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Identification of Rice Kinesin 13A Interacting Proteins through Yeast Two-Hybrid Screening

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Abstract

By using full-length rice kinesin 13A protein (OsKIN13A) as a bait construct and rice cDNA library as a prey, we obtained several interacting proteins through yeast two-hybrid (Y2H) screening. These interacting proteins were selected by beta-galactose filter assay after growing on SD-TLH media containing 25 mM of 3-aminotriazole (3-AT). All of selected interacting proteins were confirmed by sequencing and annotated according to the rice genome database, and grouped into seven functional categories, i.e. cellular processes, transport and binding protein, metabolic activity, signal transduction, molecular chaperone and other category. This study suggests that OsKIN13A protein is associated with wide range of biological processes such as development, signaling and immunity.

Keywords: *OsKIN13A*, Y2H, binding domain, cDNA library

Introduction

Kinesins have been known as family of motor proteins that move along microtubules. Initially, kinesin was identified in squid, and has been reported as a microtubule-based protein with high motility[1,2]. Afterwards, plant kinesins were also discovered in tobacco organs such as pollen tubes and phragmoplasts[3-5]. The members of kinesins family have unique features, i.e., the existence of conserved motor domain with ATP- and MT-binding sites. However, this family has relatively less- and non-conserved coiled-coil region and tail domain, respectively.

Comprehensive genomic study has classified kinesins into 14 subfamilies, according to the conserved motor domain[6]. In the genome of *Arabidopsis thaliana*, at least 61 genes have been identified to encode microtubule-based motor kinesins[7]. Some of them have been characterized physiological- and genetically using mutant lines. For example, fragile mutant (*FRA1*), an *Arabidopsis* kinesin-4, is involved in oriented deposition of cellulose microfibrils in fiber cell walls[8]. Kinesin 12A and 12B play prominent roles in cytokinesis during male gametogenesis[9]. Likewise, *Arabidopsis* kinesin-14 is indispensable for early spindle formation[10]. Recently, kinesin-like protein 1 (KP1) has been reported to interact with voltage-dependent anion channel 3 (VDAC3), a mitochondrial outer membrane protein[11].

In contrast to *Arabidopsis*, rice genome evolved less number of kinesins. Current study reported that the rice cultivar (cv.) japonica contain 41 kinesins, while in cv. indica has larger number (45 kinesins)[12]. Functional studies in rice were also reported within the recent years. For example, *Pollen Semi-Sterility1* (*PSS1*) encodes a kinesin-1-like protein, is crucial for male gametogenesis and anther dehiscence[13]. *Brittle Culm12* (*BC12*), a gene encoding a rice kinesin-4 protein, is involved in maintaining plant mechanical strength through regulation of cell wall composition[14]. *BC12* also play important role in cell elongation by regulating GA biosynthetic pathways[15]. In addition, BC12 has been found to interacts with CDKA;3, a cell cycle protein, through yeast two hybrid analysis.

The yeast two-hybrid system (Y2H) is one of the most powerful and versatile methods to study protein-protein interaction as well as characterizing a protein's functions. This method is able to identify novel proteins interact with the bait construct through the recovery of yeast GAL4 transcription[16]. Using this strategy, we aimed to identify proteins that interact with rice kinesin 13A (OsKIN13A).

In spite of its critical function in maintaining microtubule-related processes, kinesins have been reported to play role in many other biological processes. Functional studies in plant revealed that kinesins are indispensable for plant architecture and development. Although research about kinesins has been started since the last decade, the investigation of its member is still plenteous due to the huge number of this family in the plant. In this study, we characterize the rice kinesin 13A (OsKIN13A) in the respect of protein-protein interaction.

Material and Methods

Sequence Analysis: The nucleotide and amino acid sequences of OsKIN13A were retrieved from the rice genome database (http://rice.plantbiology.msu.edu/). Alignment between cDNA and genomic DNA was observed through Spidey program (http://www.ncbi.nlm.nih.gov/spidey/index.html). Molecular

weight (MW) and isoelectric point (pI) was calculated using the prediction site: http://web.expasy.org/compute_pi/. In addition, protein domain feature was determined with Prosite (http://prosite.expasy.org/cgi-bin/prosite/). A phylogenetic tree was constructed as mentioned previously[17,18].

RNA Isolation and RT-PCR Analysis: Total RNA was isolated from leaves, roots, flower, and seeds using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA). First-strand cDNA was synthesized at 37°C for 1 h in a total reaction volume of 25 µl containing 2 µg of total RNA, 1.5 µg oligo $(dT)_{18}$ primer, 2.5 mM dNTP and 200 units of M-MLV reverse transcriptase (Promega, Madison, WI, USA). For studying the expression of *OsKIN13A*, 1.0 µl cDNA sample from the RT reaction was used for PCR in 25 µl total reaction mix containing 10 picomole of *OsKIN13A*-RTF (5′-AAC TCT AGG ACG GTG ATG ATC TCT TG-3′) and *OsKIN13A*-RTR (5′-GTT GCT GTT GCA ACT GCT TTG CCT G-3′) primers. The PCR mixture was initially denatured at 95°C for 5 min and then subjected to 27 cycles of the following conditions: 95°C for 15 s, 58°C for 15 s, 72°C for 1 min with a final extension at 72°C for 5 min. Amplification of rice *Actin* was used to validate equal amounts of cDNA per reaction. Primers for *Actin* were: *Actin*-RTF (5′-AAC TGG TAT GGT CAA GGC TGG GTT-3′) and *Actin*-RTR (5′-GCA ATC CAC ATC TGC TGG AAT GTG C-3′). PCR conditions were: 95°C for 5 min, followed by 23 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. Detection of PCR product was carried as mentioned previously[19-21].

Yeast Two-hybrid Assay: For bait vector construction, the ORF of *OsKIN13A* was PCR-amplified using *Pfu* DNA polymerase with the following primers: 5′-GAT GGG GGA CTC CGG GGA CGC CGT CAT-3′ and 5′-GGA TCC TTA TCT GGA AGA TTT CTT ACG GCT G-3′ (the start codon and *Bam*HI site is underlined). The pGBT9 vector was sequentially digested with *Sma*I and *Bam*HI. Subsequently, the PCR-amplified product of *OsKIN13A* was digested with *Bam*HI and ligated into pGBT9 vector to create pGBT9-*OsKIN13A*. The cDNA library (prey) was made from rice according to the HybriZAP two-hybrid cDNA Gigapack cloning kit manual (Stratagene, La Jolla, CA, USA). Total cDNA of the phagemid form was obtained by the mass *in vivo* excision method. The yeast (*Saccharomyces cereviseae*) strain YRG-2 (genotype: *MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4-542, gal80-538,* LYS::UAS_{GAL1}-$TATA_{GAL1}$-HIS3, URA3:: $UAS_{GAL417mers(x3)}$- $TATA_{CYC1}$-*lacZ*) was transformed with the binding domain pGBT9-*OsKIN13A*. The yeast cells containing the binding domain were co-transformed with 100 µg of the HybriZAP cDNA library plasmid DNA and salmon sperm carrier DNA by the lithium acetate method[22]. Transformants were selected on the synthetic dropout medium lacking tryptophan, leucine, and histidine (SD-TLH). The transformants, which appeared after 3-5 days incubation at 30°C, were then grown on an SD-TLH plate containing 5 mM and then 25 mM 3-aminotriazole (3-AT). Yeast colonies which still survived on media containing 25 mM 3-AT were tested for the beta-galactosidase activity by the filter assay[23]. The colonies that turned blue in less than 6 h were collected and used as the template in colony PCR reaction as described previously[24]. The primers for amplification were GAL4 AD-forward and -reverse with following PCR procedure: the denaturation condition was 7 min at 95°C, followed by the amplification reaction for 1 min at 95°C, 1 min at 55°C, and 3 min at 72°C for 35 cycles, prior to a final extension for 7 min at 72°C and storage at 4°C. The PCR product was then purified with a QIAEX II kit (Qiagen, Valencia, CA, USA) and confirmed by DNA sequencing. Nucleotide sequences obtained from DNA sequencing were used for gene annotation by BLAST against NCBI (http://www.ncbi.nlm.nih.gov) and rice genome database (http://rice.plantbiology.msu.edu/).

Results and Discussion

Expression Pattern, Molecular Structure and Sequence Analysis of *OsKIN13A*: The expression pattern of *OsKIN13A* was investigated through RT-PCR with gene-spesific primers, based on the steady-state mRNA levels in different rice tissues and /or organs. The results showed that this gene is indeed expressed in all tissues /or organ examined (figure-1). *OsKIN13A* gene transcript was highly expressed in flower and seed. The expression level was lower in leaf and root, respectively. The *OsKIN13A* is most likely expressed constitutively through the rice life cycle. This result indicates that *OsKIN13A* might play potential role during vegetative and reproductive development.

The molecular structure of *OsKIN13A* is shown in figure-2A. *OsKIN13A* consists of nineteen exons and eighteen introns. The *OsKIN13A* contained an open reading frame (ORF) of 3,300 bp encoding a polypeptide of 1,099 amino acid residues with a calculated molecular mass of 122.22 kDa and pI of 6.14. Kinesin motor domain was determined to be located at amino acid between 188 and 458 (figure-2B). More specifically, a short motif (GKISFIDLAGSE) located at amino acid number 426 to 437 was predicted as a core motor domain signature. Glycine-rich region was found in amino acid residue 27-50. In addition, nuclear localization signal (NLS) was determined at amino acid residue 965-979. Database search revealed that *OsKIN13A* is located at chromosome number 5 (LOC_Os05g06280). Phylogenetic tree was generated by comparing OsKIN13A protein with other previously reported kinesins in rice. As shown in figure-2C, OsKIN13A has high identity with LOC_Os01g43580.

Screening Proteins that Interact with OsKIN13A: In general, proteins interact with other biomolecules to execute their functions. With yeast-two hybrid (Y2H) system, proteins that interact with OsKIN13A were determined and classified

according to their function. These proteins may functions as co-activator, inhibitor or substrate.

The Y2H was conducted to identify proteins that interact, as partner or target proteins, with OsKIN13A. More than 1000 transfromants were grown under SD-Trp-Leu-His (SD-TLH) plate. To reduced the amount of transformants, the second screening was conducted by transferred about 500 colonies into SD-TLH containing 5 mM and 25 mM of 3-aminotriazole (3-AT). Approximately, more than 100 transformants were able to grow under media with 25 mM 3-AT. The transformants were also tested for *LacZ* activation. Finally, we observed 81 interacting proteins (table-1). To make better understand, all candidate proteins were classified into several groups due to their functional role in biological processes (table-1, figure-3). These proteins were grouped into six functional categories, i.e. cellular processes (CP), transport and binding protein (TB), metabolic activity (MA), signal transduction (ST), molecular chaperone (MC), and other category. The example of proteins that is involved in cellular processes are elongation factor, expansin, tubulin and membrane protein. Cyclin-dependent kinase C was also included in this category. The example of proteins that is involved in transport and binding are transferase, reductase, and zinc finger protein. Metabolic activity group consist of several functional proteins such as cellulose, carboxylase, dehydrogenase, and synthase. Proteins such as PGIP2, B12D, and Myb transcription factor were grouped into signal transduction category. Three heat shock protein family (DnaJ, Hsc70, Hsp81) and an immunophilin were categorized into molecular chaperone.

Of seven categories, proteins involved in transport/ binding are mostly interacts with OsKIN13A. Interestingly, four proteins appeared twice during screening process. These are zinc finger (C3HC4-type RING finger) protein, heat shock cognate 70 kDa protein, senescence-associated protein (B12D) and ferredoxin thioredoxin reductase. Hence, OsKIN13A likely has notable interaction with these proteins. Zing fingers are protein that can adapt one or more zinc ions for their folding stabilization. In addition, most of zing finger proteins in plant are transcription factors and involved in several numbers of biological processes such as interactions with other molecules and stress-tolerance regulation[25,26]. More specifically, C3HC4-type RING finger proteins play a key role in ubiquitination pathway[27,28]. Heat shock cognate 70 kDa protein (Hsc70) is a member of heat shock protein 70 family that can binds nascent polypeptide to facilitate correct protein folding[29]. Like other heat shock proteins, Hsc70 also has ATPase activity[30].

Plant senescence related with hormonal signaling and maturation or aging. In addition, senescence can be induced by environmental stresses or pathogen infection associated with programmed cell death (PCD)[31,32]. Our result identified senescence-associated protein (B12D) interacts with OsKIN13A. B12D has been reported to be accumulated during seed maturation and leaf senescence[33,34]. Ferredoxin thioredoxin reductase is potent candidate that interacts with OsKIN13A. This enzyme involved in photosynthetic action, especially during light-dependent reaction i.e. coverts ferredoxin into thiol group.

Another interesting finding is OsKIN13A interacts with Cyclin-dependent kinase C (CDKC). CDKC is orthologues of Cdk9, a human protein that critical for embryo development. In *Arabidopsis*, CDKC and its partners (CYCT1) regulate immunity responses against *Cauliflower mosaic virus* (CaMV), responsible for normal growth morphology, trichome development, and flowering time regulation[35]. OsKIN13A also interacts with polygalacturonase inhibiting protein 2 (PGIP2). PGIP have been reported to restrain fungal and bacterial invasion in *Arabidopsis*, tomato, grapevine, tobacco and cabbage[36-40]. Interestingly, of five to six PGIP members, PGIP2 is the most powerful molecule to inhibit polygalacturonase activity secreted by fungal pathogens[39,41].

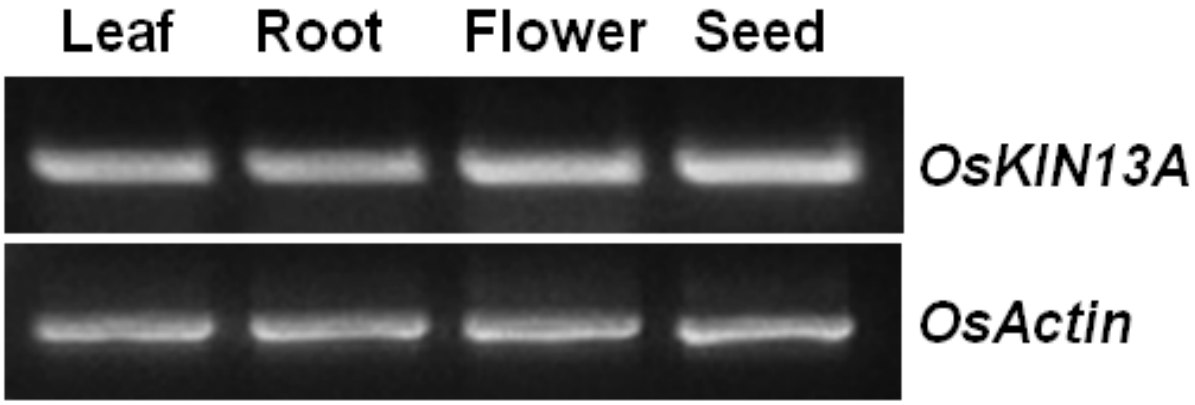

Figure-1
Expression pattern of the *OsKIN13A OsActin* was included as an internal control

Table-1
List of OsKIN13A interacting proteins obtained from yeast two-hybrid screening

Category: Transport and binding protein (TB)[a]	
Colony number	**Gene annotation/ putative role**
1	Calnexin 1 (CNX1)
11	Dihydrolipoamide S-acetyltransferase (LTA2)
32, 200	Zinc finger (C3HC4-type RING finger) protein[b]
61	Speckle-type POZ protein
82	Lipid transfer protein-related
85	N-methyl-D-aspartate receptor-associated protein
132	Zinc finger (GATA type) protein
144	FtsJ-like methyltransferase protein
151	Monodehydroascorbate reductase
158	Choline kinase
169	Clathrin adaptor
172	SAR DNA-binding protein-1
181	Selenium-binding protein
218	Glutathione S-conjugate ABC transporter (MRP1)
223	Ubiquitin-conjugating enzyme E2-17 kDa
229	Methyltransferase
230	Glycosyl transferase protein
268	ADP-ribosylation factor
309	Adapter protein SPIKE1 (SPK1)
328	Polyadenylate-binding protein (PABP)
350	Heavy-metal-associated domain-containing protein
Category: Metabolic activity (MA)[a]	
Colony number	**Gene annotation/ putative role**
5	Endo-1,4-beta-glucanase / cellulase (CEL2)
26	Dienelactone hydrolase family protein
31	Ribulose-bisphosphate carboxylase
43	D-alanine ligase
59	ATP synthase
72	Glyceraldehyde 3-phosphate dehydrogenase
189	Pyruvate decarboxylase
197	Anthocyaninless2 (ANL2)
264	3-hydroxy-3-methylglutaryl-CoA reductase 1
267	Glucan phosphorylase
326	Cinnamyl-alcohol dehydrogenase
329	Glycosyl hydrolase family 17 protein
352	Pyruvate kinase
246	Omega-6 desaturase
Category: Cellular processes (CP)[a]	
Colony number	**Gene annotation/ putative role**
6	Cyclin-dependent kinase C (CDKC)
22	Pentatricopeptide (PPR) repeat-containing protein
25	Elongation factor 2 (EF-2)
41	Kinesin motor protein
48	Tubulin gamma-1 (TUBG1)
54	Histone H1
70	Hydroxyproline-rich glycoprotein protein
124	Integral membrane protein
127	F-box family protein / SKP1 interacting partner 3-related
136	Beta-expansin
141	Vesicle-associated membrane protein (VAMP)
143	Armadillo/beta-catenin repeat protein
161	Elongation factor 1 (EF-1)
187	Histone H3
194	DEAD box RNA helicase
252	Tubulin alpha-2
269	Actin-depolymerizing factor
280	Disulfide isomerase
343	Histone H4
220	Alpha-tubulin
Category: Molecular chaperone (MC)[a]	
Colony number	**Gene annotation/ putative role**
35	DnaJ heat shock protein
67, 182	Heat shock cognate 70 kDa protein[b]
119	FK506-binding protein (FKBP12) / immunophilin
260	Heat shock protein 81-2 (HSP81-2)
Category: Signal transduction (ST)[a]	
Colony number	**Gene annotation/ putative role**
46	Dehydration-induced protein (ERD15)
73	Calmodulin-7 (CAM7)
78	Serine protease HTRA2
107	SPX domain-containing protein (NUC-2)
110	Wall-associated kinase 4
183	Calcium-dependent protein kinase
191	AP2 domain-containing protein
199	Ethylene-responsive transcriptional coactivator
201, 204	Senescence-associated protein (B12D)[b]
212	Senescence-associated protein 5
228	Myb family transcription factor
254	3-phosphoinositide-dependent protein kinase
283, 284	Ferredoxin thioredoxin reductase[b]
310	Transcriptional coactivator p15 (PC4)
313	Floral homeotic protein APETALA1 (AP1)
316	Polygalacturonase inhibiting protein 2 (PGIP2)
341	Nascent polypeptide-associated complex protein
Category: Other	
Colony number	**Gene annotation/ putative role**
126	ARF GTPase-activating domain-containing protein
184	Nodulin
315	Early nodulin-related
321	Endomembrane protein 70
348	Iron-sulfur cluster assembly complex protein

[a]Classified according to their function in biological processes.
[b]Appeared twice during the Y2H screening.

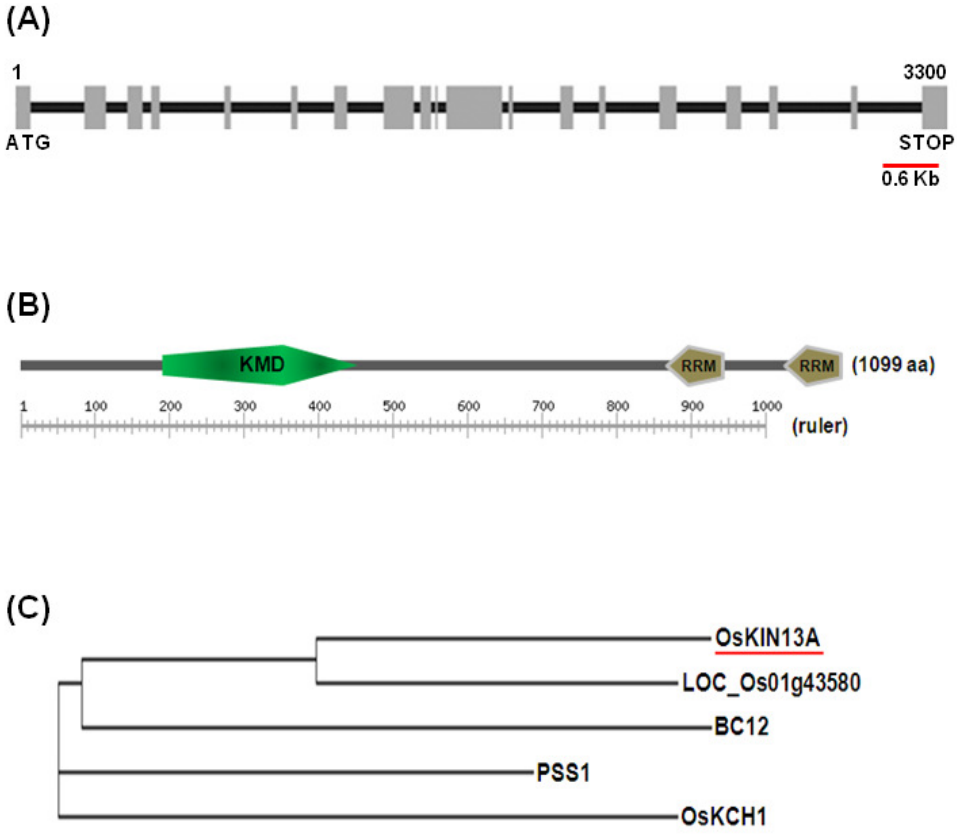

Figure-2
Structure and phylogenetic tree of rice kinesin (*OsKIN13A*)
A, Molecular structure of OsKIN13A with nineteen exons (gray-filled boxes) and eighteen introns (inter-lines); B, Location of motor domain signature (KMD, green color) in OsKIN13A; C, Phylogenetic of OsKIN13A with other kinesins from rice.

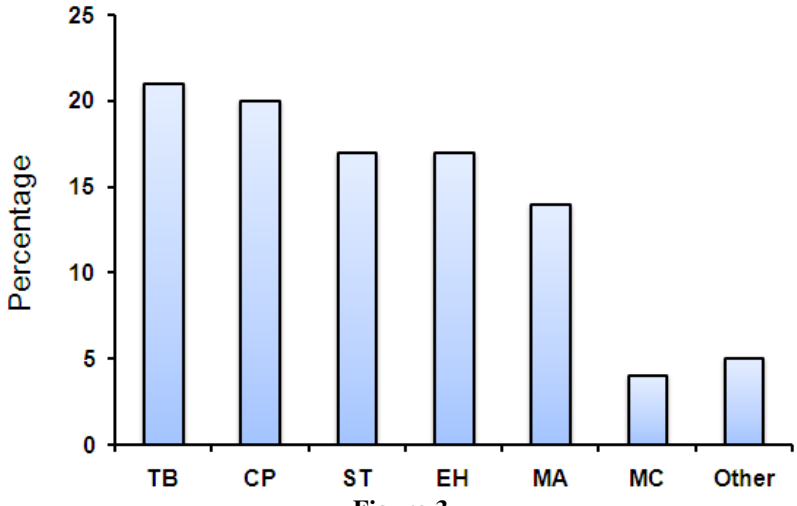

Figure-3
Classification of OsKIN13A interacting-proteins according to their role in biological processes

Conclusion

This study underlines a critical function of OsKIN13A that involved in wide ranges of biological processes, not only related with cellular mechanism and morphology determination, but also associated with protein folding and plant immunity.

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