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Chapter 4

Hd3a Florigen Recruits Different Proteins to Reveal Its Function in Plant Growth and Development

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Additional information is available at the end of the chapter

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Abstract

The nature of Hd3a protein in rice and its ortholog FT in *Arabidopsis* as a florigen has been proposed. However, molecular mechanism of its function still remains to be investigated. Therefore, it is important to search their interaction partners to better understand their signaling in flowering. As a long-distance signal that moves along leaf cells and the vascular system of leaves and stem and exerts its action in apical buds, it is important to determine the possible mediators of such common responses activated by Hd3a. To search Hd3a interactor, yeast two-hybrid screening have performed by using a cDNA library. A wide range of Hd3a interacting proteins involved in signaling were identified, including GF14c, OsKANADI and the BRI1 kinase domain interacting protein 116b (BIP116b). To reveal its function, Hd3a recruits different protein in plant developmental stage. It is possible that Hd3a and its partner(s) may form a platform for cross-talk between signal transduction pathways. Another homolog of Hd3a in many plants was identified and sugessted that Hd3a/FT has versatile role in plant development. This role depend on its partner and interaction to achieve its function. Our understanding in floral transition in rice would make for better crop management in future.

Keywords: Hd3a, florigen, interacting proteins

1. Introduction

Transition from vegetative phase to flowering involves many genetic pathways that interact with the external signals, such as day length and temperature, and internal signals such as hormones and developmental controls. One of the most important factors controlling flowering plants is response to daylight or photoperiod [1]. Based on photoperiodism, two

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model plants, *Arabidopsis thaliana* and *Oryza sativa* are used to study on regulation of flowering time of long-day plant (LDP) and short-day plant (SDP), respectively. Three genes, which constitute a major genetic pathway in the photoperiodic regulation of flowering in rice, have recently been isolated. *O. sativa GIGANTEA* (*OsGI*), an ortholog of *Arabidopsis GI*, *Heading date 1* (*Hd1*), an ortholog of *Arabidopsis CO* (*CONSTANS*), and *Heading date 3a* (*Hd3a*), an ortholog of *FLOWERING LOCUS T* (*Arabidopsis FT*) are shown to form the main pathway for photoperiodic regulation of flowering in rice. These three genes were conserved between rice and *Arabidopsis*; however, the differences in their regulation results in either SDP or LDP (**Figure 1**). The major difference between rice, a SDP, and *Arabidopsis*, a LDP, was shown to be the regulation of *Hd3a*/*FT* by *Hd1*/*CO*. Under LD conditions, this regulation is positive in *Arabidopsis* while negative in rice [2].

In *Arabidopsis*, *GI* encodes a nuclear protein [3]. Expression of *GI* mRNA exhibits a circadian rhythm and *gi* mutants defects in clock function, indicating that *GI* is closely associated with the clock itself. *CO* encodes a transcription factor with B-box type zinc fingers thought to mediate protein-protein interaction. *FT* encodes a protein with homology to Raf kinase inhibitor protein (RKIP). It is a powerful promoter of flowering, activating the floral meristem identity gene, *APETALA1* (*AP1*) and is the target of several pathways controlling flowering time [4].

Hd3a was initially identified by quantitative trait locus (QTL) promoting flowering under short day-length condition. Using map-based cloning, *Hd3a* was determined as an ortholog of *FT* in *Arabidopsis* [5]. *Hd3a* overexpression under 35S promoter, native promoter, and vascular-specific promoters showed early flowering phenotype [6]. On the other hand, the suppression of *Hd3a* by RNA interference exhibited delayed flowering in rice [7]. These results strongly suggest that Hd3a protein function for flowering promotion.

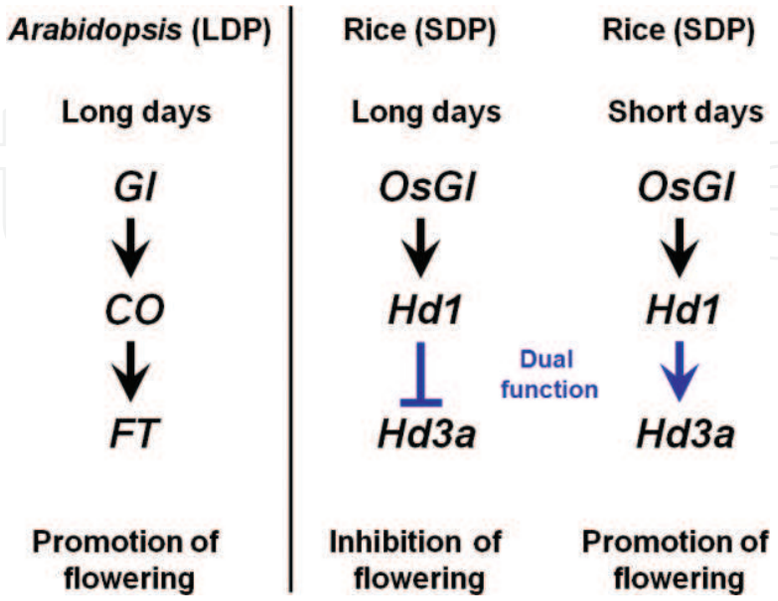


Figure 1. Flowering pathway regulation in *Arabidopsis* and rice.

Hd3a shares sequence similarity with the mammalian phosphatidylethanolamine-binding protein (PEBP or RAF1 kinase inhibitor protein—RKIP) (**Figure 2**). The PEBP family regulates signaling pathways to control growth and differentiation. The PEBPs seem to act biochemically as inhibitors, binding signaling components to modulate the flux through their pathways.

The crystal structure of PEBP from human and bovine sources, CEN protein from *Antirrhinum* [8] and that of the Terminal Flowering Locus (TFL) and FT proteins from *Arabidopsis* [9] have been determined. In **Figure 3**, the molecular model of Hd3a protein was built using the Swiss-Prot automated comparative protein modeling server, based on its sequence homology to two members of the RKIP protein family whose structures have been determined by X-ray crystallographic methods (*Arabidopsis* FT and TFL1, protein databank accession numbers 1WKP and 1WKO, respectively). Structural analysis of these proteins indicated the accessibility of the ligand-binding pocket to interact with the proteins partner.

PEBPs might also act as either scaffolds for or regulators of signaling complexes, as showed by the finding that Self-Pruning (SP) and Single-Flower Truss (SFT), as a tomato homolog of Terminal Flowering Locus 1 (TFL1) and Flowering Locus T (FT), respectively, can interact with a range of diverse proteins [10, 11]. Several studies of protein interactions involving FT/Hd3a orthologs have been published. In *Arabidopsis*, FT interacts with the basic/leucine zipper (bZIP) transcriptional factor FD [12, 13]. Interestingly, the bZIP transcription factor Self-pruning G-box protein (SPGB) in tomato, a homolog of FD, interact with SP and SFT as well. Moreover, FT interacts with FD and 14-3-3 proteins (**Table 1**) [11–13]. However, no Hd3a interacting proteins have yet been identified in rice.

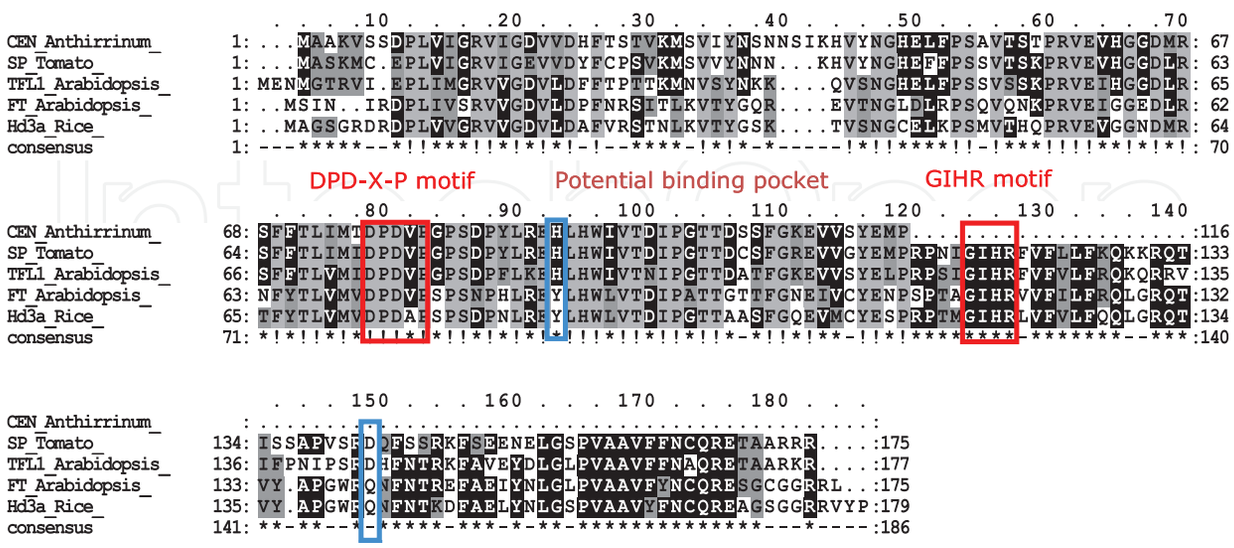


Figure 2. Hd3a shares high homology with other phosphatidylethanolamine binding protein or Raf kinase inhibitor protein (PEBP/RKIP) in various plant species. Hd3a and FT has 73% homology. The blue box indicates the amino acid difference which is responsible for its function as flower inducer (tyrosin in FT and Hd3a) or repressor (histidine in CEN, SP, and TFL1).

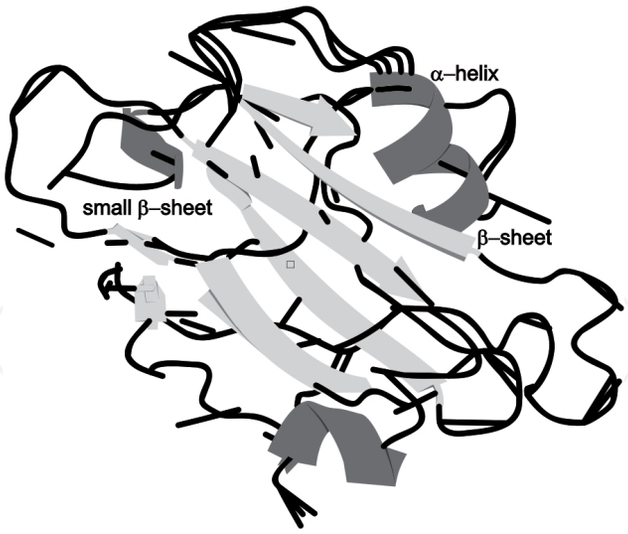


Figure 3. Molecular model of Hd3a. Hd3a protein contains a large central β-sheet (yellow ribbons), which is flanked on one side by a smaller β-sheet and on the other by an α-helix (red ribbons).

In *Arabidopsis*, it is believed that the combination of interacting proteins, resolved crystal structures, and mutant phenotype analysis will lead to a comprehensive understanding of the mechanisms that facilitate the switch from vegetative phase to reproductive phase. It seems likely that Hd3a/FT is involved not only in flowering, but also in other aspects of growth and development in plant architectures [11, 15–18]. This function will be achieved by interacting with its partners. Hd3a might recruit different proteins to perform its roles in plant growth and development, particularly during floral transition.

In this chapter, we will discuss the regulation of Hd3a florigen in rice and the identification of novel interaction partners for rice Hd3a protein using yeast two-hybrid screening. The interaction between Hd3a and its partners was further confirmed by several methods, such as yeast two-hybrid assay using full-length cDNA, *in vitro* pull-down assay, co-immunoprecipitation, and bimolecular fluorescence complementation (BiFC). The expression pattern and subcellular localization of each Hd3a interacting partner provided important insights into its function. To further characterize the function of Hd3a interacting proteins in plant growth

Protein	Interacting proteins	References
Mammalian PEBP	Raf1	[14]
Self-Pruning (SP) tomato	14-3-3 (adapter protein)	[10]
	SPGB (a putative bZIP transcription factor)	
	SPAK (SP-associated kinase)	
Flowering Locus T (FT) *Arabidopsis thaliana*	FD (bZIP transcription factor)	[12, 13]
Single-Flower Truss (SFT) tomato	SPGB	[11]
	14-3-3/74	

Table 1. PEBP/RKIPs interacting proteins.

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and development, particularly during the floral transition, a reverse genetics approach was used to generate gain of function mutants (overexpression)/knockdown mutants using RNAi or knockout mutants.

2. Flowering regulation in rice

In rice (*O. sativa* L.), flowering is mainly induced by photoperiod. *GIGANTEA* in rice (*OsGI*) is a clock-regulated gene, which was first identified by the differential display method, and its expression is high in the middle of day [2]. Under inductive SD conditions, *OsGI* promotes the expression of *Heading date 1* (*Hd1*), and *Hd1* activates *Hd3a* expression. However, under noninductive LD conditions, *Hd1* suppresses *Hd3a* expression [2]. *OsGI* acts as the primary upstream regulator of *Hd1* expression [19]. OsGI is a large protein that is present in both the nucleus and cytoplasm of rice cells [12]. Suppression of *OsGI* by RNAi or antisense expression caused late flowering and reduced *Hd1* transcription under SD conditions [2].

Several unique genes in rice were isolated. *Early heading date1* (*Ehd1*) encodes a B-type response regulator and is a unique flowering time gene [20]. *Ehd1* promotes floral transition preferentially under SD conditions, even in the absence of functional alleles of *Hd1*. Expression analysis revealed that *Ehd1* functions upstream of *Hd3a*, *RFT1*, and some MADS-box genes [20]. More recently, *Ghd7* (for grain number, plant height, and heading date 7), which encodes a *CO*, *CO-LIKE*, and *TIMING OF CAB1* (*CCT*)-domain protein, was isolated from natural variants in rice [21]. *Ghd7* affects transcript levels of *Ehd1* and *Hd3a* under LD conditions and delayed the flowering. In the same condition, *Ghd7* does not affect *Hd1*. Therefore, rice have different flowering pathway: *Hd1* pathway and *Ehd1* pathway. These two different pathway integrated with environmental condition, in this case is photoperiod and regulate the FT-like genes to initiate or delay the flowering [22]. Another unique flowering gene in rice, *RID*/*OsId1*/*ehd2*, yields an extremely late flowering phenotype under both SD and LD conditions. This gene encodes a putative transcription factor with a zinc finger motif, and is an ortholog of *INDETERMINATE1* (*ID1*), which promotes flowering in maize (*Zea mays*). Specifically, it promotes the floral transition, mainly by upregulating *Ehd1* and genes downstream of *Ehd1*, such as *Hd3a* and *RFT1* [23–25].

Hd3a expression under SD conditions is also regulated by phytochrome. In the *se5* (the photoperiodic sensitivity 5) mutant, which lacks a functional gene encoding heme oxygenase [26], an enzyme that is required for loss-of-function alleles in one of the three rice phytochromes [27], rice exhibited early in flowering. *Hd1* expression is not affected by *se5* or *phyB* mutations; thus, phytochrome represses *Hd3a* expression downstream or independently of *Hd1* expression under SD conditions.

During the vegetative phase in rice, the shoot apical meristem (SAM) produces a series of leaves. The vegetative parts of the rice plant, consisting of root, culm, and leaves, form a tiller. A dramatic change occurs during the transition from vegetative to reproductive stages, with the tiller terminating to produce leaf and the panicle (inflorescence) being generated on the uppermost internode of the culm (**Figure 4**). The development stage of the young panicle is also related to the timing of leaf emergence. The differentiation stage of the young panicle almost

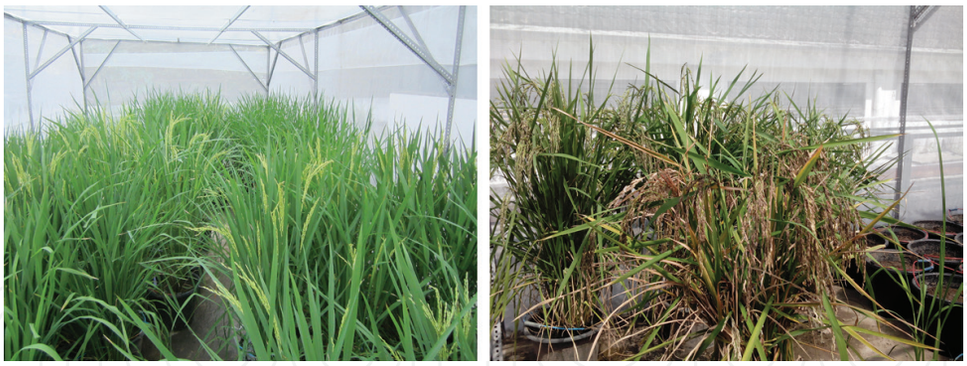


Figure 4. Rice plant during the developmental stages: vegetative phase, early reproductive phase (left panel) and during seed maturation (right panel).

directly correlates with the start of emergence of the fourth leaf (counted downward from the flag leaf). At the time of flag leaf (small last leaf) emergence, the glumes flower primordial has already differentiated and pollen mother cells are being formed [28]. The flag leaf, contributes largely to the filling of grains because it supplies photosynthetic products mainly to the panicle.

Flowering time in rice is indicated by the emergence of the flag leaf or the panicle (heading date). The panicle is initiated when the first bract primordium differentiates on the shoot apex, approximately 30 days before panicle emergence (heading). The start of differentiation of the glumes flower primordial follows after the rachis-branches differentiation has occurred (24 days before heading). The pistil and stamen differentiate 20 days before heading. Meiosis in the anther occurs 12 days before heading, and flower organ completion occurs 1–2 days before heading.

3. Yeast two-hybrid screening for searching Hd3a-interacting proteins

An important element in the characterization of Hd3a function is the identification of other proteins with which it interacts. To reveal the function of Hd3a in rice, yeast two-hybrid system was used as a tool for screening Hd3a-interacting proteins. This system consist of a fusion of protein interest to a DNA-binding domain (DBD-Hd3a) as a bait, and a fusion of cDNA library to a transcriptional activation domain (AD-cDNA library) as a prey (**Figure 5**). The DNA binding domain (DBD) recognizes a specific sequence in the DNA upstream of a promoter and the activation domain (AD) stimulates transcription by binding to RNA polymerase. If the two domains interact, they will activate transcription. In the two-hybrid system, both the DBD domain and the AD domain are fused to two other proteins. If the bait captures the prey, means that proteins are interacting, a complex will be formed and the reporter gene will be activated. The reporter gene is used to monitor for a successful interaction.

As a tool for searching Hd3a interactors, the construction of a bait and prey is important. cDNA library were constructed from leaf blades harvested 35 days after sowing at ZT 0, 2, and 4 when transition from vegetative to reproductive phase occurred and *Hd3a* was highly expressed [7]. The Hd3a full-length cDNA was constructed as a bait and expressed in yeast system (**Figure 6**).

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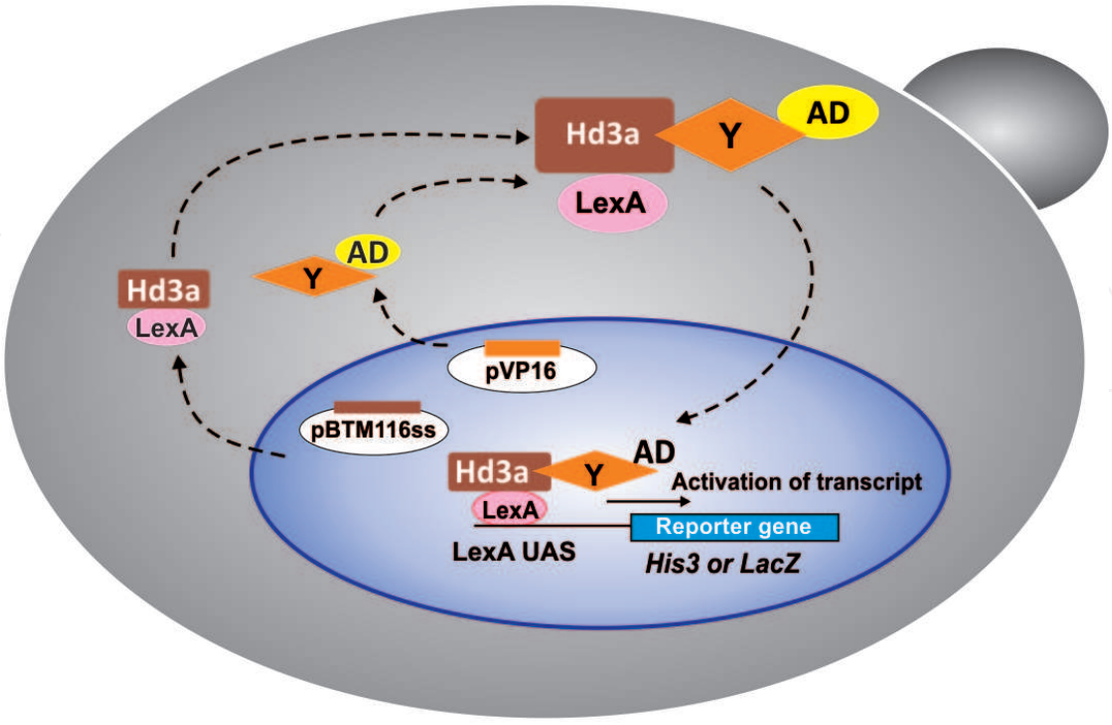


Figure 5. Yeast two-hybrid system. Positive interactions can be detected by selecting on plates lacking histidine, followed by screen for β-galactosidase expression.

For the large-scale yeast two-hybrid screen, a total of ~1.6 × 10^6 transformants were screened for activation of histidine synthase 3 (HIS3) onto selective medium plates without amino acids leucine (L), tryptophan (W) and histidine (SC-His). In the first screening on media SC-His, 354 colonies were obtained. The next screening on medium SC-His containing 3-AT, a total of 96 colonies' unique proteins were identified as positive clones. To eliminate false positive, we used 2.5 mM 3-AT, a competitive inhibitor of the *His3* gene product (histidine synthase),

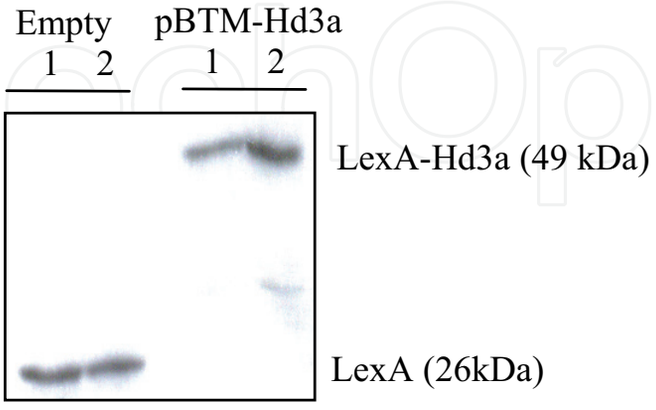


Figure 6. The Hd3a protein expression (pBTM-Hd3a) in yeast strain L40. The protein was run on 10% SDS-PAGE and electrotransferred onto an immobilon-P membrane followed by detection of Hd3a-LexA fusion using the anti-LexA antibody. Molecular weight of Hd3a-Lex A and Lex A are 49 and 26 kDa, respectively.

which is the reporter gene for the interaction in the yeast two-hybrid system. The 96 positive clones with the size ranged between 600-2000 bp then were checked to confirm the presence of "in frame" cDNA-AD fusion. Some clones were found in full-length of cDNA, but the other clones were not. They lack of N-terminal coding region. The clones were then retransformed into yeast containing Hd3a bait to confirm their interaction on selective medium SC-His plus 3-AT (**Figure 7**). The flowchart of yeast two hybrid screens is presented in (**Figure 8**). For the result, a diverse range of interactor proteins can be identified in this yeast two hybrid system (**Table 2**).

The main group of proteins identified in this yeast two-hybrid screen belongs to the class of signal transduction pathway components (57%). Others are classified as proteins that are involved in carbohydrate metabolism, protein/RNA/DNA synthesis, and proteins with unknown function. Interestingly, our yeast two-hybrid screening identified a diverse range of proteins that are mainly involved in signaling. In this chapter, we focus on three candidates: (i) GF14c (G box factor 14c), a 14-3-3 protein identified as a protein that is involved in signaling pathway. The recent results revealed that Ha3a, 14-3-3 and FD form a hexameric florigen activation complex (FAC) and in shoot apical meristem cells, 14-3-3 protein act as intracellular receptor for florigen [29]; (ii) BRI1-kinase domain (KD)-interacting protein 116b, and (iii) a novel myb transcription factor-like protein, namely OsKANADI1.

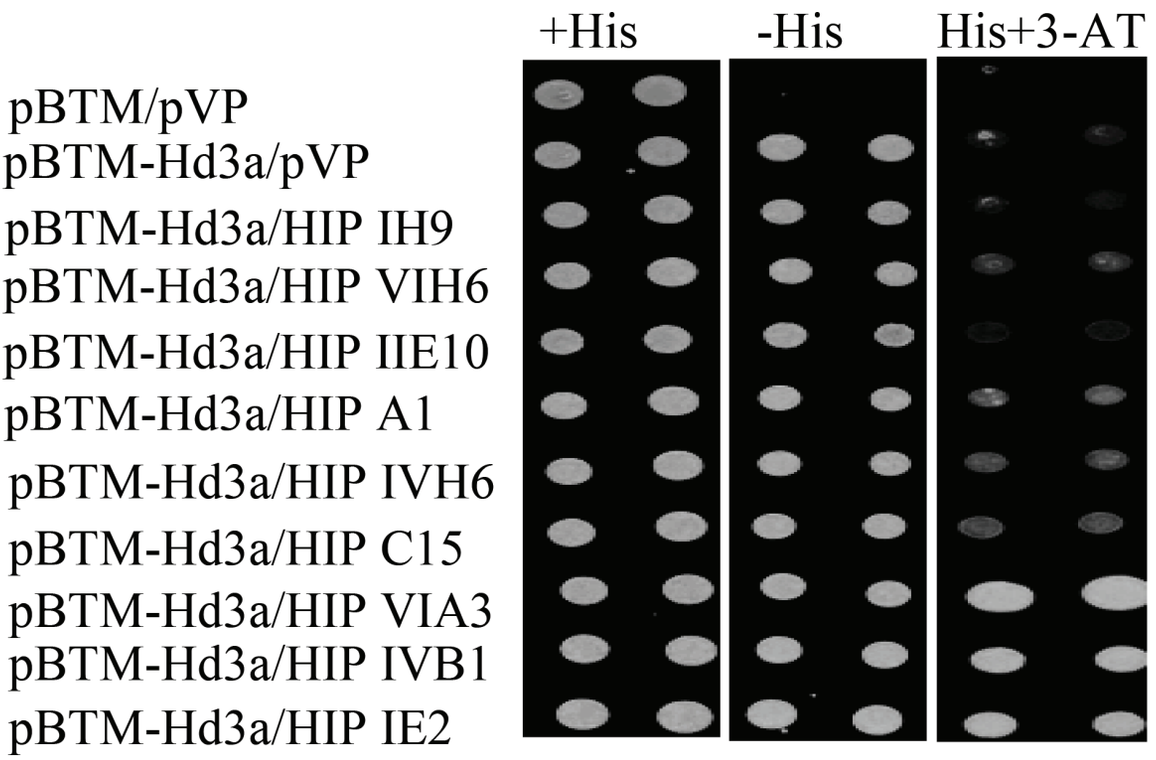


Figure 7. Interaction of Hd3a with its partners in yeast two-hybrid system. The growth of yeast colonies on the plate (LWH) lacking leucine (L), tryptophan (W), and histidine and with 2.5 mM 3-AT (3-aminotriazole) indicates a positive interaction between Hd3a and the particular Hd3a-interacting proteins. 3-AT is a competitive inhibitor of the *HIS3* gene product (histidine synthase), which is the reporter gene for the interaction in the yeast two-hybrid system. Each clone of Hd3a interacting protein (HIP) was spotted onto selective plate.

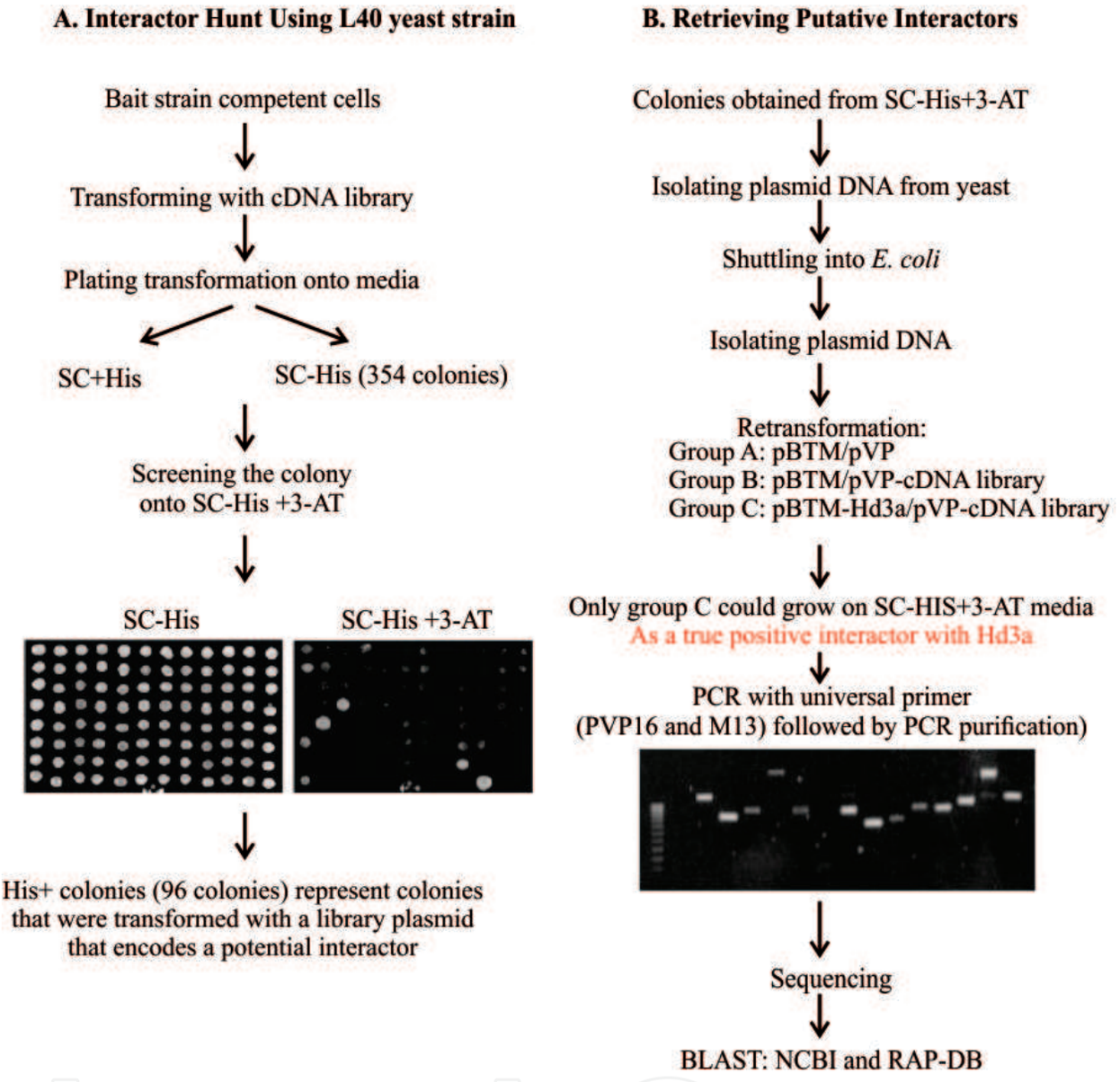


Figure 8. Flowchart of yeast two-hybrid screening. (A) Interactor hunt using L40 yeast strain; (B) Retrieving putative interactors.

Homologous proteins	Accession number	Two-hybrid*	Amino acids**	Domain/Function
Carbohydrate metabolism				
Ribulose 1,5-biphosphate carboxilase (RuBisCO) small subunit	gi|3063524	150	175	RuBisCO
RuBisCO activase large isoform precursor protein	gi|77552725	162	466	RuBisCO
Protein synthesis/DNA and RNA binding proteins				
Putative RNA helicase	gi|34897325	127	756	–
Putative 40S ribosomal protein	gi|50939279	211	305	–

Homologous proteins	Accession number	Two-hybrid*	Amino acids**	Domain/Function
Translation initiation factor 5A/eIF5A	gi|50918889	161	161	–
Linker histone	gi|50921921	185	188	–
Signaling				
GF14c (G-box factor 14-3-3c protein)	**gi|50903393 AK122149/Os8g0430500**	**115**	**256**	**14-3-3, adaptor, chaperone, scaffolding**
BRI1-KD interacting protein 116b/ BIP116b	**–**	**112**	**366**	**TPX2 domain**
OsKANADI1	**gi|41053181 Os02g0696900**	**147**	**361**	**GARP domain**
Beta cyanoalanine synthase	gi|55233175	377	377	–
Putative endonuclease/phosphatase	gi|37532222 AK100514/Os10g0203000	209	483	phosphatase
Putative anthranilate phosphoribosyltransferase	gi|50905035	121	141	–
Putative NADPH-dependent retinol dehydrogenase/reductase	gi|50726535	170	253	–
Putative SF16 protein	gi|50931997	240	500	–
Putative heat shock 70 KD protein (HSP70)	gi|27476086	157	657	chaperone
Ser/Thr kinase	gi|34894358	205	461	kinase
Putative glutathione S-transferase	gi|50900303	142	223	–
Thioredoxin H-type (TRX-H)	gi|50934769	204	350	–
Unknown				
Unknown protein	gi|50938743	68	68	–
Unknown protein	gi|54287657	91	91	–

* Length in amino acids identified from yeast two-hybrid screening.

** Length in amino acids of protein.

Table 2. Proteins identified by yeast two-hybrid screening.

4. Rice flowering regulation by Hd3a and GF14c interaction

14-3-3 proteins in general bind to phosphoserine-modified proteins, as well as to some non-phosphorylated proteins such as exoenzyme S, which has no phosphorylated residue in its binding motif. They regulate the activities of a wide array of targets via direct protein-protein interactions, and effect changes in the client proteins. These changes can vary from inactivation or activation of the enzymatic activity of a target protein, to degradation or protection from degradation of the target, to movement of the target from one cellular location to another (usually nuclear-cytoplasmic shuttling). Plant 14-3-3 proteins bind a range of TFs and other signaling proteins, and have pivotal roles in regulating developmental and stress responses.

To reveal the biological functions of 14-3-3 in plants, the common approaches of generating overexpression, RNA suppression, or knockout plant lines probably have limited potential, for two reasons. First, because they interact with so many different targets, multiple pleiotropic effects would be expected. Second, 14-3-3s are encoded by a gene family with at least 12 expressed members in *Arabidopsis* [30], 8 members in rice [31, 32], and similar numbers in other plants, such as tomato and tobacco, which adds the complicating issues of isoform specificity and redundancy. However, working with a family of proteins that play so many roles will be interesting, given the challenge of trying to identify a particular protein-protein interaction associated with a phenotype using 14-3-3 mutants (overexpression or knockout mutants). Rather than focusing on 14-3-3s themselves, more targeted and informative approaches would be to identify specific interactions using biochemical or yeast two-hybrid methods, followed by *in vivo* confirmation and directed investigation of the potential 14-3-3 binding sites in target proteins.

Several studies of protein interactions involving FT/Hd3a orthologs have been published. In *Arabidopsis*, FT interacts with FD and 14-3-3 proteins [12, 13]. There are several SP interacting proteins in tomato, including a 14-3-3 family member, protein kinase and bZIP transcription factor [10]. SFT, another tomato ortholog of FT/Hd3a, also interacts with 14-3-3 as well as bZIP [11]. In rice, GF14c (G-box factor 14-3-3c protein) has been identified as a partner of Hd3a to activate particular gene in certain pathway. Since the 14-3-3 family members known to interact with various protein, the role of GF14c in Hd3a signaling is remarkable to elucidate. Functional analysis using knockout and overexpression approach were performed and the result indicate that *GF14c* is a negative regulator by interacting with *Hd3a* [33]. No diurnal changes or developmental patterns of *GF14c* expression were observed, indicating that *GF14c* is expressed independently of the photoperiod and abundantly throughout plant development.

4.1. Subcellular localization of Hd3a and GF14c

To identify the intracellular localization of Hd3a and GF14c, a fusion construct to express mCherry fluorescent protein-linked Hd3a under the ubiquitin promoter, and GFP-linked GF14c driven by the CaMV 35S promoter were made. These constructs were introduced into rice protoplasts. In all of the rice protoplasts observed in this experiment, Hd3a-mCherry localized in both cytoplasm and nucleus; however, GF14c-GFP was predominantly visualized in the cytoplasm (75% of rice protoplasts observed) (**Figure 8**). The predominant cytoplasmic localization of 14-3-3 proteins has led to the hypothesis that they might act as cytoplasmic anchors that either block import into the nucleus or other organelles, or promote export from organelles into the cytoplasm [34].

4.2. *In vitro* and *in vivo* interaction of Hd3a and GF14c

The interaction between Hd3a and GF14c was confirmed using several methods, including a GST pull-down assay, yeast two-hybrid and a co-immunoprecipitation [33]. A GST-Hd3a fusion protein was pulled down with His-tagged GF14c, indicating that Hd3a interacted with GF14c *in vitro*. Results of this experiment were thus consistent with the results of the yeast two-hybrid experiment. An *in vivo* interaction was also demonstrated by a co-immunoprecipitation experiment using rice suspension culture cells overexpressing myc-tagged Hd3a.

4.3. Bimolecular fluorescence complementation (BiFC) of Hd3a and GF14c

To determine the distribution of Hd3a and GF14c *in vivo*, the BiFC technique was performed. The expression vector of Hd3a and GF14c fused to the N-terminal half of mVenus (Vn) and C-terminal half of mVenus (Vc), respectively. Those vectors were then transiently expressed on rice cell protoplasts. Plasmid mChery was used as a marker for transformed cells. The transformed rice protoplasts showed an interaction between Hd3a and GF14c as observed by strong green fluorescence that concentrated in the cytoplasm. The combination treatment of expression vectors using GUS to confirm the interaction cleary showed that the venus fluorescence only observed up to 10% of the transformed cells. This, indicated Hd3a only interact with GF14c mainly in cytoplasm [33].

4.4. Confirmation of Hd3a interacting protein GF14c by mutant generation

Several methods has been used to confirm the interaction between Hd3a and GF14c. All the methods used (yeast-two hybrid, in vitro pull-down, and BiFC assays) clearly showed that Hd3a and GF14c have strong interaction in rice. The subcellular distribution of Hd3a as well as GF14c demonstrated their localization in both the cytoplasm and nucleus.

GF14c acts as negative regulator of flowering and the overexpression of GF14c caused late flowering phenotype. Since the expression of Hd3a was observed both in the cytoplasm and nucleus, whereas GF14c is mainly in the cytoplasm, the possible interaction of these two protein is interesting to study. The mechanism of interaction of which GF14c would inhibit shuttling of Hd3a from the cytoplasm into the nucleus were confirmed by BiFC experiment. Based on data obtained it is clearly showed that Hd3a-GF14c is localized in cytoplasm. This indicate Hd3a-GF14c interaction lead to Hd3a cyoplasmic retention. The increasing of cytoplasmic retention of Hd3a can be explained as a model of late flowering by overexpression of GF14c. An example in *Arabidopsis*, the floral transition initiation in SAM, FT interacts with FD (bZIP transcription factor) that localized in the nucleus to induce the expression of target gene such as *AP1* [12]. In rice, this process could be attenuated by the cytoplasmic retention of Hd3a by GF14c. Several lines of evidence indicate a function for 14-3-3 proteins in nuclear-cytoplasmic shuttling in the signal transduction pathway [35, 36].

Another possible mechanism which can be explain the phenotype of GF14c overexpression and knockout mutant lines is the interaction between GF14c and Hd3a inhibit the movement of Hd3a from leaf to SAM. When GF14c expression level is low, Hd3a protein is capable to move along the floem to the SAM, since HD3a is small protein (about 20kDa) that is below of the size limit of plasmodesmata [37, 38]. The *gf14c* knockout mutant exhibited early flowering compare to WT. Remarkably, this mutant shown another phenotype such as dwarfism and increased tiller number. These pehotype suggesting that GF14c has another function independenlty with respect of flower induction at SAM.

According to Taoka [29] finding, 14-3-3 protein acts as intercellular receptor for Hd3a in the shoot apical cells. When Hd3a protein is transported to SAM, it will interact with 14-3-3 and form florigen activation complex (FAC). This FAC complex will be translocated into nucleus and binds to *OsFD1* (*FD* homolog of *A. thaliana*). The expression of *OsFD1* will lead to activation

of flowering identity genes, resulting in the flowering. This finding helps us to understand the flowering mechanism in rice and also offers new approaches to manipulate the plants flowering in future.

5. Hd3a interaction with BIP116b and OsKANADI

Analysis of Hd3a interacting proteins in yeast two-hybrid screening identified a novel protein homolog of BIP116 (brassinosteroid-insensitive 1 [BRI1] kinase domain interacting protein 116) and a novel putative transcription factor belonging to the KANADI domain protein family, namely OsKANADI1 (OsKAN1).

5.1. Hd3a interact with BIP116b

BRI1 has an extracellular domain (containing an N-terminal signal peptide, and LRR-RK and island domains), a transmembrane domain, a kinase domain and a C-terminal peptide [39]. Only a partial C-terminal region fragment was identified initially from yeast two-hybrid screening. An experiment using full-length Hd3a and full-length BIP116b, either as bait or as prey, further confirmed that Hd3a and BIP116b interact in the yeast system. To further study the interaction between Hd3a and BIP116b *in vitro*, a GST pull-down assay was performed. A GST-BIP116b fusion protein was pulled down with His-tagged Hd3a. However, an interaction could not be found between Hd3a and BIP116b, even though both proteins were expressed in *Escherichia coli*. One possible explanation for this discrepancy is that posttranslational modification of BIP116b may be necessary for binding.

The transgenic plant for either overexpression or RNAi mutant were generated. The results showed that the BIP116ab RNAi plants exhibited delayed flowering compared to wild-type plant, and the overexpressed plant showed branching and increasing tiller number similar to Hd3a overexpressed plants. Therefore, further characterization in mutant plants will be interesting to get an insight into its function.

5.2. Hd3a interact with OsKANADI1

In *Arabidopsis*, *KANADI* genes function in lateral polarity in organs including roots, leaves, and flowers. *KANADI* is required for abaxial identity in both leaves and carpels. It encodes a nuclear-localized protein in the GARP family of putative transcription factors [40]. GARP homologs constitute a large family of DNA-binding proteins in plants that may be needed for a variety of key cellular functions including regulation of transcription, phosphor transfer signaling, and differentiation. A GARP motif was also found in the identified *KANADI* product [41].

KANADI acts antagonistically to the *class III HZ-Zip* genes. *KANADI* genes are expressed in a pattern complementary to that of the *class III HD-Zip* genes in the shoot; *KANADI* expression occurs in the phloem and abaxial regions of lateral organs early in development [41–43]. While *KANADI* genes do not appear to be required for proper meristem function, they are needed for pattern formation of organs produced by the shoot apical and vascular meristems.

Sequence analysis showed that OsKAN1 consists of six exons. Phylogenetic and comparative genetic analysis indicated that OsKAN1 is an ortholog of AtKANs and is most closely related to AtKAN4. The predicted OsKAN1 polypeptide contains a potential phosphorylation site in the C-terminal region which may be important for interaction with other proteins involved in signaling. The overall identity between *KANADI* family members is low, but they are nearly identical within the GARP domain. The sequence similarity found in this subset of GARP genes may indicate that they have overlapping or partially redundant functions.

Suppression of OsKANADI1 by RNAi displayed branching and increasing tiller number in several lines [44]. This phenotype resembles to the Hd3a overexpressed plants indicating that they possibly function in similar pathway. Further characterization using the OsKANADI mutants plants in the next generation remains to be investigated.

6. Hd3a homolog in plant development

The role of Hd3a as flowering mobile signal in rice and *Arabidopsis* leads to investigation of its function in another plants. The diverse role of FT was identified in many plants. FT protein involved in vernalization, stomatal opening, dormancy and tuberization [45–48]. Navarro et al. [49] provided a new insight in understanding the florigen as graft-transmissible signal and designated its role as tuberi. Hd3a homolog were overexpressed in potato under noninductive long-day condition and resulting in tuberization and flower development. A grafting experiment using tobacco florigen as scion and potato as rootstock to test the hypothesis confirmed that FT proteins were transmitted from scion to rootstock and induced tuberization. This experiment suggests that FT/Hd3a is a strong tuberization inducer.

The flowering and tuberization pathway are regulated in different pathways, indicating that FT has many homolog in potato. This hypothesis was confirmed by Navarro et al. [49]. In potato, there are three homolog of FT. StSP3A regulate the tuber formation, StSP3D regulate the flowering, and StSP5G as the repressor of tuberization. Expression of *StSP6A* gene is strong in leaves and stolon of short-day induced plants. Interestingly, 30-minute night break was reported to inhibit tuberization. The *StSP3D* expression is necessary for flowering induction. However, this gene is not required for normal tuberization. Interestingly, the StSP5G is an antagonist for StSP6A. The expression of StSP5G was high in long-day condition [47]. The role of Hd3a/FT protein in potato flowering and tuberization is well understood. This photoperiodic pathway is conserved in potato, rice, and *Arabidopsis*.

Recent study in onion tuberization reveals that its induction is controlled by Hd3a/FT homolog in the photoperiodic pathway. Onion is a biennial plant that forms tuber during the summer and flower after the winter in the next summer. There are many Hd3a/FT homologs found in onion and the pattern is the same with potato. There are Hd3a/FT homologs that regulate the tuber/bulb formation (AcFT1 and AcFT4) and flowering (AcFT2) [50]. As seen in potato tuberization, AcFT4 in onion has the same role as StSP5G in potato that inhibits onion tuber formation; while AcFT1 is a strong inducer for tuberization (tuberigen) under long-day condition. This finding suggests that Hd3a/FT has a versatile role in plant development.

Tuberigen and florigen are known to be mobile/graft-transmissible signals. Several experiments showed that this signal is interchangeable between species in Solanaceae family (potato/tobacco, and potato/tomato) and can induce tuberization of potato and onion, but cannot in other plant species. As example, the ability to form a tuber is limited in potato species among Solanaceae family. Due to those fact, there must be an unknown mechanism present in the tuberization process in potato. The underlying mechanism that involves FT roles needs further investigation.

Another Hd3a role in rice as mobile signal that induced branching was reported by Tsuji et al. [18]. Their findings suggest that Hd3a was moved along the xylem to promote branching in rice lateral meristem. The formation of florigen activation complex (FAC) for the developmental process other than flowering is not well understood. The experiment proved that Hd3a protein was transported to lateral meristem in the lateral bud and its transport is required for branching. This finding suggests that Hd3a has the function besides flowering.

Our work in local black rice namely "Cempo Ireng" which has very long harvest period also reveal that Hd3a gene is conserved. The regulation of flowering pathway of rice in short- and long-day condition is well understood. However, when rice are planted in neutral day condition such as tropical condition, the photoperiodic pathway might be affected. Our result suggests that Hd3a, which normally regulate flowering under short-day condition, is expressed in neutral day condition earlier compared to its homolog RFT1, which is responsible for flowering induction in long-day condition. In "Cempo Ireng," two flowering genes are expressed. The expression pattern of both genes are redundant to induce the flowering under neutral day condition. RFT1 and Hd3a are known to have different induction pathways. Our result suggests that RFT1 and Hd3a may have independent pathway in rice flowering regulation under neutral day condition according to their expression pattern (data not shown). The Hd3a/RFT1 interactor of black rice that involved in flowering induction is of interest for future study to understand the flowering mechanism under neutral day condition and shortening the harvest period of black rice "Cempo Ireng."

7. Conclusion and future prospects

As the floral stimulus that controls floral transition in the SAM, Hd3a has the capacity to traffic from cell to cell and move long distances via the phloem. To reveal its function, Hd3a recruits different proteins in plant developmental stage. A wide range of proteins that interact with Hd3a were identified using yeast two-hybrid screening. GF14c (a 14-3-3 homolog), OsKANADI (a novel transcription factor containing a GARP domain), and the BRI1 kinase domain-interacting protein 116b (BIP116b) are the Hd3a interactors of interest. It is possible that Hd3a and its partner(s) may form a platform for cross-talk between signal transduction pathways. Another homolog of Hd3a in many plants was identified and it was suggested that Hd3a/FT has versatile role in plant development. This role depends on its partner and interaction to gain its function. Further study using reverse genetics to obtain mutants, either gain-of-function mutants or suppression mutants by RNA interference of Hd3a partners to get insight into the function in plant growth and development, particularly during floral transition is important. Our understanding in floral transition in rice would make for better crop management in future and represent an important addition to our knowledge about FT signaling in plants.

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