *Oryza staiva*, being the second highly produced and consumed staple food. Understanding of OsCBL-OsCIPK interaction network is crucial for our knowledge of rice plant's behavior to cope with multiple abiotic stresses. Recently, few studies have presented structural and functional modalities of interaction between OsCBL and OsCIPKs. On the other hand a number of investigations have highlighted various other partners up and down stream in OsCBL-OsCIPK pathway in *Arabidopsis thaliana* responsible for *A thaliana*'s response to diverse types of stresses (de la Torre *et al.*, 2013; Z. Y. Li *et al.*, 2013; Tripathi *et al.*, 2009). These studies sought to investigate role of CBL-CIPK signaling in the regulation of salt ion homeostasis, the plant's response to hormones, soil environment and growth and development (L. Li *et al.*, 2006; Pandey *et al.*, 2007; Xu *et al.*, 2006). However, many questions remain unanswered particularly with reference to rice plant's response to hypoxic stress. This study has sought to address the question of rice plant's response to hypoxia triggered by CIPK-CBL interaction, finding OsCBL10 to be responsible for triggering plant's response to hypoxia in submerged conditions.

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