Identification and characterization of Hd3a interacting proteins in rice (*Oryza sativa* L.)

Yekti Asih Purwestri

Graduate School of Biological Sciences
Nara Institute of Science and Technology, JAPAN
January 26, 2009

A Doctoral Dissertation
submitted to Graduate School of Biological Science
Nara Institute of Science and Technology
in partial fulfillment of the requirements for the degree of
Doctor of SCIENCE

Yekti Asih Purwestri

Thesis Committee:

Prof. Ko Shimamoto	(Supervisor)
Prof. Masao Tasaka	(Member)
Prof. Takashi Hashimoto	(Member)
Prof. Seiji Takayama	(Member)

バイオサイエンス研究科　博士論文要旨

所属 （主指導教員）	Laboratory of Plant Molecular Genetics (Ko Shimamoto)		
氏名	Yekti Asih Purwestri	提出	平成 2008 年 12 月 22 日
題目	Identification and characterization of Hd3a interacting proteins in rice		

要旨

Most recently, the nature of Hd3a and its ortholog as a florigen, a mobile flowering signal, has been proposed. The next question which should be addressed is the mechanism of Hd3a function. One important step in the characterization of Hd3a function is to identify other proteins with which it interacts. We focus here on searching for Hd3a interacting proteins using yeast two-hybrid screening, which has been widely used to identify protein-protein interactions. In this study we have performed a yeast two-hybrid screen to search for Hd3a partners in a cDNA library made from rice leaf blades harvested 35-40 days after sowing under SD conditions, a period when the transition from vegetative phase to reproductive phase was occurring and *Hd3a* was being highly expressed.

Several studies of protein interactions involving FT/Hd3a homolog have been published. In *Arabidopsis*, FT interacts with FD and 14-3-3 proteins. A study in tomato revealed several SP interacting proteins including a 14-3-3 family member, protein kinase and bZIP transcription factor. SFT, another tomato ortholog of FT/Hd3a, also interacts with 14-3-3 as well as bZIP. However, no Hd3a interacting proteins have yet been identified in rice.

In this study, we identified diverse proteins which mainly involved in signaling. We focused on three candidate proteins: GF14c (14-3-3 isoform protein), OsKANADI (transcription factor) and BIP116b (BRI1 kinase domain interacting protein 116b). Since 14-3-3 family members in plant interact with diverse proteins, it was of interest to understand their role in Hd3a signaling. *In vitro* and *in vivo* experiments, using a combination of pull-down assay, co-immunoprecipitation and bimolecular fluorescence complementation, confirmed the interaction between Hd3a and GF14c.

Functional analysis using either GF14c overexpression or knockout transgenic rice plants indicated that this interaction plays a role in flower regulation. GF14c-overexpressing plants exhibited a delay in flowering and, surprisingly, the knockout mutants displayed early flowering that was comparable to wild-type plants under short-day conditions. These results suggest that GF14c acts as a negative regulator of flowering by interacting with Hd3a. Since the 14-3-3 protein has been shown to interact with FT protein in tomato and Arabidopsis, our results in rice provide important findings about FT signaling in plants. The functional characterization of other Hd3a interactors should be a priority for future research.

Achievement

Publication:

Purwestri, Y.A., Ogaki, Y., Tamaki, S., Tsuji, H. and Shimamoto, K. (2009) The 14-3-3 protein GF14c acts as a negative regulator of flowering in rice by interacting with the florigen Hd3a . Plant Cell Physiol. (in press).

履　　歴　　書

本籍 INDONESIAN

現住所（〒　6310033）1-3-19　Ayameike Minami, Nara

ふりがな

氏　名 Yekti Asih Purwestri

生年月日　昭和 1971 年 05　月　23 日生

学　　歴

1977-1983 Elementary School (SD Kristen 2 Salatiga)

1983-1986 Junior High School (SMP Negeri 1 Salatiga)

1986-1989 High School (SMA Negeri 1 Salatiga)

1989-1996 Undergraduate (Faculty of Biology Gadjah Mada University, Yogyakarta, Indonesia)

2000-2003 Master Course (Biotechnology, Gadjah Mada University, Yogyakarta, Indonesia)

研　究　歴

-

職　　歴

1996 - present : Assistant Professor (lecturer) Faculty of Biology Gadjah Mada University, Yogyakarta Indonesia

賞　　罰

-

上記のとおり相違ありません。

平成 2009　年　01 月 22　日

氏　名　Yekti Asih Purwestri

CONTENTS

List of Figures

List of Supplemental Figures

List of Tables

Appendix

Acknowledgements

Thanks God for everything you provide for me.

I would like to express my gratitude to Prof. Ko Shimamoto for his excellent supervision, guidance and advice during my study.
I would also like to show my great application to Prof. Masao Tasaka, Prof. Takashi Hashimoto, Prof. Seiji Takayama (my committee members) for their time and attention.
I wish to thank Dr. Shuji Yokoi who teach me some technical works in molecular biology and Dr. Hiroyuki Tsuji for his valuable discussion during this project.
I wish to express my gratitude also to Dr. Hann Wong Ling, Dr. Tsutomu Kawasaki, Dr. Yoji Kawano, Dr. Letian Chen, Dr. Sylvie Meissouneve for their valuable discussions and encouragement during my PhD course.
I wish to express my sincere appreciation to Dr. Ian Smith for his efforts in assisting me on scientific writing.

Thanks to all of Plant Molecular Genetics lab members for their valuable discussion, expecially flowering team members for sharing the data. Thanks to Ms Yuka Ogaki for allowing me to site her data in this theses. I also wish to thank to my friends, Ebru, Jane, Susan, Onuma, Meiow for sharing my happiness and sadness on this journey, and all of my Indonesian friends for their friendship and sharing experiences to enjoy life beside science in Japan.

My special thank to Ms. Sachiko Iida who support me by providing the Iida Scholarship during my PhD course. Her encouragement, attention and treated me as a daughter are really appreciated.

I wish to thank Prof. Sukarti Moeljopawiro and all Biochemistry lab's staff, Fac of Biology, Gadjah Mada University for their support throughout my study.

Finally, I am very grateful to my beloved husband, Indrianto Adiatmo and my lovely daughter, In Kristi Tikam Tirani for their loves, understanding, support and patient throughout my study. My special thanks to all of my family in Indonesia for their support and pray.

Abbreviations

3-AT; 3-aminotriazole
BiFC; bimolecular fluorescence complementation
GST; glutathione S-transferase
HIS; histidine
MBP; maltose binding protein
LD; long day
QTL; quantitative trait loci
SAM; shoot apical meristem
SD; short day
ZT; zeitgeber time

Proteins:
BIP116b; BRI1 kinase domain interacting protein 116b
CO; CONSTANS
FT; FLOWERING LOCUS T
GF14c; G box factor 14-3-3 c
GI; GIGANTEA
Hd1; Heading date 1
Hd3a; Heading date 3a
OsGI; *Oryza sativa* GIGANTEA
OsKAN1; *Oryza sativa* KANADI1

CHAPTER 1
Introduction

1.1 Regulation of flowering

Flowering is critical for growth and reproduction in plants and is controlled by both environmental and endogenous conditions. One of the most important factors that control flowering is the plant's response to daylight or photoperiod (Imaizumi and Kay, 2006). In addition to the photoperiodic pathway, the regulation of flowering time involves complex signaling pathways, such as the vernalization, autonomous flowering and gibberellins pathways. Photoperiod is a measure of the season, and most plants time their reproductive development to the appropriate season. Most annuals flower in late summer and fall, when daylight becomes short, and are called short-day (SD) plants. Biennials or perennials have sufficient vegetative growth in spring, and flower in spring or early summer to take advantage of the resourceful summer for reproductive development. These are called long-day (LD) plants. Some plants flower at a certain developmental stage regardless of the photoperiod, and are called autonomous-flowering or day-neutral plants.

1.1.1 Flower regulation in rice, a model short-day plant

In rice (*Oryza sativa* L.), flowering is mainly induced by photoperiod. In the photoperiod pathway, three conserved genes in rice and *Arabidopsis* are responsible for this process. *OsGI* (*GIGANTEA* in rice) is a clock-regulated gene, which was first identified by the differential display method (Hayama et al. 2002), and its expression is high in the middle of day (Hayama et al. 2003). Under inductive SD conditions, *OsGI* promotes the expression of *Hd1* (*Heading date 1*), and *Hd1* activates *Hd3a* expression

(Hayama et al. 2003). However, under non-inductive LD conditions, *Hd1* suppresses *Hd3a* expression (Hayama et al. 2003). *OsGI* acts as the primary upstream regulator of *Hd1* expression (Hayama et al. 2002). OsGI is a large protein that is present in both the nucleus and cytoplasm of rice cells (Abe et al. 2008). Supression of *OsGI* by RNAi or antisense expression caused late flowering and reduced *Hd1* transcription under SD conditions (Hayama et al. 2003).

In QTL analysis using the japonica cultivar Nipponbare and the indica cultivar Kasalath, *Hd1* (Yano et al. 2000) and *Hd3a* (Kojima et al. 2002) were identified and cloned by a map-based cloning strategy. *Hd1* was found to be a homolog of *Arabidopsis CO*. *Hd3a* is the primary target of Hd1, a B-box zinc finger CCT protein that plays a central role in photoperiodicity. As a short-day plant, flowering time in rice is triggered when the plant perceives light shorter than a critical day length. Under SD conditions, *Hd1* activates *Hd3a* in vascular tissue (phloem). The Hd3a protein then moves through the phloem and enters the shoot apical meristem to promote the transition from vegetative phase to reproductive phase. In *Arabidopsis*, FT, an ortholog of Hd3a, interacts with FD (Flowering locus D), a shoot apical meristem-specific gene, to activate expression of the MADS box meristem identity gene *APETALA1* (*AP1)* (Abe et al. 2005; Corbesier et al. 2007; Wigge et al. 2005). There is no evidence to date for an ortholog of FD in rice. Further attempts to identify an ortholog of FD, and its interaction with Hd3a in the shoot apical meristem to activate floral identity genes such as *OsMADS14*/ *OsMADS15*, two rice orthologs of *Arabidopsis AP1*, will be important to yield a more complete understanding of floral induction in rice (Tsuji et al. 2008). Overexpression of Hd3a under a phloem-specific promoter and the native promoter induces an early flowering phenotype, suggesting that Hd3a protein acts as a floral

promoter (Tamaki et al. 2007). On the other hand, the suppression of *Hd3a* by RNA interference (RNAi) results in a delay in flowering. Moreover, recent study in suppression of both *Hd3a* and *RICE FLOWERING LOCUS T1* (*RFT/FTL1)* by RNAi abolished the flowering phenotype (>300 days) under inductive conditions (Komiya et al. 2008). In the absence of *Hd3a* and *RFT1* expression, transcription of *OsMADS14* and *OsMADS15* was strongly reduced, suggesting that they act downstream of *Hd3a* and *RFT1*. This result indicates that Hd3a and RFT1 act as floral activators under SD conditions (Komiya et al. 2008).

Several unique genes in rice were isolated. *Ehd1* (*Early heading date1*) encodes a B-type response regulator and is a unique flowering time gene (Doi et al. 2004). *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 (Doi et al. 2004). More recently, *Ghd7* (for grain number, plant height, and heading date 7), which encodes a *CCT* (*CO*, *CO-LIKE*, and *TIMING OF CAB1*)-domain protein, was isolated from natural variants in rice (Xue et al. 2008). *Ghd7* affects levels of *Ehd1* and *Hd3a* transcripts, but does not affect *Hd1* mRNA levels. *Ghd7* represses *Ehd1* and *Hd3a* expression under LD conditions, leading to delayed flowering. Therefore, two independent floral pathways are present in rice: the conserved *Hd1* pathway and a unique *Ehd1* pathway that may integrate environmental photoperiod signals into the expression of *FT-like* genes (Izawa, 2007). 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 *ID1* (*INDETERMINATE1*), 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* (Matsubara et al. 2008, Park et al. 2008, Wu et al. 2008a).

Hd3a expression under SD conditions is also regulated by phytochrome. In the *se5* mutant, which lacks a functional gene encoding heme oxygenase (Izawa et al. 2000), an enzyme that is required for loss-of-function alleles in one of the three rice phytochromes (Takano et al. 2005), 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. The development stage of the young panicle is also related to the timing of leaf emergence. The differentiation stage of the young panicle almost 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 (Hoshikawa 1989). The flag leaf, contributes largely to the filling of grains because it supplies photosynthetic products mainly to the panicle (Hoshikawa, 1989).

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.

In rice cultivar Norin 8 (N8), the main rice background used in our experiments, the transition period from vegetative phase to reproductive phase occurs approximately 35-40 days after sowing (das) when grown under SD conditions, and the flowering time or heading date is around 60-65 das.

1.1.2 Flower regulation in *Arabidopsis*, a model long-day plant

Arabidopsis is a facultative long day plant that flower earlier under long days of 16 h of light than under short days of 8-10 h of light. The flowering time is controlled by several pathways, which integrate environmental signals with the developmental status of a plant (Boss et al. 2004; Komeda et al. 2004). There are four pathways corresponding to the signals for the initiation of flowering in *Arabidopsis*: the gibberellin (GA), autonomous, vernalization and photoperiod pathways. These multiple floral promoting signals regulate the expression of a common set of genes that converge in floral pathway integrator genes: *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*, also known as *AGL20*) and *LEAFY* (*LFY*) (Kardailsky et al. 1999, Kobayashi et al. 1999, Samach et al. 2000). Floral-pathway integrators activate floral-meristem identity genes, and these trigger the transition from the vegetative to the reproductive phase (Boss et al. 2004, Komeda 2004).

In the photoperiod-dependent pathway, *GIGANTEA* (*GI*), *CONSTANS* (*CO*) and *FT* are the key elements that mediate the effect of day length on flowering. The

photoperiod-pathway mutants *co* and *gi* exhibit a strong late-flowering phenotype under LD conditions (Putterill et al. 1995, Koornneef et al. 1998).

CO is expressed in leaves, yet can activate the expression of two genes, *LFY* and *APETALA1* (*AP1*), expressed in the reproductive meristem, that directly control the initiation of flower development. Therefore, *CO* could regulate the synthesis of a single molecule that might fit the definition of florigen. Alternatively, *CO* could control the levels of several molecules which together regulate the flowering transition.

CO activity is regulated at the transcriptional level. *CO* is an important activator of *FT* and is expressed in leaf phloem tissue (Takada et al. 2003). CO protein seems to directly regulate *FT* and its paralog *TWIN SISTER OF FT* (*TSF*), because both FT and TSF are up-regulated by dexamethasone-induced activation of CO in the presence of the translation inhibitor cycloheximide (Samach et al. 2000, Yamaguchi et al. 2005). The activity of CO is also regulated by protein stability. CO protein is destabilized in darkness by CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1), which is counteracted by the blue-light photoreceptor CRYPTOCHROME. A PhyB-mediated signal also destabilizes CO at dawn (Jang et al. 2008, Laubinger et al. 2006, Liu et al. 2008).

The vernalization pathway represents the promoting activity of prolonged cold treatment, as it naturally occurs in winter. In the vernalization pathway, the MADS-box transcriptional regulator *FLOWERING LOCUS C* (*FLC*) represses expression of *FT* in the leaf and of *FD* and *SOC1* in the shoot apex (Searle et al. 2006). Vernalization downregulates *FLC*, thus allowing *FT* to be expressed in the leaves and increasing the competence of the shoot apex to respond to *FT*. Overexpression of *FT* in non-vernalized

Arabidopsis promotes early flowering without affecting *FLC* mRNA levels, indicating that *FT* acts downstream of *FLC*.

As a quantitative LD plant, in *Arabidopsis* the transition to flowering under SD conditions is predominantly mediated by the GA-dependent pathway. Gibberellin byosnthesis and signaling mutants show delayed floral transition under SD (Wilson et al. 1992, Jacobsen and Olszewski 1993). Applied GA in SD induces expression of *SOC1* and the floral meristem identity gene *LFY*, but has no effect on *FT* expression. Constitutive expression of *FT* in the GA-deficient *ga1-3* mutant in SD induces very early flowering. These results suggest that GA and FT act independently on flowering in *Arabidopsis*. Observations in Lolium and *Arabidopsis* suggest that GA and FT function redundantly; they are both leaf-produced mobile signals that induce flowering (King et al. 2006). However the application of GA in many species fails to induce flowering, so the effect is not general.

The autonomous pathway was identified from the behavior of mutants that display photoperiod-independent late flowering and strongly accelerated flowering in response to prolonged exposure to cold (Koornneef et al. 1991).

1.1.3 Flower regulation in other plants

The role of *FT* homologs in other plants including tomato, wheat and maize seems to be similar to that of *Hd3a* and *FT* in rice and *Arabidopsis*, respectively. In cereals, which are more closely related to rice, overexpression of *TaFT* (*Triticum aesticum* L. *FT*) in transgenic wheat plants accelerates flowering relative to wild type plants, suggesting a conserved role as a flowering promoter (Yan et al. 2006). *TaFT* and *HvFT* (*Hordeum vulgare* L. *FT*) are upregulated by long days, and increased transcript level correlate

with accelerated flowering time (Turner et al. 2005, Yan et al. 2006). Expression of the *FT* target gene *AP1* in *Arabidopsis* is not detected in the leaves and is very low in the vascular tissues of cotyledons (Abe et al. 2005). Conversely, in wheat and barley, *AP1* homolog *VRN1*, are expressed in the leaves at high level. In addition, in rice the target of *Hd3a*, *OsMADS14*, is expressed at low level in the leaves.

Li and Dubcovsky (2008) showed evidence that *TaFT2* is regulated by *TaFT* and that the products of these genes interact with different *TaFDL* partners. Only one of the *TaFDL* proteins that interact with *TaFT* was able to interact with the *VRN1* promoter.

1.2 Hd3a as a mobile flowering signal in rice

Florigen represents a signal that triggers flowering, and was thought to be a specific hormone. Early grafting experiments showed that photoperiod is sensed by leaves, yet affects flowering and the SAM. Therefore, a signal must be produced in the leaves and transported to the SAM. More recently, others have proposed that substances are transmitted from leaves to the SAM and together trigger flowering (Imaizumi and Kay 2006, Zeevart 2006).

In plants which show a light-responsive pattern of floral induction, the perception of light by phytochrome occurs in the leaves rather than the apical meristem. The plants respond to inductive photoperiod and form florigen (hormone) as a floral stimulus in the leaves which is translocated through the phloem to the apical meristem.

According to Chailakhyan's theory, flowering occurs under the influence of a hypothetical hormonal stimulus (florigen). The postulates of Chailakyan's theory are:

1. The stimulus of flowering (florigen) is synthesized in leaves under the influence of certain external and internal factors (photoperiod, temperature, plant age, etc.).
2. Floral stimulus is a long-distance signal that moves along leaf cells and the vascular system of leaves and stem and exerts its action in apical and axilary buds located rather far from the leaf.

   The rate of signal movement along the phloem was found to be 2.4 – 3.5 mm/hour in various plant species.
3. The formation of flowers requires the accumulation of a certain critical (threshold) amount of floral stimulus.

If the floral stimulus level is insufficient, which may arise from an incomplete number of inductive photoperiodic cycles, flower formation does not begin or, during the initial stages of flowering, a so-called reversion (return to vegetative growth) may occur.

The function of Hd3a as a florigen fulfils the above criteria for a florigen. Results of the overexpression of Hd3a under the vascular tissue-specific promoter (*rolC*) in *Chrysanthemum* demonstrated induction of flowering in LD conditions, under which the wild type plant never flowers, indicating the universality of Hd3a as a florigen (H. Asao et al., unpublished data). Under inductive conditions for flowering, since *Hd3a* mRNA accumulates in the leaf blades, with very little in the leaf sheath, and levels remain very low in shoot apex, it is considered unlikely that *Hd3a* mRNA moves from the leaves to the shoot apex (Tamaki et al. 2007). Moreover, activity of the *GUS* transgene driven by the *Hd3a* promoter was detected in the vascular bundles, but not in the shoot apex.

Several reports provide no evidence that *FT* mRNA functions as the phloem-mobile signal for floral induction. Huang et al. (2005) initially reported that a single heated leaf of a transgenic plant expressing FT under the control of a heat shock promoter induced flowering in the *Arabidopsis ft-7* mutant in SD, and that FT mRNA was detected in the shoot apex by RT-PCR. However, this work was later retracted (Böhlenius et al. 2007). In grafting experiments with tomato overexpressing SINGLE-FLOWER TRUSS (SFT, ortholog of FT in tomato), the floral stimulus did cross the graft union, but *SFT* mRNA could not be detected in the flowering receptor shoots (Lifschitz et al. 2006). In contrast, several studies detected the Hd3a/FT proteins and their family members in phloem sap (Aki et al. 2008, Lin et al. 2007, Giavalisco et al. 2006). Evidence in *Arabidopsis* also supports the florigen activity of FT protein. Phloem-expressed FT-GFP and Myc-tagged FT move to the apex and cause early flowering, but FT protein with a nuclear localization signal or 3xYFP does not, probably because of immobilization (Corbesier et al. 2007, Jaeger et al 2007, Mathieu et al. 2007)

Future questions after the identification of FT as a mobile flower-inducing signal concern the mechanisms of its production, transport and action. FT is a small protein of 20 kDa, which is below the selective exclusion limit of 67 kDa for companion cell plasmodesmata, so it should easily diffuse into the phloem stream. Beside a role in floral induction, FT can also effect vegetative growth independently of flowering. Rice provides the evidence that Hd3a, an ortholog of FT, moves along the vasculature through the phloem into the shoot apical meristem.

FT and its orthologs share high amino acid similarity. They also share sequence similarity with the mammalian phospatidylethanolamine-binding protein (PEBP) or RAF1 kinase inhibitor protein (RKIP).

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) (Fig. 1-1).

Studies of RKIP family members in diverse organisms show that some of them have biochemical function as inhibitors, binding signaling component to modulate the flux through their pathway. Hanzawa et al. (2005) demonstrated that a single amino acid was critical in FT and TFL1 protein for converting a repressor to an activator of flowering. By a swapping experiment they revealed that His and Tyr residues are important for the function of FT and TFL family member proteins, respectively, as an activator or repressor of flowering.

Based on current data, the regulation of FT production and mobility may vary among species, but the end product, FT, is always the same, strongly suggesting FT protein as the universal florigen. However, the question remains whether FT is the only component of florigen, or whether another component(s) associated with FT in the phloem is also essential for flowering (Zeevart 2008). FT protein in the phloem stream may require a chaperone-like protein for protection against proteolysis and for unloading at the shoot apex (Lough and Lucas, 2006). If a protein were identified that was invariably associated with FT in the phloem and that was also essential for flowering (a mutation in the gene encoding this hypothetical protein would result in a late-flowering phenotype), it would have to be considered a component of florigen (Zeevart 2008).

1.3 FT-interacting proteins

In *Arabidopsis*, FT is the primary target of CONSTANS (CO), a B-box zinc finger CCT protein that plays a central role in the photoperiod pathway (Putterill et al. 1995, Robson et al. 2001, Wigge et al. 2005). Under LD condition, CO activates FT in the leaf vascular tissue (phloem) (An et al. 2004). The FT protein then moves through the phloem to the SAM, where it interacts with the bZIP transcription factor FD to activate expression of the MADS-box meristem identity gene *APETALA1* (*AP1*) (Abe et al. 2005, Corbesier et al. 2007, Wigge et al. 2005). The FT-FD protein interaction is regulated spatially by the preferential expression of FD in the SAM, and temporally by the integration of various environmental signals that converge to regulate FT (Wigge et al. 2005). According to this model, FD provides specificity in recognition of the DNA target, and FT acts in concert with FD to transcriptionally activate *AP1* (Wigge et al. 2005). Under inductive photoperiods, the FT protein is expressed in the phloem and travels to the shoot apex where it interacts with FD (Corbesier et al. 2007, Jaeger and Wigge 2007, Mathieu et al. 2007, Tamaki et al. 2007, Notoguchi et al. 2008).

A study of the CETS protein family (CEN/CENTRORADIALIS, TFL/TERMINAL FLOWER1, and SP/SELF PRUNING) in *Antirrhinum*, *Arabidopsis* and tomato, respectively, supports the view that to regulate shoot architecture, CETS proteins are recruited to function as a hub of signaling systems that have the inherent flexibility and potential to integrate a wide variety of developmental cues. RKIP interacts with two members of the MAPKKK family, NIK and TAK1, to modulate the response of NF-κB pathway to TNF-α and other signals (Yeung et al. 2001). The study of SP interactions in tomato, by yeast two-hybrid screening using a cDNA library made from apices containing the second and third shoot segments, revealed that SP interacts

with a plant kinase of the NIMA class, adapter factors of the 14-3-3 family, a transcription factor of the G-box binding family, and a novel protein, SIP4. The authors conclude that the CETS genes encode a new family of modulator/adapter proteins analogous to those of the 14-3-3 families.

Several studies of protein interactions involving FT/Hd3a orthologs have been published. In *Arabidopsis,* FT interacts with FD and 14-3-3 proteins (Abe et al. 2005, Wigge et al. 2005). A study in tomato revealed several SP interacting proteins including a 14-3-3 family member, protein kinase and bZIP transcription factor (Pnueli et al. 2001). SFT, another tomato ortholog of FT/Hd3a, also interacts with 14-3-3 as well as bZIP (Lifschitz et al. 2006). However, no Hd3a interacting proteins have yet been identified in rice.

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, because overexpression of Hd3a/FT produces phenotypes such as branching, stem elongation, dwarfs, leaf shape alteration, early release of lateral shoots, and induced dormancy (Endo et al. 2005, Teper-Bamnolker and Samach 2005, Lifschitz et al. 2006, Bohlenius et al. 2006, Tsuji et al. unpublished data). The diverse functions of Hd3a need to be explored to find activators or repressors that may be necessary for its function. As a florigen, Hd3a may function to inactivate the floral repressor and activate floral meristem identity genes during the transition from vegetative to reproductive phase. This function will be achieved by interacting with its partners.

We hypothesize that Hd3a recruits different protein partners to perform its roles in plant growth and development, particularly during floral transition.

1.4 Objectives of this study

To investigate the Hd3a protein function, we searched for Hd3a interacting partners.

1.4.1 Identification of Hd3a interacting proteins using yeast two-hybrid screening

The yeast two-hybrid method was used as a tool for searching potential Hd3a partners.

1.4.2 Characterization of Hd3a and its partners (GF14c, BIP116b and OsKANADI)

We focused on three Hd3a interacting proteins. These are GF14c (a 14-3-3 protein family member), BIP116b (BRI1 kinase domain interacting protein 116b) and OsKANADI1 (a transcription factor). 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 BiFC (bimolecular fluorescent complementation). The expression pattern and subcellular localization of each Hd3a interacting partner provided important insights into its function.

1.4.3 Functional characterization of Hd3a interacting partners using mutant (overexpressing, knockdown and/or knockout) plants

To further characterize the function of Hd3a interacting proteins in plant growth 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 knock out mutants.

CHAPTER 2

Materials and Methods

2.1 Plant materials and growth conditions

Japonica rice cultivars 'Norin 8' (N8) and 'Dongjin' were used as wild-type plants. The transgenic overexpressing plants (35S::GF14c, 35::OsKANADI1 and 35S::BIP116b) and RNAi plants were generated by *Agrobacterium*-mediated transformation in an N8 background. T-DNA insertion alleles of *GF14c* in a 'Dongjin' background (IB-10131) were obtained from Dr. Gynheung An, POSTECH, Korea. To confirm insertion sites, the sequences flanking the right border of T-DNA were determined using primers designed from sequence information provided in the POSTECH database. To determine flowering-time phenotypes, all plants were grown in climate chambers with 24-h temperature cycles (10h, 30°C during subjective day; 14h, 25°C during subjective night). The humidity was 70%. The fluence of light was ~300 $\mu mol^{-2}s^{-1}$ (400-750 nm) under SD conditions.

2.2 cDNA library

Poly $(A)^+$ RNA was isolated from wild-type N8 rice leaf blades, harvested at 35-40 days after sowing at ZT 0, 2, 4 with an RNA Easy Prep kit (Takara) and used to construct a cDNA library with a cDNA synthesis kit (Stratagene). The resultant cDNAs were inserted into the pVP16 vector.

2.3 Hd3a bait construct

The pBTM116ss vector was used to obtain Hd3a bait construct, using the *Sal*1 and *Spe*1 restriction sites. The *Hd3a* open reading frame (ORF) was amplified using forward primer 5'gtcgacATGGCCGGAAGT3' and 5'CTAGactagtACCTAGGGGT3' as reverse primer (lower case letters indicate the restriction enzyme recognition sites) and PrimeStar polymerase (Takara), followed by Zero Blunt TOPO cloning (Invitrogen) in *Escherichia coli*. The construct was checked for correct DNA sequence by performing appropriate sequence reactions. The resulting plasmid was digested with *Sal*1 and *Spe*1 and subcloned in pBTM116ss to produce an Hd3a bait construct.

2.4 Yeast transformation and protein expression from yeast

The Hd3a bait construct was used to transform yeast strain L40. Yeast transformations were performed using Frozen-EZ Yeast TransformationII^{TM} (Zymo Research) according to the supplier's instruction manual. Yeast cultures containing the constructs of Hd3a were grown in 50 ml in selective medium, without the amino acids leucine (L), tryptophan (W) and histidine (SC-His), overnight at 30°C until OD_{600} = 0.4-0.6. Cells were collected by centrifugation at 1,000*g* (4°C for 5 min), and the pellet was resuspended in 100 µl pre-heated extraction buffer (8M urea, 5% sodium dodecyl sulfate (SDS), 40 mM Tris-HCl pH 6.8, 0.1 mM EDTA, 0.4 mg/ml Bromophenol Blue). The cell suspension was transferred to a 1.5-ml tube containing 80 µl of glass beads, incubated at 70°C for 10 min and then mixed by vortex for 1 min. The suspension was centrifuged at 14,000 rpm for 5 min at RT. The supernatant was transferred to a new tube and stored at -40°C or subsequently used for western blot analysis.

For western blot analysis, equal amounts of protein extracts were separated by 10% SDS-PAGE and electrotransferred for 1 h onto an Immobilon-P membrane (Millipore Corporation) in transfer buffer (25 mM Tris 192 mM glycine, 10% MeOH, 0.1% SDS). The membrane was blocked for 1 h in TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Triton X-100) containing 5% skim milk powder, washed three times with TBST, incubated for 1 h with anti-LexA (diluted 1:2500) antibody (Santa Cruz) in TBST containing 0.5% skim milk, and washed five times with TBST. The membrane was then incubated for 1 h in TBST containing 0.5% skim milk with anti-mouse IgG conjugated to horseradish peroxidase (diluted 1:10,000; GE Healthcare) and then washed as described above. Detection was performed using ECL-plus western blotting detection reagents (GE Healthcare) according to the manufacturer's instructions and visualized using an LAS-1000 Imager (Fujifilm).

2.5 Yeast two-hybrid screening

Independent clones (1.6×10^6) were screened for interaction with Hd3a. Double transformants were spotted on SC+His, SC-His and SC-His containing 2.5 mM 3-aminotriazole (3-AT). Clones that showed the activation of *His3* (histidine synthase), and were therefore able to grow in SC media without His and containing 3-AT, were selected, and DNA was isolated according to Zymo Research's protocol. To retrieve positive interactors, the DNA isolated from yeast was electroporated into *E. coli*, and DNA minipreps were performed (Invitrogen). The resulting plasmids were retransformed into yeast strain L40 and the plasmid DNA was isolated from yeast. The DNA was amplified by PCR using forward and reverse primers 5'AGTTTGAGCAGATGTTTACC3' and 5'CGACGTTGTAAAACGACGGCCAGT3',

respectively. The PCR products were purified according QIAGEN's protocol for sequencing reactions. The resulting sequences of the cDNA clones were searched by BLAST against the Rice Annotation Project Data Base/RAP-DB (http://rapdb.dna.affrc.go.jp/) and the NCBI database (http://blast.ncbi.nlm.nih.gov/). A swapping experiment, using full-length constructs of each Hd3a interactors (GF14c, BIP116b, OsKANADI1) and Hd3a either as bait or as prey, was performed. Interactions were tested on SC medium lacking histidine (-H) or lacking histidine and containing 2.5 mM 3-AT.

2.6 Full-length cDNA construction

Since only partial cDNAs were obtained from yeast two-hybrid screening, we constructed full-length GF14c, OsKANADI1 and BIP116 cDNA based on sequence information in the rice DNA database. We designed forward and reverse primers (see Appendix Table 1) to produce a full-length PCR fragment using cDNA as a template. The PCR fragment was then subcloned into the pENTR/D-TOPO cloning vector to obtain an entry clone.

Because of the GC-rich content in the full-length cDNA of *OsKAN1*, two steps of cloning were performed as presented in Fig. 5-1. First, a partial cDNA fragment from the start codon to halfway along the *OsKAN1* cDNA (+1- 641 base pairs (bp)) was amplified using forward and reverse primers 5'CACCATGGAGCTGTTCCCGG CAA3' and 5'TCTCTCGTGGCCTCCGAGAA3', respectively, and then cloned into pENTR-D TOPO vector. The other half of the cDNA fragment to the stop codon (position 591- 1095 bp) was amplified using forward and reverse primers, 5'CACCATGCGGTGGACGACGTCGCT3' and 5'TCATGGCGCTGACAGTCGTC3',

respectively, and cloned into the pENTR-D TOPO vector. Each entry vector was digested with *Asc*I and *Aat*II and ligated according to the manufacturer's instructions.

Full-length *GF14c*, *OsKAN1* and *BIP116b* constructs without stop codons were generated using site-directed mutagenesis for stop codon deletion. Forward and reverse primers (see Appendix Table 1) were used to amplify *GF14c*, *OsKAN1* and *BIP116b* pENTR-TOPO plasmids. The PCR conditions were as follows: initial denaturation 96^{o}C, 15s, followed by 16-18 cycles of denaturation 96^{o}C, 14s; extension 68^{o}C. The PCR product was digested overnight with *Dpn*I, which digests methylated or parental DNA.

2.7 Database analysis of GF14c, OsKANADI1 and BIP116b

Rice GF14, OsKANADI1 and BIP116b family sequences were obtained from the GenBank database and the International Rice Annotation Project Database (Rice Annotation Project, 2008). The deduced amino acid sequences were aligned using ClustalW with default parameters, and each phylogenetic tree was generated using the neighbor-joining method.

2.8 *In vitro* pull-down assays

2.8.1 *In vitro* pull-down assay of Hd3a and GF14c

Hd3a and *GF14c* were cloned into pDEST15 and pDEST17 vectors (Invitrogen), respectively. GST, GST-Hd3a and His-GF14c were expressed in *E. coli* BL21 (DE3) (Invitrogen) and purified with glutathione-agarose (Sigma) and HisTrap (Amersham) columns according to the manufacturers' instructions. *In vitro* binding assays were

performed as follows. The concentration of each fusion protein was determined by Coomassie staining. Equal amounts of GST-Hd3a protein coupled to glutathione sepharose 4B beads and His-GF14c were incubated in TEDM buffer [10 mM Tris-HCl, pH7.5, 0.5 mM EDTA, pH 7.5, 1 mM DTT, 1x Complete Proteinase Inhibitor Cocktail (Roche)]. The beads were then washed four times with binding buffer. Bound proteins were eluted in 1x SDS sample buffer by boiling for 5 min, separated by 10% SDS-PAGE, transferred to PVDF membrane (Millipore) and subjected to immunoblotting with anti-His antibody (BD Biosciences Pharmingen). After washing with TBS containing 0.1% Tween, the membranes were incubated for 1 h with anti-mouse IgG conjugated to horseradish peroxidase (GE Healthcare). Detection was performed using ECL protein gel blot detection reagents (GE Healthcare) and visualized using an LAS-1000 Imager (Fujifilm).

2.8.2 *In vitro* pull-down assay of Hd3a and OsKANADI1

Hd3a and *OsKANADI1* were cloned into pMAL-c4X and pDEST17 vectors (Invitrogen), respectively. GST, MBP-Hd3a and GST-OsKANADI1 were expressed in *E. coli* BL21 (DE3) and purified with glutathione-agarose and Amylose resin high flow (New England Biolabs) columns according to the manufacturers' instructions. *In vitro* binding assays were performed as follows. The concentration of each fusion protein was determined by Coomassie staining. The equal amount of GST-OsKANADI1 protein coupled to glutathione sepharose 4B beads and MBP-Hd3a were incubated in TEDM buffer. The beads were then washed four times with binding buffer. Bound proteins were eluted in 1x SDS sample buffer by boiling for 5 min, separated by 10% SDS-

PAGE, transferred to PVDF membrane and subjected to western bloting with α-MBP antibody (New England Biolabs).

2.8.3 *In vitro* pull-down assay of Hd3a and BIP116b

Hd3a and *BIP116b* were cloned into pET-15b (Invitrogen) and pGEX4T (Amersharm Pharmacia Biotech) vectors, respectively. GST, GST-BIP116b and His-Hd3a were expressed in *E. coli* BL21 (DE3) and purified with glutathione-agarose and HisTrap (Amersham) columns according to the manufacturers' instructions. *In vitro* binding assays were performed as described above (section 2.8.1).

2.9 Co-immunoprecipitation

An expression construct of Hd3a-tagged myc under the cauliflower mosaic virus (CaMV) 35S promoter was introduced into rice calli by *Agrobacterium*-mediated transformation. Rice suspension culture cells were ground to a fine powder in liquid nitrogen, and extracted with NEB extraction buffer [20 mM HEPES pH 7.5, 40 mM KCl, 1 mM EDTA pH 7.5, 1% Triton X-100 and 1x Complete Proteinase Inhibitor Cocktail (Roche)]. The extract was centrifuged at 20,000 *g* for 10 min. The supernatant was incubated with pre-washed protein G-coupled Sepharose beads and monoclonal α–myc antibody (Nacalai Tesque) for 3 h at 4°C, and the beads were then washed four times with washing buffer [20 mM HEPES pH 7.5, 40 mM KCl, 0.1% Triton X-100 and 1x Complete Proteinase Inhibitor Cocktail (Roche)]. The immunoprecipitate was eluted with 2x SDS sample buffer by boiling for 5 min, separated on a 10% SDS-PAGE gel, transferred to PVDF membrane, and detected with monoclonal α-myc antibody (Nacalai Tesque) and α-14-3-3 antibody (kindly provided by Dr Yohsuke Takahashi,

Hiroshima University, Japan). After washing with TBST (20mM Tris, 140 mM NaCl, 0,1% Triton-X100 [pH 7.5]) the membranes were incubated for 1 h with anti-mouse and anti-rat IgG conjugated to horseradish peroxidase, respectively (GE Healthcare). Detection was performed as above.

2.10 Tissue-specific expression

Total RNA from seeds, seedlings, leaf blades, leaf sheaths, stems, shoot apices, roots and rice suspension culture cells were extracted using TRIZOL reagent (Invitrogen) and treated with DNaseI (Invitrogen). The cDNA was synthesized from 1 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen). The cDNA was subjected to semi-quantitative analysis of gene expression using PCR with primers specific for *GF14* family members, *Hd3a* and ubiquitin (see Appendix Table 1).

2.11 GF14 protein expression *in planta*

Leaves and stems from wild-type, Hd3a::Hd3a:GFP overexpressing, GF14c overexpressing and *gf14c* knockout plants were harvested and ground to a fine powder in liquid nitrogen. Proteins were extracted as described previously (Mathieu et al. 2007), using an extraction buffer containing 150 mM NaCl, 50 mM Tris pH 7.5, 0.1% Tween 20, 10% glycerol, 1 mM DTT, 1 mM Pefabloc (Roche), 1x Halt Phosphatase Inhibitor Cocktail (Pierce) and 1x Complete Proteinase Inhibitor Cocktail (Roche). The extracts were centrifuged at 15,000 rpm for 40 min at 4^{o}C. The supernatant was separated by 12.5% SDS-PAGE and subjected to immunoblotting using the first antibody (polyclonal rabbit anti-GFP antibody, Abcam) as described previously (Mathieu et al. 2007) and

anti-14-3-3 antibody as described above. After washing with TBST the membranes were incubated for 1 h with anti-rabbit and anti-rat IgG conjugated to horseradish peroxidase, respectively (GE Healthcare). Detection was performed as above.

2.12 **Subcellular localization** (performed by Yuka Ogaki)

The GFP sequence was fused to either the C or the N terminus of GF14c using the Gateway system (Invitrogen). Expression of GF14c-tagged GFP constructs and Hd3a-tagged mCherry was driven by the CaMV 35S promoter and the ubiquitin promoter, respectively. Protoplast isolation from rice Oc suspension culture (Kyozuka and Shimamoto 1991) and protoplast transformation (Chen et al. 2006b) were performed as described previously. After a 24-h incubation at 30°C, the protoplasts were examined under a confocal microscope (LSM510, Zeiss).

2.13 **Bimolecular fluorescence complementation** (performed by Yuka Ogaki)

For constructs used in BiFC experiment, vectors containing N-terminal and C-terminal halves of mVenus were kindly provided by S. Takayama (NAIST). Hd3a, GF14c and GUS coding regions were cloned into the BiFC vectors and purified by the Purelink Plasmid Midiprep Kit (Invitrogen). The purified BiFC plasmids were introduced into rice protoplasts as described previously (Kyozuka and Shimamoto 1991, Chen et al. 2006b). mCherry expression plasmid was introduced at the same time as the marker for transformed cells. The number of cells with reproducible mVenus fluorescence in the mCherry-expressing cells was scored.

2.14 Transgenic plant (overexpression and RNAi) construction

We generated mutant plants overexpressing GF14c, BIP116b and OsKANADI1 under the control of 35S CaMV promoter and suppressing plants by RNAi. Full-length GF14c, BIP116b and OsKANADI1 were subcloned into the pENTR/D-TOPO cloning vector. These constructs were introduced by LR clonase into pGWB2 vector for GF14c and BIP116b, and GWB17 vector to obtain OsKANADI1-tagged 4xmyc under the CaMV 35S promoter. These constructs were introduced into rice calli by *Agrobacterium*-mediated transformation.

To generate RNAi transgenic plants, the gene sequences of *BIP116b* and *OsKANADI1* (including 383- and 309-bp fragments, respectively, as 3' untranslated /UTR regions), for which inverted repeats were made, were amplified using specific primers (see Appendix Table 1) and subcloned into the pENTR/D-TOPO cloning vector to yield entry vectors (pENTR-BIP116b and pENTR-*OsKANADI1* RNAi). To suppress the expression of both BIP116a and BIP116b, the 3'UTR region of BIP116a and BIP116b were fused and used as a target fragment for RNAi.

The final RNA silencing vectors were produced by an LR clonase reaction between each entry clone RNAi and pANDA (Miki and Shimamoto 2004). Transgenic rice plants were generated by *Agrobacterium*-mediated transformation of rice calli in N8 background, performed according to a published protocol (Hiei et al. 1994).

CHAPTER 3
Identification of Hd3a Interacting Proteins

3.1 Introduction

Yeast two-hybrid screens have been widely used to identify protein-protein interactions (Fields and Song 1989, Ito et al. 2001). To study Hd3a function, we focused on searching for Hd3a interacting proteins using a yeast two-hybrid approach. The advantage of yeast two-hybrid screening is that large numbers of cDNA clones can be tested with relative ease in a short time. In this study we have performed yeast two-hybrid screening to search for Hd3a partners by using a cDNA library from rice leaf blades, which were harvested 35-40 days after sowing (at Zeitgeber Time/ZT 0, 2 and 4) under SD conditions when the transition from vegetative to reproductive phase was occurring and *Hd3a* was being highly expressed. In this chapter, we will discuss the validity of our yeast two-hybrid screening and the results of the identification of Hd3a partners. These experiments indicated that Hd3a interacts physically with a wide range of proteins which are mainly involved in signaling.

3.2 Results

3.2.1 Novel interaction partners for rice Hd3a protein identified in a yeast two-hybrid screen.

To identify proteins which interact with Hd3a, we took advantage of the sensitive yeast two-hybrid protein-protein interaction assay to screen a rice leaf cDNA library. To begin with, we constructed an Hd3a full-length cDNA as bait and expressed it in the

yeast system (Fig. 3-1). A cDNA library was constructed from leaf blades harvested when the transition from vegetative to reproductive phase occurred, a time at which *Hd3a* is highly expressed (Komiya et al. 2008).

3.2.2 Validation of isolated clones

For large scale yeast two-hybrid screening, a total of ~1.6 x 10^6 transformants were screened for activation of HIS3 (histidine synthase 3) on selective medium plates without the amino acids leucine (L), tryptophan (W) and histidine (H). In the first screening on SC-His, 354 colonies were obtained. From the next screening, on SC-His containing 3-AT, a total of 96 colonies synthesizing unique proteins were identified as positive clones. To eliminate false positives, we used 2.5 mM 3-AT, a competitive inhibitor of the *His3* gene product HIS3, which is the reporter gene for interactions in the yeast two-hybrid system. All 96 positive clones were checked for the presence of an "in frame" cDNA-AD fusion. The sizes of the clones varied from approximately 600 bp to 2000 bp. In general, clones lack the N-terminal part of the coding region, although some were found to contain full-length cDNA. The clones were then retransformed into yeast containing Hd3a bait, to confirm their interaction on the selective medium SC-His containing 3-AT (Fig. 3-2). The flowchart of our yeast two hybrid screens is presented in Fig. 3-3.

3.2.3 Hd3a interacts with a variety of proteins involved in signaling

As shown in Table 3-1, 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.

We identified GF14c (a homolog of 14-3-3 family members), which was previously identified as SP (SELF PRUNING), a paralog of Hd3a/FT, in tomato (Pnueli et al. 2001). The presence of a 14-3-3 protein as an Hd3a interacting protein in our screen can be considered as a positive control.

3.3 Discussion

An important step to reveal the function of Hd3a is the identification of other proteins with which it interacts. It has been suggested that Hd3a orthologs might also act as either scaffolds for or regulators of signaling complexes, based on the finding that SP (SELF PRUNING) and SFT (SINGLE FLOWER TRUSS), as tomato homologs of TFL1 (TERMINAL FLOWERING LOCUS 1) and FT (FLOWERING LOCUS T), respectively, can interact with a range of diverse proteins (Pnueli et al. 2001, Lifschitz et al. 2006). In *Arabidopsis*, FT interacts with the bZIP transcriptional factor FD (Abe et al. 2005, Wigge et al. 2005). Interestingly, the bZIP transcription factor SPGB in tomato, a homolog of FD, also interacts with SP and SFT.

Interestingly, our yeast two-hybrid screening identified a diverse range of proteins that are mainly involved in signaling. In this study 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 in a wide range of organisms (Robert 2003). 14-3-3 proteins function as major regulators of primary metabolism and cellular signal transduction in plants, generally acting as adaptors, chaperones, activators or repressors,

and interacting physically with phosphorylated target proteins to execute important steps in signal transduction and metabolism; (ii) BRI1-kinase domain (KD)-interacting protein 116b, a homolog of a protein that can be phosphorylated by the BRI1-KD brassinosteroid receptor and likely has a function in brassinosteroid signal transduction (Hirabayashi et al. 2004); and (iii) a novel myb transcription factor-like protein, namely OsKANADI1, which encodes a nuclear-localized protein in the GARP (GOLDEN 2 in maize, *Arabidopsis* response-regulator-ARR and the PsrI protein from *Chlamydomonas*) family.

The presence of a putative protein kinase and endonuclease (phosphatase) in our screen also needs to be explored. Phosphatase and kinase have an important role in signal transduction. Signaling networks often rely on the formation of dynamic protein complexes, with kinases/phosphatase determining the strength of protein-protein interaction through (de)phosphorylation. An endonuclease is defined as an enzyme that is capable of cleaving the phosphodiester bonds between nucleotides. It is becoming apperent that simple phosphorylation is not enough to effect a change in protein function. In many cases, the 14-3-3 proteins play the major role of bringing the signal transduction event to completion (Sehnke et al. 2002a, Ferl 2004).

In addition, as a long-distance component of florigen, the stability of Hd3a, which is generated in the leaves and translocated to the SAM via plasmodesmata, is important. Many proteins function as chaperones to maintain correct protein folding. Chaperones are vital in the crowded conditions of the cytoplasm, since they prevent newly synthesized but improperly folded protein chains from associating with the wrong partners. Among the proteins identified in our yeast two-hybrid screening that

might function as chaperones is a heat shock protein, HSP70. However, the existence of an Hd3a/HSP70 complex must await future confirmation.

It is interesting to note we identified proteins that presence in rice leaves phloem sap, such as glutathione S-transferase (GST) (Fukuda et al. 2004), eukaryotic initiation factor 5A (eIF5A)(Aoki et al. 2005), and thioredoxin h (TRXh) (Fukuda et al. 2005). This finding might provides new insight the mechanism of Hd3a long-distance transport via the phloem.

Taken together, the results of our screen have revealed many established and novel polypeptides as putative Hd3a interacting proteins. In this study, a wide range of target proteins which function in signal transduction has been identified in rice. It is possible that Hd3a and its partner(s) may form a platform for cross-talk between signal transduction pathways. Further functional studies using reverse genetics to obtain mutants (either gain or loss of function mutants), or knock-down mutant using RNAi of Hd3a partners, should yield insights into the function of Hd3a in plant growth and development, particularly during the floral transition.

CHAPTER 4
Functional Characterization of GF14c

4.1 Introduction

In the mid-1960s, Moore & Perez first identified 14-3-3 proteins as acidic, soluble proteins within the mammalian brain. The names were assigned according to their fractionation on DEAE cellulose and electrophoretic mobility in starch gels. In native conditions, they form a dimer with a molecular weight of ~60 kDa (Ferl 1996).

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). In animal cells, the majority of their known targets are involved in signal transduction and transcription. *In planta*, they primarily participate in the regulation of the plasma membrane H^+-ATPase and enzymes of carbon and nitrogen metabolism. Plant 14-3-3 proteins bind a range of TFs and other signaling proteins, and have pivotal roles in regulating developmental and stress responses. An outstanding question is: what is the mechanism of their regulation? They may function in shuttling proteins between different cellular locations and act as scaffolds for the assembly of larger signaling complexes.

There are two key features of 14-3-3 protein interactions (Roberts 2003). First, their interactions are regulated by the phosphorylation status of their targets, and they

can interact with either one or two targets at the same time. A short amino acid motif on the target protein containing phosphoserine and/or phosphothreonine is responsible for interactions. It has been demonstrated that 14-3-3 proteins bind to a phosphorylated serine residue in the motifs RSXpSXP and RXY/FXpSXP (where pS indicates a critical phosphoserine, and X is any amino acid) in their target proteins (Muslin et al. 1996; Yaffe et al. 1997). Second, binding of 14-3-3 to a target can affect the function of that protein, either in terms of its activity, its interaction with other proteins, or its localization within the cell.

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* (Rosenquist et al. 2001), 8 members in rice (Chen et al. 2006a, Yao et al. 2007) 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.

Instead of identifying target proteins, it may be preferable to understand what roles are played by different 14-3-3 protein isoforms. Evidence indicates that the *Arabidopsis* 14-3-3 isoforms are differentially expressed in different cell types, but are also distributed differently within single cells in the cytoplasm, nucleus, chloroplast and mitochondrion (Sehnke et al. 2002b). Identifying the kind of dynamic localization and co-localization with its protein targets will be important to address the function of a particular 14-3-3 protein (Roberts 2003).

The 14-3-3 proteins are a family of highly conserved regulatory molecules, expressed in all eukaryotic cells, and multiple isoforms have been found in different tissues. In the bovine brain seven isoforms have been identified, were given Greek letter designations (α, β, γ, δ, ε, ζ, and η) on the basis of their order of elution during reversed phase chromatography (Ferl 1996). Accumulating evidence indicates that the 14-3-3 proteins play an important role in the regulation of cell differentiation, proliferation and transformation. They can bind to a wide variety of proteins involved in signal transduction, cell cycle control, vesicular transport, DNA replication and apoptosis. Each isoform seems to have a distinct tissue localization and specific function.

Several studies of protein interactions involving FT/Hd3a orthologs have been published. In *Arabidopsis*, FT interacts with FD and 14-3-3 proteins (Abe et al. 2005, Wigge et al. 2005). There are several SP interacting proteins in tomato, including a 14-3-3 family member, protein kinase and bZIP transcription factor (Pnueli et al. 2001). SFT, another tomato ortholog of FT/Hd3a, also interacts with 14-3-3 as well as bZIP (Lifschitz et al. 2006). Here, we identified GF14c (G-box factor 14-3-3c protein) as an Hd3a interacting protein both *in vitro* and *in vivo*. Since 14-3-3 family members interact

with diverse proteins, it was of interest to understand their role in Hd3a signaling. Functional analyses using both knockout and overexpressing plants indicate that GF14c acts as a negative regulator of flowering in rice by interacting with Hd3a. Since 14-3-3 proteins have been shown to interact with FT in tomato and *Arabidopsis*, the results described below for rice provide important further findings about FT signaling in plants.

4.2 Results

4.2.1 Hd3a interacts with GF14c in the yeast two-hybrid system

In our yeast two-hybrid screen, three independent clones were identified as GF14c, an isoform of the rice 14-3-3 gene family (Table 4-1). Only a partial fragment corresponding to the C-terminal region (115-256) was identified from the initial yeast two-hybrid screen. An experiment using full-length Hd3a and full-length GF14c, each either as bait or as prey, further confirmed that Hd3a and GF14c interact in the yeast system (Fig. 4-1). In plants, 14-3-3 proteins are known to interact with Hd3a orthologs: FT in *Arabidopsis* and SP and SFT in tomato (Pnueli et al. 2001). GF14 family members have roles in both disease resistance and development in rice (Cooper et al. 2003). We were interested in further characterizing the molecular function of GF14c in association with Hd3a throughout rice development, particularly during the floral transition.

Table 4-1 Isolation of GF14c, an Hd3a interacting protein, in a yeast-two hybrid screen

Number	Accession number	Homologous protein	Two-hybrid*	Amino acids*
IV-B1 VI-G2 VI-A3	gi 50903393 AK122149/Os08g0430500	GF14c protein [(*Oryza sativa* (japonica cultivar-group)] 14-3-3 homologous	141 (115-256)	256

*Length in amino acids

4.2.2 Tissue-specific expression of Hd3a and GF14s in rice

From the International Rice Annotation Project Database (Rice Annotation Project, 2008), we identified GF14 family members. GF14s belong to the 14-3-3 protein group and have eight isoforms, designated *GF14a-h* in rice (Chen et al. 2006a, Yao et al. 2007) that share 85-95% amino acid identity (Fig. 4-2A). To determine whether the *GF14* genes are expressed in rice organs, we performed semi-quantitative RT-PCR using specific primers (Fig. 4-2B). The *GF14s* were transcribed in all tested organs, suggesting that they have broad functions in rice growth and development. The expression of GF14a-f seems to be abundant in comparison with that of GF14g and h. No diurnal changes or developmental patterns of *GF14c* expression were observed (Fig. 4-3), indicating that *GF14c* is expressed independently of the photoperiod and abundantly throughout plant development. We also examined the distribution of Hd3a and 14-3-3 proteins in rice plants. Plants overexpressing Hd3a under its native promoter (*Hd3a::Hd3a:GFP*) (Tamaki et al. 2007) and non-transgenic control plants, co-expressed Hd3a and 14-3-3 proteins in both leaf blades and stems (Fig. 4-2C).

4.2.3 Subcellular localization of Hd3a and GF14c

To gain insights into the molecular function of Hd3a and its partner GF14c, we made a fusion construct to express mCherry fluorescent protein-linked Hd3a under the ubiquitin

promoter, and GFP-linked GF14c driven by the CaMV 35S promoter, to identify the intracellular localization of Hd3a and GF14c. 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) (Supplemental Fig. 1). 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 (Muslin and Xing 2000).

4.2.4 *In vitro* and *in vivo* interaction of Hd3a and GF14c

To further study the interaction between Hd3a and GF14c, we performed a GST pull-down assay. A GST-Hd3a fusion protein was pulled down with His-tagged GF14c, as shown in Fig. 4-4A, 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 (Fig. 4-4B). Results of this experiment were also thus consistent with the results of the yeast two-hybrid experiment.

4.2.5 The Hd3a and GF14c complex is localized in the cytoplasm by BiFC (results from Yuka Ogaki)

We used bimolecular fluorescence complementation (BiFC) to determine the distribution of Hd3a and GF14c *in vivo*. The expression vectors for Hd3a protein fused

to the N-terminal half of mVenus (Vn) and GF14c protein fused to the C-terminal half of mVenus (Vc) were transiently introduced into rice protoplasts, with the mCherry expression plasmid serving as a marker for transformed cells. As shown in Supplemental Fig. 2, 95% of the transformed rice protoplasts observed in this experiment showed Hd3a and GF14c interaction in both protein fusion combinations, as documented by a strong green fluorescence concentrated in the cytoplasm. Hd3a-Vn/GUS-Vc or GUS-Vn/GF14c-Vc combinations yielded remarkably few cells with Venus fluorescence (0-10% of the transformed cells). These results clearly demonstrate that Hd3a interacts with GF14c mainly in the cytoplasm.

4.2.6 GF14c-overexpressing and *gf14c* mutant plants

To characterize the function of GF14c, a GF14c overexpression (ox) construct driven by the CaMV 35S promoter was generated and used to transform rice plants (Fig. 4-5A). T1 generation transgenic plants (n=15) were used for the phenotypic analysis of GF14c. GF14c-ox plants were delayed by 5 to 20 days in flowering relative to wild-type plants (Fig. 4-5B and C). A t-test confirmed that the flowering time was significantly different between GF14c-ox and wild-type plants (p = 0.0046). Other phenotypes observed in this mutant were erect leaves and longer culms compared to the wild-type plants (data not shown). In GF14c-ox plants, overexpression of GF14c at both the mRNA and the protein level was confirmed (Fig. 4-5D and E).

To further study GF14c function in flowering, we also used *gf14c* T-DNA insertion mutants in japonica rice cv ‘Dongjin’, generated by Postech, Korea. One independent heterozygous line (Fig. 4-6B) possessed a T-DNA insertion in the second exon of *GF14c* (Fig. 4-6A and D). We germinated 50 seeds derived from the *gf14c* T-

DNA mutants and found that 24 were heterozygous, 7 were wild-type, and 19 did not germinate which were likely to be homozygous, suggesting that the mutation may be lethal. The *gf14c* plants flowered earlier than wild-type by ca. 14 days (12-17 days depending on plants) (Fig. 4-6C). A t-test confirmed that the flowering time was significantly different between wild-type and *gf14c* (p = 0.0387, n=5). GF14c expression at both mRNA and protein levels in these mutants plants was lower than wild type-plants (Fig. 4-6E and F). These results clearly indicate that GF14c is involved in regulation of flowering time in rice.

4.3 Discussion

Hd3a is thought to move along leaf cells and the vascular system of leaves and stems, and exerts its action in the SAM. Since the tissue localization of Hd3a protein was determined using transgenic rice plants expressing an Hd3a-GFP fusion protein driven by the *Hd3a* promoter, and since Hd3a protein is present in vascular tissue from the leaf blades and transported through the phloem (Tamaki et al. 2007), we prepared a cDNA library from leaf blades to search for Hd3a interacting proteins in a yeast two-hybrid screen. We identified GF14c, a member of the 14-3-3 protein family that is highly conserved in all eukaryotes (Ferl 1996) and known to be involved in eukaryotic signaling pathways. In plants, 14-3-3 proteins have been shown to interact with FT and TFL1 in *Arabidopsis* and with SP and SFT in tomato (Pnueli et al. 2001, Lifschitz et al. 2006). More recently, a comprehensive study using yeast two-hybrid screening and affinity chromatography with mass spectrometry provided a large number of novel putative 14-3-3 interacting partners in barley (Schoonheim et al. 2007). Interestingly, a wide range of 14-3-3 targets have functions in various signal transduction pathways and

might form a platform for cross-talk between signal transduction pathways. Binding of 14-3-3 proteins regulates their partner proteins through a variety of mechanisms, such as altering their catalytic activity, subcellular localization, stability or interactions with other proteins (Roberts 2003).

Identification of GF14c as an Hd3a partner has been confirmed here by several methods. The results from yeast two-hybrid assays using a full-length construct, *in vitro* pull-down and BiFC assays, demonstrated the interaction between Hd3a and GF14c in rice. The subcellular distribution of Hd3a as well as GF14c demonstrated their localization in both the cytoplasm and nucleus. The 14-3-3 proteins are highly conserved throughout the animal and plant kingdom. In *Arabidopsis*, there are at least twelve isoforms, whereas eight isoforms exist in rice. These proteins are known as a component associated with a number of different proteins in signal transduction pathways, including plant hormone signaling pathways.

Overexpression of GF14c caused late flowering, suggesting that 14-3-3 functions as a negative regulator of flowering. Hd3a protein is localized in both the cytoplasm and the nucleus; however, GF14c is mainly localized in the cytoplasm, and only a very weak fluorescent signal was observed in the nucleus (Fig. 4-2D). To address the question of whether the interaction between Hd3a and GF14c would inhibit shuttling of Hd3a from the cytoplasm into the nucleus, we performed a BiFC experiment. The results demonstrate that the Hd3a-GF14c protein complex is mainly localized in the cytoplasm (Fig. 4-4C). This strongly suggests that Hd3a and GF14c interaction increases Hd3a cytoplasmic retention. Therefore, the delayed flowering phenotype in GF14c-ox plants can be explained by the increased cytoplasmic retention of Hd3a by GF14c. In *Arabidopsis*, to initiate floral transition in the shoot apex, FT

interacts with FD, a basic/leucine zipper (bZIP) transcription factor that localizes in the nucleus to induce target meristem identity genes such as *AP1* (Abe et al. 2005). 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 (Igarashi et al. 2001, Ishida et al. 2004).

Another mechanism which could possibly explain the phenotypes of GF14c-ox plants and the *gf14c* knockout mutants is an interaction of Hd3a with GF14c which inhibits movement of Hd3a in its long-distance trafficking through the phloem. Interestingly, the *gf14c* knockout mutants exhibited an early flowering phenotype. These mutants also showed a dwarf phenotype and increased tiller numbers (Fig. 4-7), suggesting that GF14c might also function independently of from flower induction at the SAM . When expression levels of GF14c are low in the leaf, Hd3a protein may be capable of moving long distances freely, from companion cell to the phloem sieve elements, since Hd3a is a small protein that is below the size exclusion limit of plasmodesmata (Lough and Lucas 2006, Giakountis and Coupland 2008). In contrast to our results in rice, loss of 14-3-3 functions in *Arabidopsis* led to a slight delay in flowering (3- 5 days) in comparison to wild-type (Mayfield et al. 2007). These workers also tested the interaction of 14-3-3 with components of the photoperiodic pathway, such as CO (a central regulator), GI, ZTL, PIF3 and TOC1; however, the interaction with FT was not tested. Therefore, while our study demonstrates an interaction between Hd3a and GF14c, and indicates that GF14c acts as a negative regulator of flowering in rice, the molecular mechanisms of GF14c function in floral transition and development remain to be elucidated.

CHAPTER 5

Functional Characterization of OsKANADI1

5.1 Introduction

The SAM plays a central role in the formation of lateral organs such as leaves and floral organ primordia. The adaxial-abaxial axis is fundamental for the subsequent asymmetric growth of the leaf and lamina expansion (McConnell and Barton 1998).

Analysis of Hd3a interacting proteins in yeast two-hybrid screening identified a novel putative transcription factor belonging to the KANADI domain protein family, namely OsKANADI1 (OsKAN1). 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 (Riechmann et al. 2000). 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 (Kerstetter et al. 2001).

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 (Kerstetter et al. 2001, Emery et al. 2003, Eshed et al. 2004). 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.

Class III HD-Zip gene family members (*PHABULOSA* [*PHB*], *PHAVULOSA* [*PHV*], *REVOLUTA* [*REV*], *ATHB8* and *ATHB15*) are putatative transcription factors that have been shown to be required for the establishment of the apical meristem, and for proper pattern formation in lateral organs and vascular tissue in the aerial portion of the plant body. Their expression in the shoot is confined to the apical meristem, the adaxial portions of lateral organs, and procambium and xylem tissue (McConnell and Borton, 1998).

In *Arabidopsis*, adaxial identity is specified by class III homeodomain leucine zipper (HD-ZIPIII) transcription factors, the transcription factor ASYMMETRIC LEAVES2 (AS2), and the transacting siRNA tasiARF, whereas abaxial identity is specified by KANADI (KAN), YABBY (YAB), AUXIN RESPONSE FACTOR (ARF), and LITTLE ZIPPER (ZPR) transcription factors and by the miRNAs miR165/miR166. Genetic analysis indicates that many of these genes interact antagonistically: loss-of-function mutations in adaxial genes typically produce an abaxialized phenotype that is accompanied by the expanded expression of abaxial genes, whereas loss of function mutations in abaxial genes produce an adaxialized phenotype that is associated with the expanded expression of adaxial genes (Wu et al. 2008b).

KANADI and *class III HD-Zip* genes families are expressed in complementary patterns in developing lateral roots, and play functional roles in lateral root formation (Hawker and Bowman 2004). Recently, Wu et al. (2008b) reported that *Arabidopsis* KANADI1 (AtKAN1) promotes abaxial identity by directly repressing the transcription of *AS2* in abaxial tissue. AtKAN1 binds to a site in the promoter of *AS2*, and that a single nucleotide mutation in this site interferes with AtKAN1 binding and produces an

adaxialized phenotype that is associated with the inappropriate expression of *AS2* in abaxial tissue.

Few reports have been published about the function of KANADI family members in rice (Luo et al. 2007, Yan et al. 2008) and maize (Candela et al. 2008). *RL9* (*Rolled leaf 9*) was identified by map-based cloning in two rice allelic rolled-leaf mutants which displayed very similar phenotypes, with completely adaxialized leaves and malformed spikelets. *RL9* encodes a GARP protein, and is an ortholog of *Arabidopsis KANADIs*. Therefore, further studies of KANADI family members in rice and their interaction with Hd3a will contribute to assessment of the possible function of OsKANADIs in Hd3a signaling.

5.2 Results

5.2.1 OsKANADI is a novel transcription factor that interacts with Hd3a

In a yeast two-hybrid screen, we identified a myb transcription factor-like protein as an Hd3a interacting protein in a BLAST search, and designated it as OsKAN1 because of its amino acid similarity with other KANADIs in *Arabidopsis* within the GARP domain (Fig. 5-2A). From the rice database we found that there are seven members of the *OsKANADI* family, and phylogenetic analysis placed OsKAN1 in the same clade as *AtKAN2*, *AtKANI3* and *AtKAN4* (Fig. 5-2B).

Sequence analysis showed that OsKAN1 consists of 6 exons. 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.

To study the expression pattern of *OsKAN1*, semiquantitative RT-PCR was performed with various wild-type tissues in the floral transition stage. The results demonstrated that *OsKAN1* is expressed in all organs of wild-type plants, but is highest in roots and leaves (Fig. 5-3A)

Previous studies indicated that the *Arabidopsis* KAN1 protein and GARP domain-containing proteins are localized in the nucleus (Kerstetter et al. 2001, Hosada et al. 2002). We hypothesize that OsKAN1 is a transcription factor in rice because it contains a GARP domain and posses a nuclear localization signal. To determine whether OsKAN1 encodes a nuclear protein, full-length OsKAN1 fused to GFP was introduced into an expression vector. Vector isolated from cells expressing the OSKAN1-GFP fusion protein was introduced into onion epidermis cells by particle bombardment. This revealed that OsKAN1 is localized in the nucleus, suggesting that OsKAN1 may be a transcription factor (Fig. 5-3B).

5.2.2 Hd3a and OsKAN1 interact *in vitro* and in the yeast system

Only partial fragment the C-terminal region (220-364) was identified from the initial yeast-two hybrid screen. An experiment using full-length Hd3a and full-length OsKAN1, either as bait or as prey, further confirmed that Hd3a and OsKAN1 interact in the yeast system (Fig. 5-4).

To further study the interaction between Hd3a and OsKAN1 in *vitro*, we performed a GST pull-down assay. A GST-OsKAN1 fusion protein was pulled down with MBP-tagged Hd3a, as shown in Fig. 5-5, indicating that Hd3a interacts with

OsKAN1 *in vitro*. Results of this experiment were thus consistent with the results of the yeast two-hybrid experiment.

5.2.3 OsKAN1 overexpressing and suppressing transgenic plants

We generated mutant plants overexpressing OsKAN1 (OsKAN1-ox) fusion with 4xmyc tag under the control of the 35S CaMV promoter (Fig. 5-6A), and OsKAN1-suppressing plants by RNAi (Fig. 5-7A). At present, we have only T0 generation plants, and therefore we have not yet analyzed the flowering time. However, we found interesting phenotypes in OsKAN1-RNAi plants. The knocked-down OsKAN plants displayed two stem folks and increased tiller number (Fig. 5-7B). This phenotype resembles that of Hd3a overexpressing plants, indicating that they may be involved in similar pathways. *OsKAN1* expression was confirmed in both T0 generation OsKAN1-ox (Fig. 5-6C) and OsKAN1-RNAi mutants plants (Fig. 5-7C). Analysis of these mutants in the next generation will be important for understanding the possible role of OsKAN1 in association with Hd3a during plant development.

5.3 Discussion

In response to floral stimulus, the apical meristem stop producing leaves to initiate floral development; this switch in morphogenesis involves a change in the identity of the primordial initiated and in phyllotaxis (Corbesier and Coupland 2006). In this study, we identified a novel putative transcription factor, OsKAN1, as an Hd3a interacting protein in rice. Sequence analysis revealed that OsKAN1 belong to GARP DNA-binding protein. Phylogenetic and comparative genetic analysis indicated that OsKAN1 is an ortholog of AtKANs and is most closely related to AtKAN4.

OsKAN1 at least has seven homologs in rice. OsKAN1 has a potential phosphorylation site for Ca^{2+} dependent protein kinase and might function as a target binding site of 14-3-3 proteins (RSXpSXP- pS motif in C-terminal region). This feature also presence in FD, a FT interacting protein in *Arabidopsis* (Abe et al. 2005). The possibility that OsKAN1, 14-3-3 proteins and Hd3a may form a complex to regulate their signaling should be interesting to address in future.

In *Arabidopsis*, members of the KAN family of GARP transcriptional regulators have been found to play essential roles in the specification of abaxial fate in leaf development. Loss of function mutations in individual genes have relatively weak effects on organ polarity (Kerstetter et al. 2001, Eshed et al. 1999), but *kan1 kan2* (Eshed et al. 2001) and *kan1 kan2 kan3* (Eshed et al. 2004) mutants are strongly adaxialized. It will be interesting to investigate possible target genes of the OsKAN1-Hd3a complex, as well as the tissue specificity of *OsKAN1* expression to determine whether expression is also localized in the phloem, similar to *Hd3a*. It has been reported that *Arabidopsis KANADI* and *class III HD-ZIP* genes exhibit complementary expression patterns in the vasculature as well as in the leaves. *KANAD*I expression is restricted to the developing phloem, positioned abaxially, and *class III HD-ZIP* expression is limited to developing xylem, positioned adaxially. While *KAN2* and *KAN3* are expressed in developing phloem throughout the plant (leaf and stem), *KAN1* expression in the phloem is largely limited to the root (Emery et al. 2003).

In *Arabidopsis* asymmetric leaf development, KANADI proteins have antagonistic function with HD-ZIP III proteins (Emery et al. 2003), and *KANADI* genes are necessary for *YABBY* expression (Eshed et al. 2004). However, in rice no *HD-ZP III* genes have yet been characterized, but four *YABBY* genes were characterized, i.e.

DROPPING LEAF, *YAB1*, *YAB3*, and *OsYAB4* (Jang et al. 2004, Yamaguchi et al. 2004, Dai et al. 2007, Liu et al. 2007). These YABBY genes in rice have been proved not to determine the abaxial cell fate, unlike their function in *Arabidopsis*.

Recent study in rice identified *RL9* as a member of OsKANADI family. The loss of function of this gene results in the rolled-leaf phenotype and malformed spikelets, suggesting that RL9 function similar to *Arabidopsis* KANADIs (Yan et al. 2008). In this study, we generated *OsKANADI1* mutants, either overexpressed or RNAi plants. Suppression of OsKANADI1 by RNAi displayed branching and increasing tiller number in several lines. This phenotype resembles to the Hd3a overexpressed plants indicating they possibly function in similar pathway. Further characterization using the OsKANADI mutants plants in the next generation remain to be investigated.

CHAPTER 6

Functional Characterization of BIP116b

6.1 Introduction

In our yeast two-hybrid screens, we identified a novel protein homolog of BIP116 (BRI1 kinase domain interacting protein 116), namely BIP116b. BIP116 (BRI 1 kinase domain interacting protein 116) in rice was initially identified in yeast two-hybrid screens due to its interaction with BRI1 (Hirabayashi et al. 2004), and we refer to it as BIP116a. BRI1 (brassinosteroid insensitive 1) is a membrane-bound LRR-RK (leucine rich repeat-receptor kinase) which perceives brassinosteroids (BRs).

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 (Vert et al. 2005). The difference between AtBRI1 and OsBRI1 is that 3-5 repeats of the LRR domain are absent in the rice ptrotein. The extracellular domain (Met1 – Leu 670) consists of 22 tandem copies of a 24-amino acid LRR motif, and 12 potential N-glycosylation sites (Asn-X-Ser/Thr) is flanked by pairs of conservatively spaced cysteines. LRR has been implicated to function in protein-protein interaction. An unusual feature of the LRR region of BRI1 is the presence of a 70-amino acid island between the 21st and 22nd LRRs (Li and Chory 1997). A highly similar feature is also present in OsBRI1, which has the same number of amino acids between the 18th and 19th LRRs, corresponding to the site of the island in BRI1 (Yamamuro et al. 2000).

OsBRI1 functions in various growth and developmental processes, including (1) elongation of internodes by inducing the formation of the intercalary meristem and the longitudinal elongation of internode cells; (2) bending of the lamina joint; and (3)

skotomorphogenesis (Yamamuro et al. 2000). Mutant *bri1* or defect of brassinosteroid metabolism in rice exhibit dwarfs, prevent both internode elongation and binding of the lamina joints (more erect leaves).

BRs control physiological and developmental processes such as stem elongation, vascular differentiation, seed size, fertility, flowering time, senescence, and resistance to biotic and abiotic stresses. Recent evidence in *Arabidopsis* suggested that BRs stimulate flowering by reducing transcript levels of the potent floral repressor *FLC* (Domagalska et al. 2007). Yu et al. (2007) provided a connection between BR signal transduction and pathways controlling floral initiation by demonstrating that a critical transcription factor required for BR-dependent gene expression directly interacts with two transcription regulators previously identified as having divergent roles in modulating time of flowering in *Arabidopsis*.

6.2 Results

6.2.1 BIP116b as a novel protein identified in yeast two-hybrid screening

We identified a novel Hd3a interacting protein that displays homology to a putative seed-specific protein (accession number gi 50934761) in the NCBI database. Sequence analysis of the full-length cDNA revealed that this gene was different from the annotated sequence because of the misprediction of splicing sites (Fig. 6-1). The gene encodes a protein that shares high homology with BIP116. These two homolog proteins, which we refer to as BIP116a and BIP116b, are located on chromosomes 7 and 3, respectively (Fig. 6-2). From the rice database we found another BIP116b homolog, namely OsXklp (Fig. 6-4).

6.2.2 Hd3a and BIP116b interact in the yeast system

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 (Fig. 6-3).

To further study the interaction between Hd3a and BIP116b *in vitro*, we performed a GST pull-down assay. A GST-BIP116b fusion protein was pulled down with His-tagged Hd3a. However, we could not find an interaction between Hd3a and BIP116b, even though both proteins were expressed in *E. coli* (data not shown). One possible explanation for this discrepancy is that post-translational modification of BIP116b may be necessary for binding.

6.2.3 Tissue-specific localization of BIP116b

To determine whether the *BIP116b* gene was expressed in particular rice organs, we performed semi-quantitative RT-PCR using specific primers. *BIP116* was transcribed in all organs tested (Fig. 6-5), suggesting that it functions in rice growth and development.

6.2.4 BIP116b overexpressing plants and BIPab RNAi plants

We generated BIP116 overexpressing plants and double transgenic BIPa and BIPb RNAi plants to further study the function this gene in rice growth and development, particularly during floral transition. For BIP116b overexpressing plants, we have obtained T0 generation plants that show interesting phenotypes: increased tiller number, emergence of new tillers from nodes, and semidwarf/dwarf size (Fig. 6-6, Table 6-1).

These phenotypes resemble those of Hd3a overexpressing plants, suggesting that BIP116b may be involved in the same signaling pathway as Hd3a.

We generated transgenic plants, either suppressed BIPa or BIPb (Fig. 6-7). Moreover, BIP116 double transgenic plants were produced, and T1 generation plants were analyzed for flowering time phenotype. The results showed that the BIP116ab RNAi plants exhibited delayed flowering compared to wild-type plants (Fig. 6-8).

6.5 Discussion

In this study, we identified a novel Hd3a interacting protein, namely BIP116b in yeast two-hybrid screens. Sequence analysis of *BIP116b* revealed that this gene was different from the annotated sequence from NCBI database because of the misprediction of splicing sites. BIP116b is predicted has a TPX2 (Targeting protein for Xklp2) domain. This family represents a conserved region approximately 60 residues long within the eukaryotic targeting protein for Xklp2. Xklp2 is a kinesin-like protein localized on centrosomes throughout the cell cycle and on spindle pole microtubules during metaphase.

BIP 116b has two homologous in rice, OsXklp like protein and BIP116b. Interestingly, only BIP116b has a potential phosphorylation site for Ca^{2+} dependent protein kinase (Fig. 6-2) similar to the site presence in OsKANADI and Arabidopsis FD.

The finding that Hd3a interacts with BIP116b in yeast two-hybrid screening, also open a new perspective the possibility of cross-talk between brassinosteroid signaling and Hd3a signaling. However, it will be necessary to confirm the interaction between Hd3a and BIP116 *in vivo* using the BiFC system and/or co-immunoprecipitation. It has been reported that the rice BRI1 kinase domain interacting

protein 116a (BIP116a) interacts with the BR receptor (Hirabayashi et al. 2004). It would be interesting to test whether BIP116b interacts with the BRI1 receptor.

Hd3a moves along the leaf cells through the phloem and enters the SAM, but the mechanism of Hd3a signaling during this movement is largely unknown. Our finding that BIP116 may interact with BR receptors should stimulate further characterization of its function. Based on our current understanding of the BRI1 receptor in *Arabidopsis* (Vert at al. 2005), BIP116 interacts with LRR 20 of the extracellular domain of BRI1. It has been reported that the expression of rice BR11 is very high in the shoot apex (Yamamuro et al. 2000). It is possible that the BIP116b-Hd3a complex interacts with BRI1 before Hd3a enters the SAM.

In the present study, we found an interesting phenotype in BIP116b overexpressed plants. They exhibited branching and increasing tiller number similar to Hd3a overexpressed plants. We could not provide the data for flowering time in this T0 generation plants. We also generated the double RNAi plants with suppressed both of BIP116b and its homolog, BIP116a. In T1 generation this mutant displayed the delayed flowering phenotype compare to the wild-type plants. Therefore, further characterization in mutant plants will be interesting to get insight its fuction.

CHAPTER 7

CONCLUSIONS AND PERSPECTIVES

In this study, using yeast two-hybrid screening, we successfully identified a wide range of proteins that interact with the florigen Hd3a in rice, and, interestingly, they are mainly involved in signaling. Our study has focused on three candidate proteins: GF14c (a 14-3-3 homolog), OsKANADI (a novel transcription factor containing a GARP domain) and the BRI1 kinase domain interacting protein 116b (BIP116b).

Since 14-3-3 proteins interact with many proteins that are involved in signal transduction, we further characterized the association between GF14c and Hd3a to explore its function in plant growth and development, particularly in flowering regulation. Transgenic plants overexpressing GF14c exhibited a delay in flowering and, conversely, the T-DNA inserted mutant plants showed an earlier flowering phenotype relative to wild-type plants. These results strongly suggest that the function of GF14c is to act as a negative regulator of flowering by interacting with Hd3a.

Our study has suggested a role for GF14c in Hd3a signaling in rice. As the floral stimulus that controls floral transition in the SAM, Hd3a has the capacity to traffic from cell to cell and to move long distances via the phloem. Hd3a is a small protein with a molecular weight of 23 kDa, and may therefore be able to move from companion cell to sieve element. Overexpression of GF14c may cause inhibition of Hd3a trafficking to the SAM and lead to a delay in flowering. In the absence of GF14c, Hd3a moves freely into the nucleus and activates floral induction. The BiFC experiment supported this hyphothesis because it revealed that the Hd3a-GF14c complex is mainly

localized in the cytoplasm. Many questions remain to be answered, however, because the mechanism of floral induction in the SAM needs to be properly explored.

Since the 14-3-3 proteins have been shown to interact with FT protein in tomato and *Arabidopsis*, our results in rice represent an important addition to our knowledge about FT signaling in plants. The functional characterization of other Hd3a interactors should be a priority for future research.

Figure 1-1. Molecular model of Hd3a. This model of the 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).

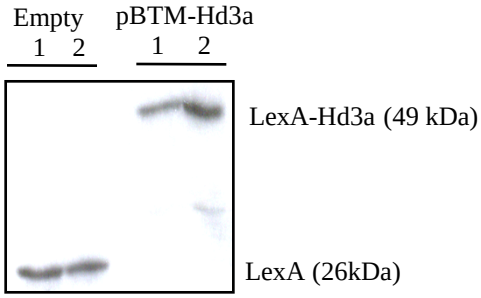


Figure 3-1. The Hd3a protein expression (pBTM-Hd3a) in yeast strain L40. The protein was run on 10% SDS-PAGE and electrotransferred onto an Immobilon-P membrane followed by detection of Hd3a-LexA fusion using the anti-LexA antibody. Molecular weight of Hd3a-Lex A and Lex A are 49 kDa and 26kDa, respectively.

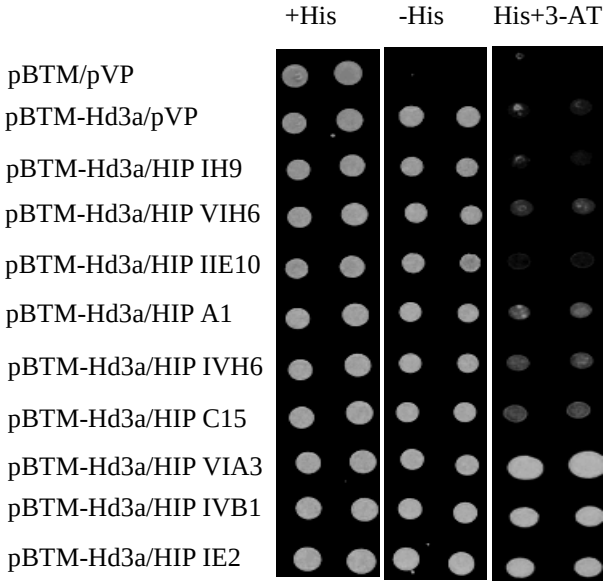


Figure 3-2. Interaction of Hd3a with its partners in yeast two-hybrid system. The growth of yeast colonies on the plate (-LWH) lacking leucine (L), tryptophan (W) and histidine and with 2.5 mM 3-AT (3-aminotriazole) indicates a positive interaction between Hd3a and the particular Hd3a-interacting proteins. 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.

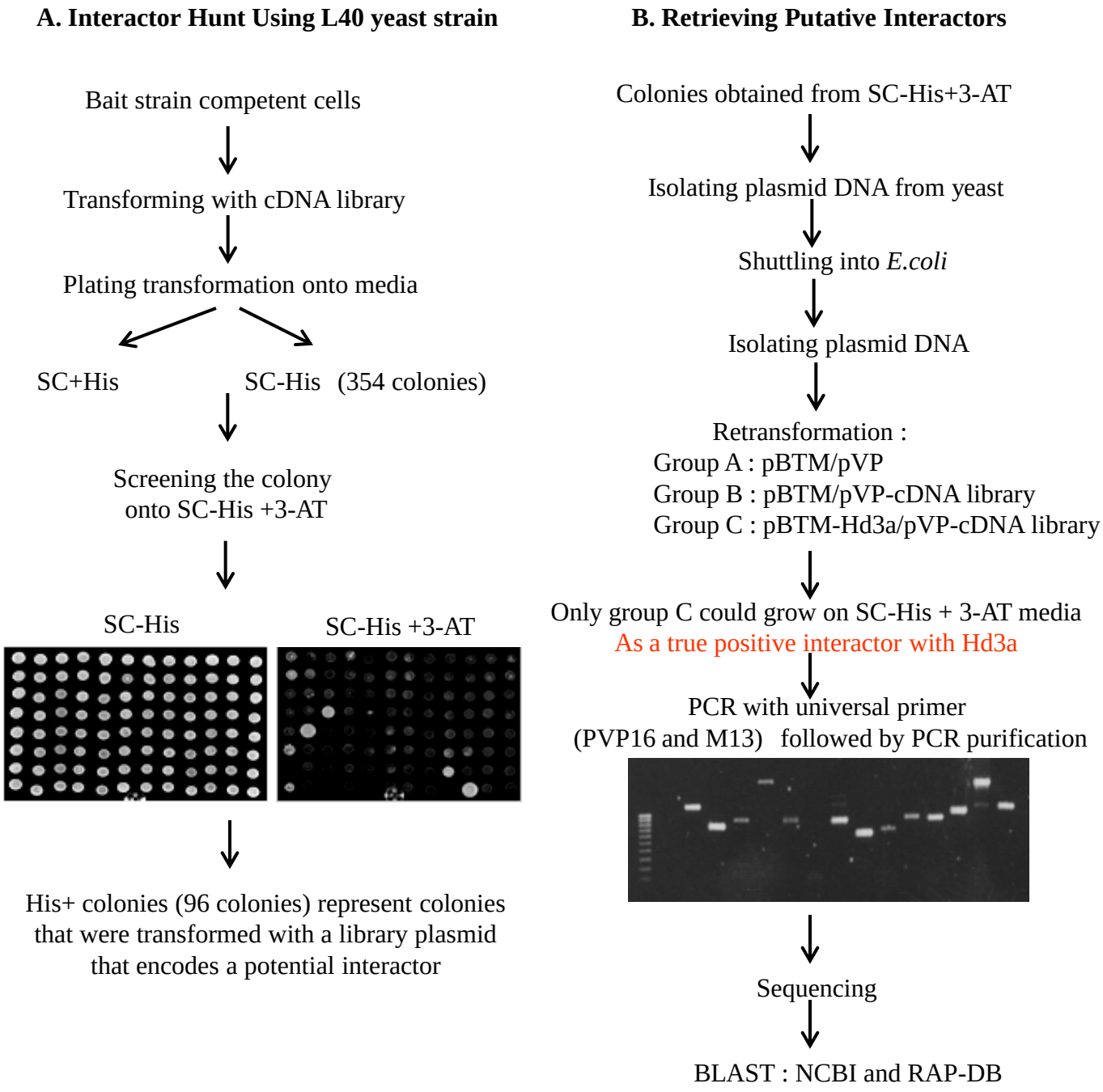


Figure 3-3. Flowchart of yeast two-hybrid screen to retrieve putative Hd3a interactors.

Table 3-1 Protein identified by yeast two-hybrid screening

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 & RNA binding proteins				
Putative RNA helicase	gi|34897325	127	756	-
Putative 40S ribosomal protein	gi|50939279	211	305	-
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,chaper-one,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

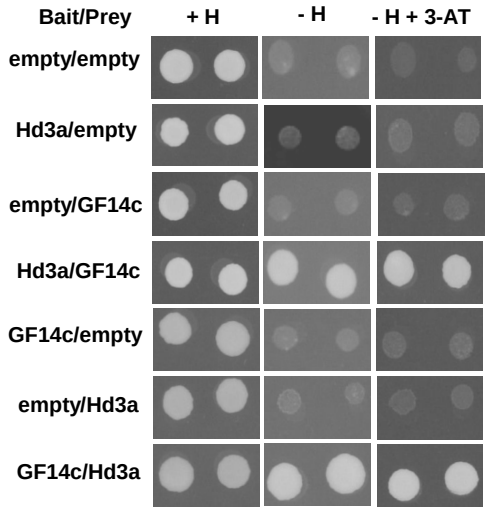


Figure 4-1. Identification of GF14c as an Hd3a interacting protein in yeast. Growth of yeast colonies on plates lacking histidine (-H) or lacking histidine containing 2.5 mM 3-aminotriazole (3-AT) indicates a positive interaction between Hd3a and GF14c. Two independent clones are shown for each treatment.

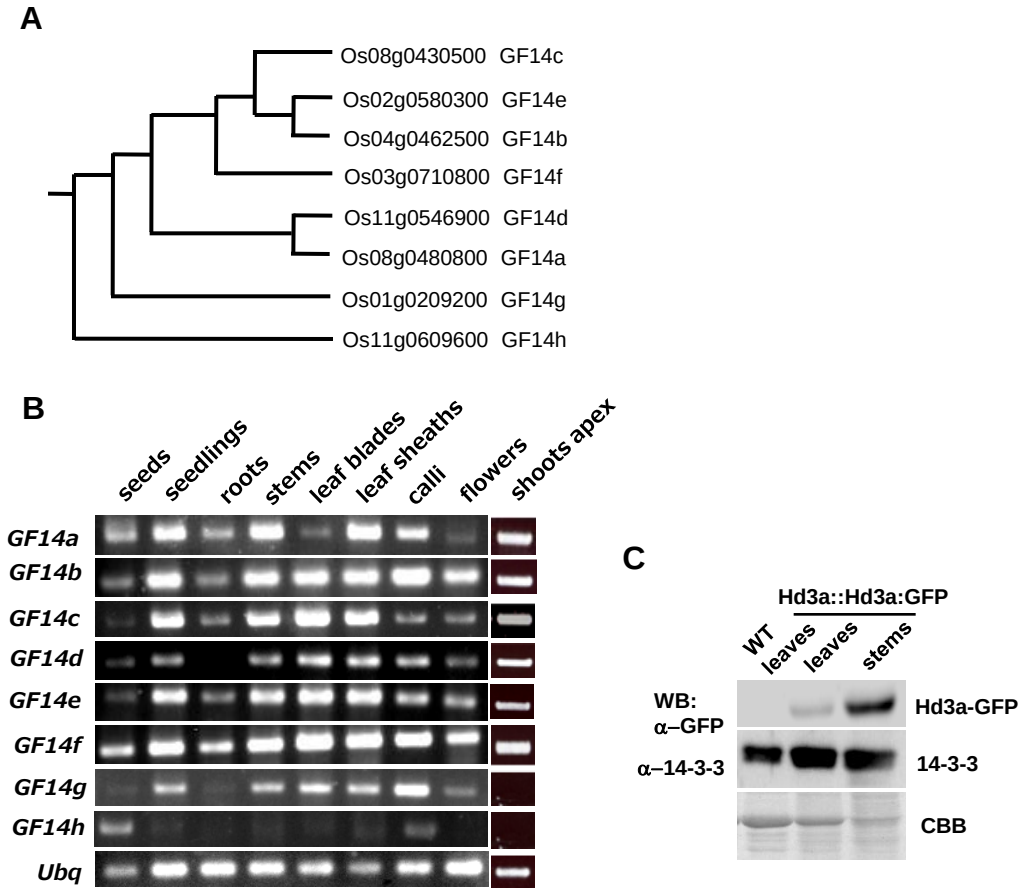


Figure 4-2. Tissue-specific localization of Hd3a and GF14s family members in rice. (A) A phylogenetic tree was constructed with the ClustalW program using the neighbor-joining method. The eight GF14 isoforms share a high degree of amino acid identity (85-95%). (B) Rice *GF14* family members are expressed in all organs tested. Using gene-specific primers, RT-PCR was performed with 1 μg of total RNA extracted from various organs of the wild-type N8 cultivar. (C) Immunoblot detection of cytosolic fractions of wild-type and Hd3a-ox plants. Hd3a and GF14s were detected by α-GFP and by α-14-3-3 antibody, respectively. Coomassie brilliant blue staining (CBB) was used as a loading control.

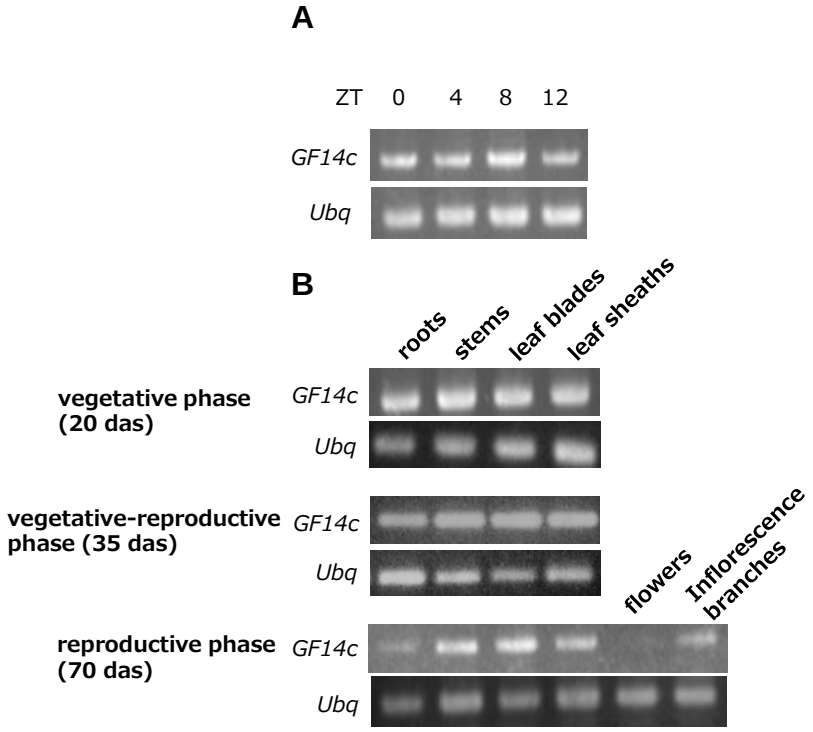


Figure 4-3. Diurnal and developmental patterns of *GF14c* expression in wild-type plants under SD conditions. (A) RT-PCR was performed with 1 μg of total RNA extracted from leaf at four hour intervals time starting with ZT 0. *GF14c* does not show a diurnal expression pattern. (B) GF14c was expressed throughout all developmental stages.

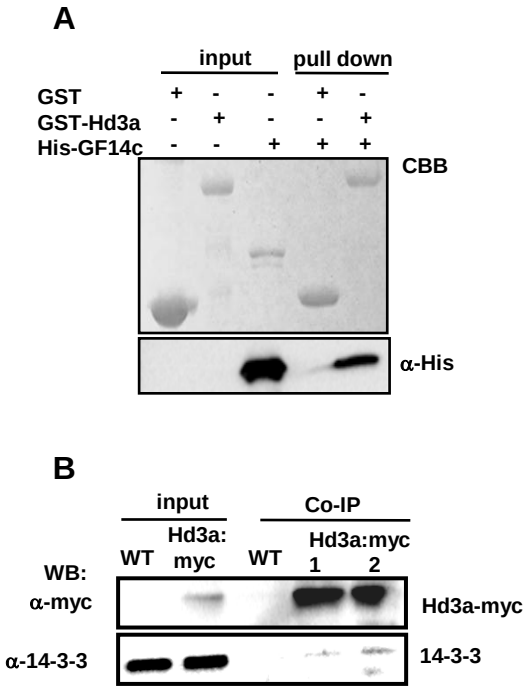


Figure 4-4. *In vitro* and *in vivo* interaction of Hd3a with GF14c. (A) Hd3a-GST interacts with GF14c-His *in vitro.* GF14c-tagged His was pulled down by Hd3a-tagged GST. GF14c was detected with an α–His antibody. (B) *In vivo* interaction of Hd3a and GF14s in rice. Western blotting of proteins precipitated from 35S::Hd3a:myc rice culture cells using α-myc antibody reveals the presence of 14-3-3 as co-immunoprecipitant with Hd3a. 1 and 2 indicate two independent rice suspension cultures.

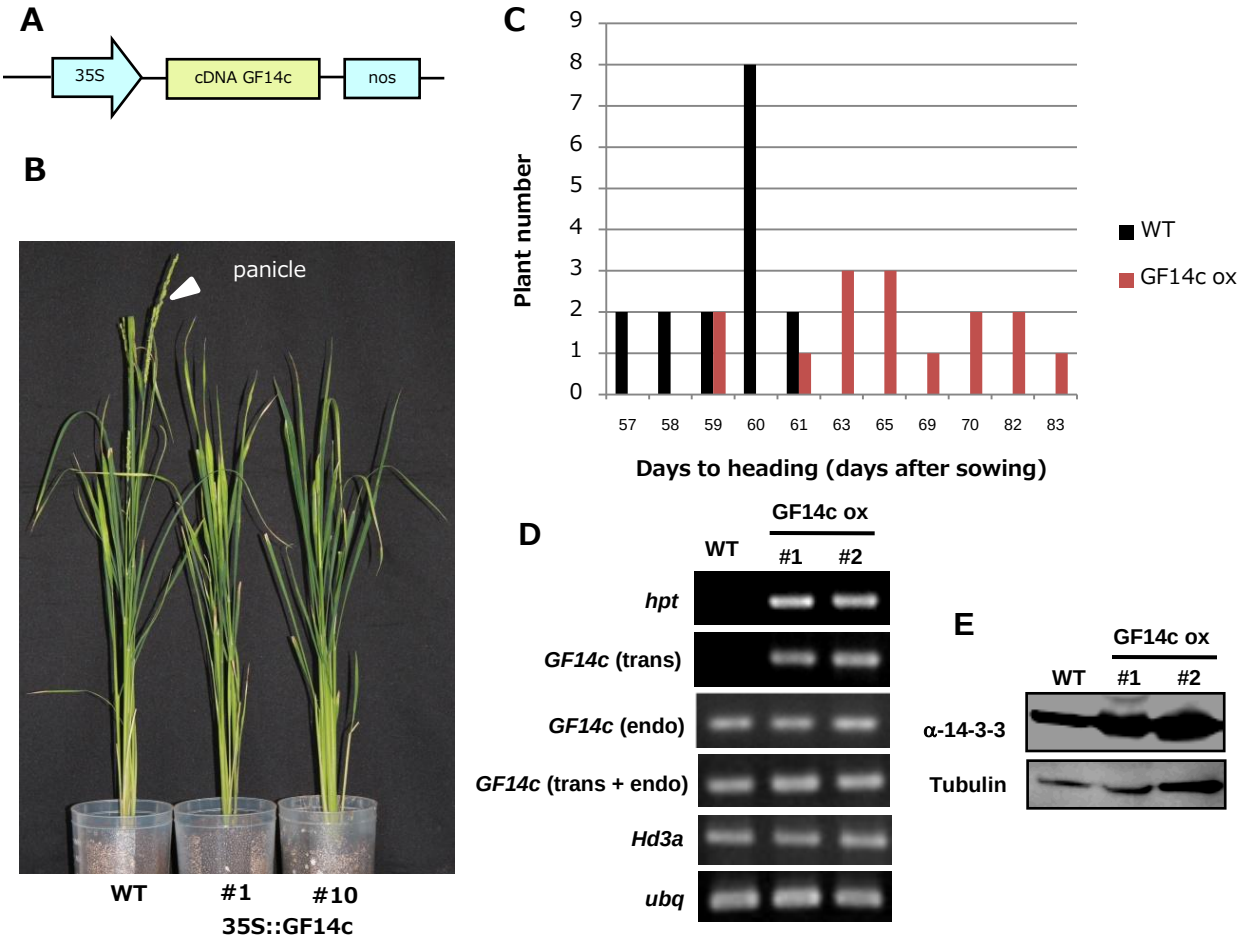


Figure 4-5. Characterization of GF14c-overexpressing (GF14c-ox) plants. (A) GF14c expression was controlled by CaMV 35S promoter. (B) Growth of N8 wild-type and GF14c-ox plants under SD conditions. The arrow indicates a panicle. (C) Flowering time of GF14c-ox plants (GF14c ox) was delayed under SD conditions. (D) *GF14c* and *Hd3a* mRNA levels in wild-type and GF14c-ox plants. 1 and 2 indicate independent transgenic lines. The *GF14c* transgene was detected in transgenic plants. *Hd3a* levels in transgenic plants were not changed compare to wild-type. (E) GF14c protein levels in GF14c-ox plants. Tubulin was used as a loading control.

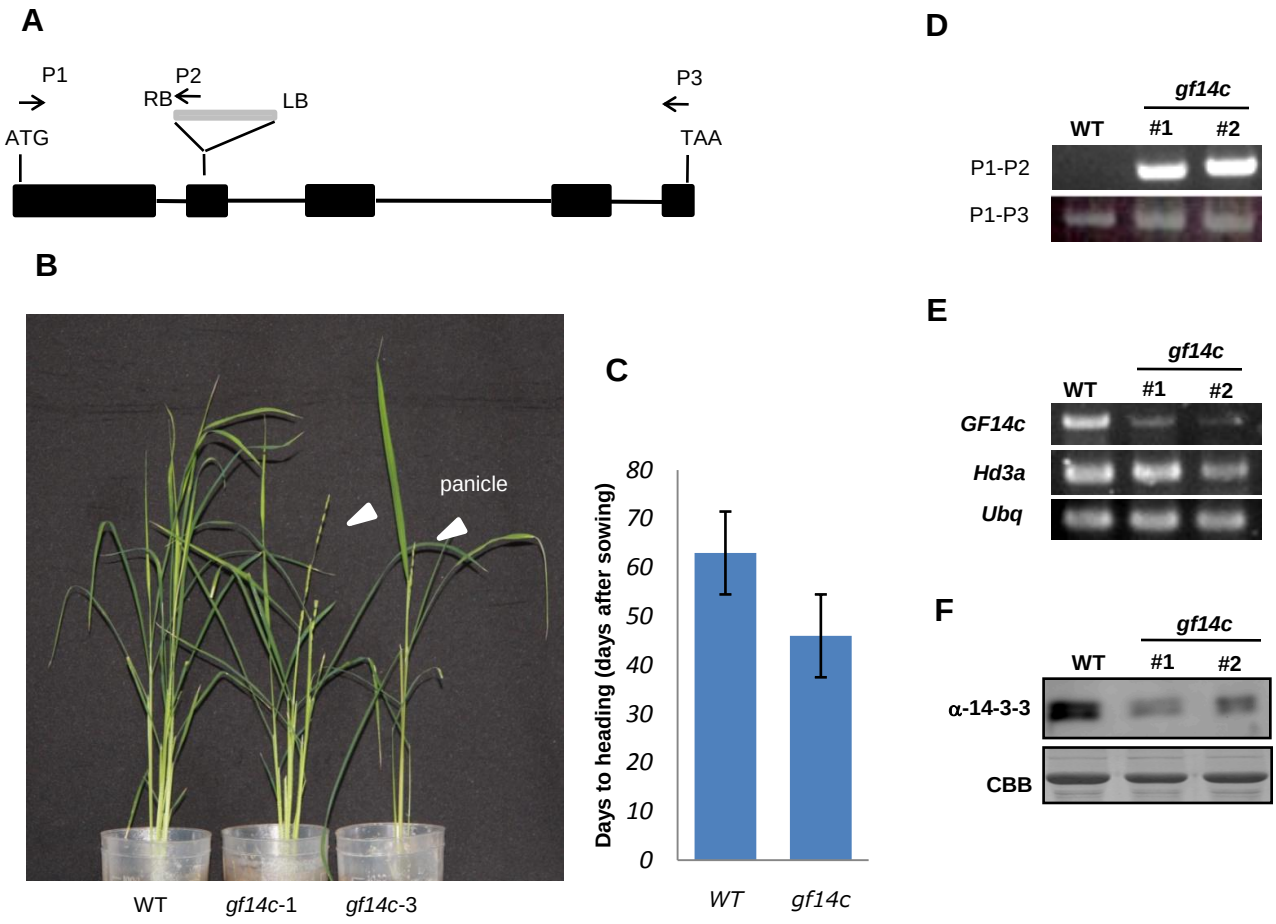


Figure 4-6. Characterization of *gf14c* T-DNA-insertion plants. (A) Structure of genomic *GF14c* (Os08g0430500) and position of T-DNA insertion. *GF14c* consists of five exons (filled boxes) and four introns (lines). The T-DNA was inserted into the second exon in the opposite orientation to the direction of *GF14c* transcription. Arrows indicate primers used for analyzing the T-DNA insertion site. LB and RB represent the left and right borders of T-DNA, respectively. (B) Growth of wild-type and two *gf14c* mutant plants (cv Dongjin) under SD conditions. Arrows indicate panicles. (C) Heading date of *gf14c* mutant and wild-type plants under SD conditions. Error bars represent standard error of the mean. (D) PCR analysis of GF14c with primers (Supplemental Table 1) indicated in (A). (E) *GF14c* and *Hd3a* expression mRNA levels in wild-type and *GF14c* expressing plants. (F) The 14-3-3 protein expression level of *gf14c* plants. Coomassie brilliant blue staining (CBB) was used as a loading control.

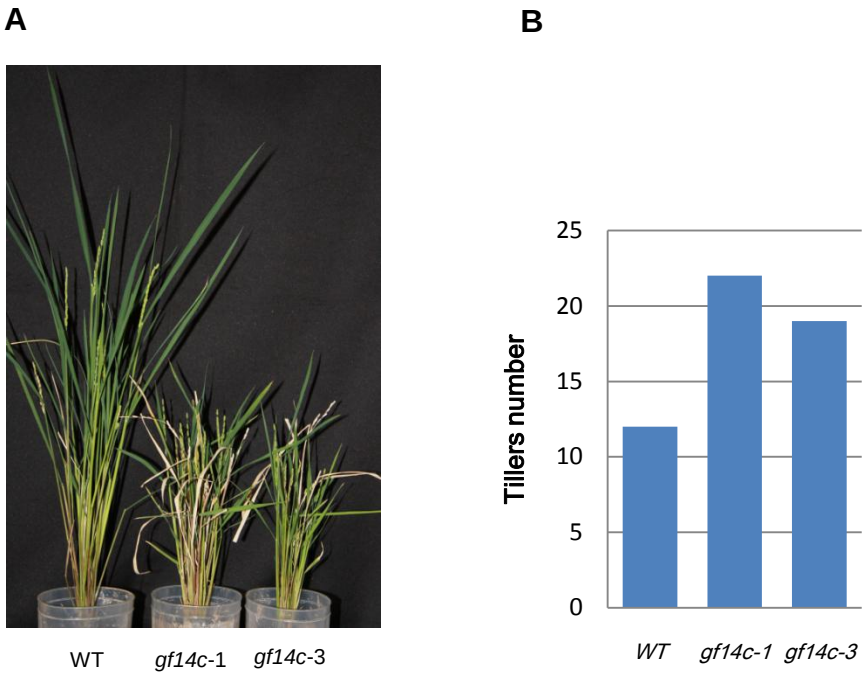


Figure 4-7. High-tillering phenotype of *gf14c* T-DNA insertion plants. (A) Growth of wild-type and two *gf14c* mutant plants (cv 'Dongjin') for 100 days under SD conditions. *gf14c* mutant plants showed increased tiller numbers and dwarf phenotype. (B) Tiller numbers of wild-type and *gf14c* mutants.

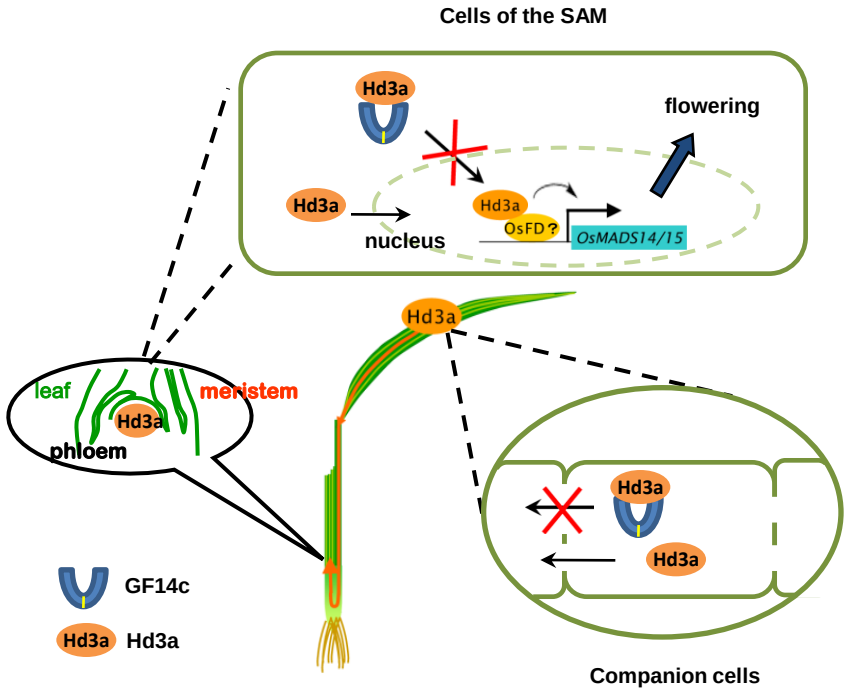


Figure 4-8. Possible role of GF14c in Hd3a signaling in rice flowering. As the floral stimulus that controls floral transition in the shoot apical meristem (SAM), Hd3a has the capacity to traffic cell to cell and move long distances via the phloem. Hd3a is a small protein with molecular weight 23 kDa, thus it may be able to move from companion cell to sieve elements. The overexpression of GF14c may cause inhibition of Hd3a trafficking to SAM and leads to a delay in flowering. In the absence of GF14c, Hd3a may move freely into the nucleus and activates floral induction.

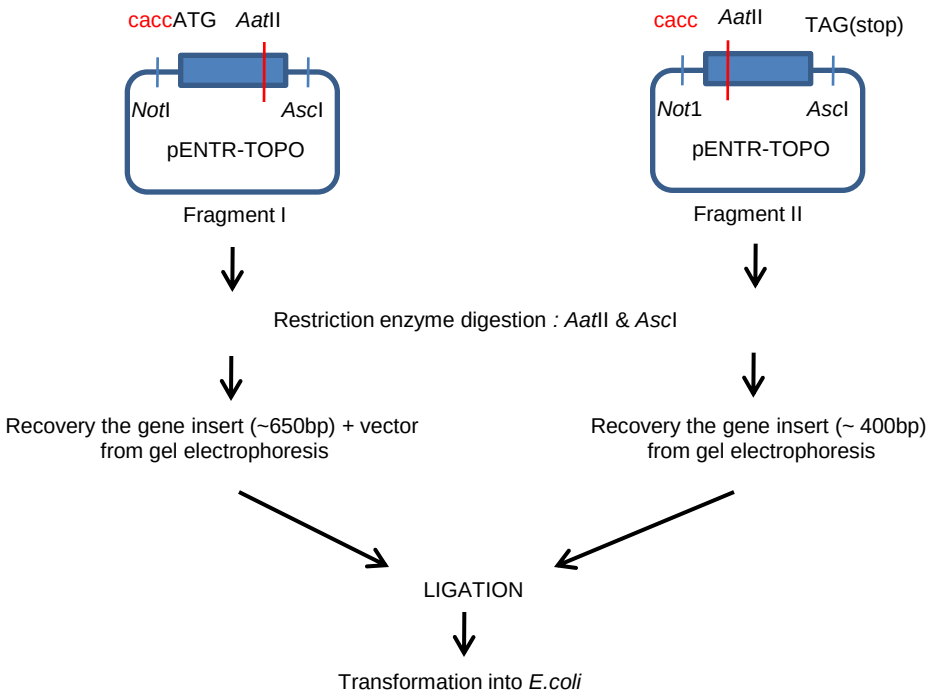


Figure 5-1. Diagram representative strategy of cloning full length *OsKANADI1*

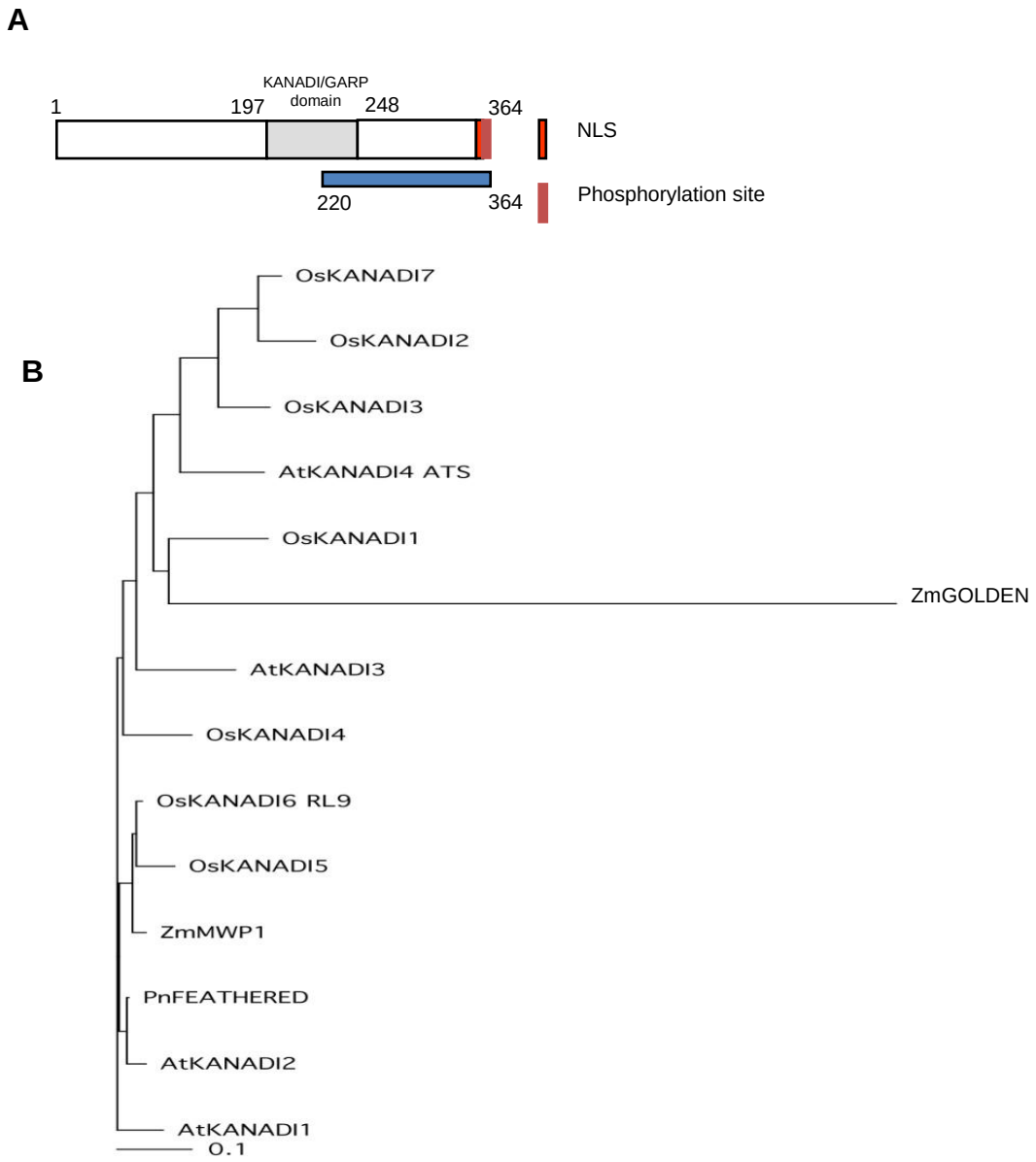


Figure 5-2. Sequence analysis of OsKANADI1. (A) OsKANADI1 predicted as myb transcription factor like that has GARP domain that important for DNA binding. C-terminal region of OsKANADI1 have nuclear localization signal (NLS) and potential phosphorylation site. (B) A phylogenetic tree was constructed with the ClustalW program using the neighbor-joining method. Accession numbers are as follows : OsKANADI 1(Os02g06996900), OsKANADI2 (Os03g0624000), OsKANADI3 (Os03g0766500), OsKANADI4 (Os08g0160300), OsKANADI5 (Os08g0426866), OsKANADI6 –RL9 (Os09g0395300), OsKANADI7 (Os12g0591600), AtKANADI1(At5g16560), AtKANADI2 (At1g32240), AtKANADI3 (At4g17695), AtKANADI4 (At5g42630), MWP1 (EU925398), InFEATHERED (AB220968).

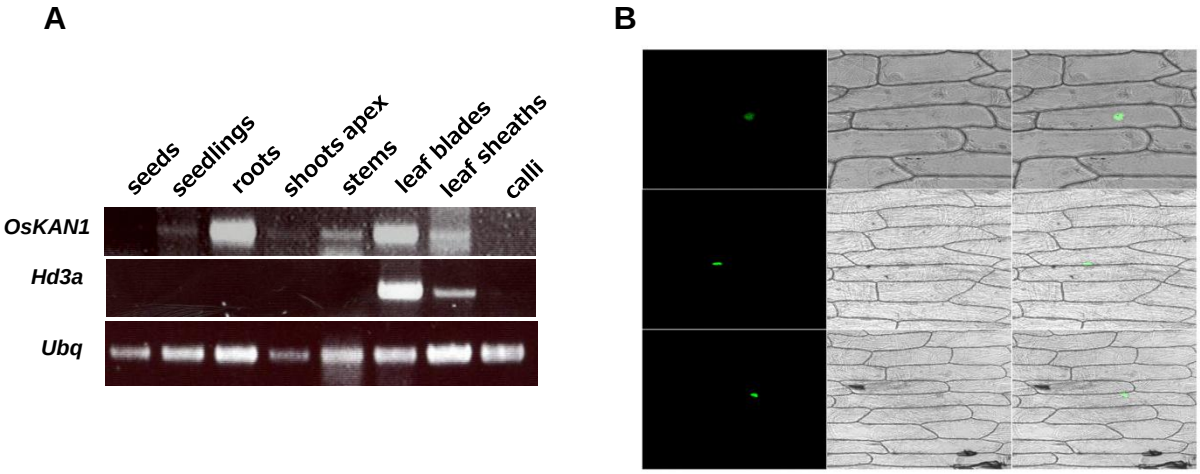


Figure 5-3. *OsKANADI1* expression and subcellular localization. (A) Semi-quantitative RT-PCR of *OsKANADI1* in various organs of wild-type N8 rice. (B) Subcellular localization of OsKAN1-GFP fusion protein in the nucleus
Note : Fig 5-3 B was provided by Yuka Ogaki

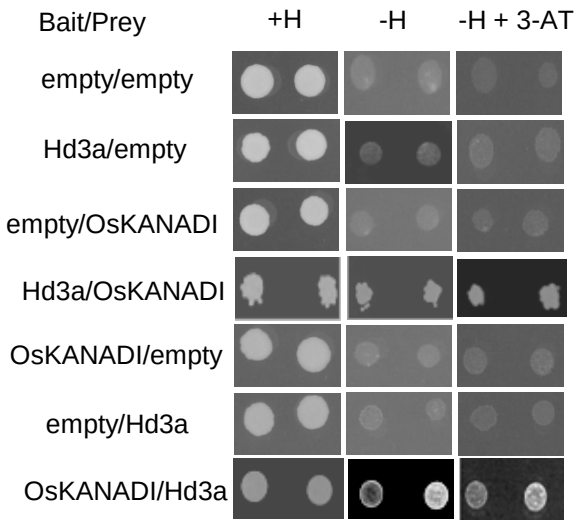


Figure 5-4. Identification of OsKANADI1 as an Hd3a interacting protein in yeast. The swapping experiment using full-length OsKANADI1, either as bait or prey suggested a strongly interaction with Hd3a. The growth of yeast colonies on the plates lacking histidine (-H) or lacking histidine plus 2.5 mM 3-aminotriazole (3-AT) indicates a positive interaction between Hd3a and GF14c. Two independent clones used as replication.

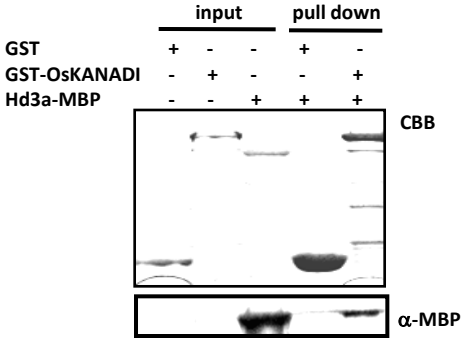


Figure 5-5. *In vitro* interaction of Hd3a with OsKANADI1. (A) Hd3a-MBP interacts with GST-OsKANADI *in vitro*. Hd3a-tagged MBP was pulled down by OsKANADI1-tagged GST. Hd3a was detected with an a-MBP antibody.

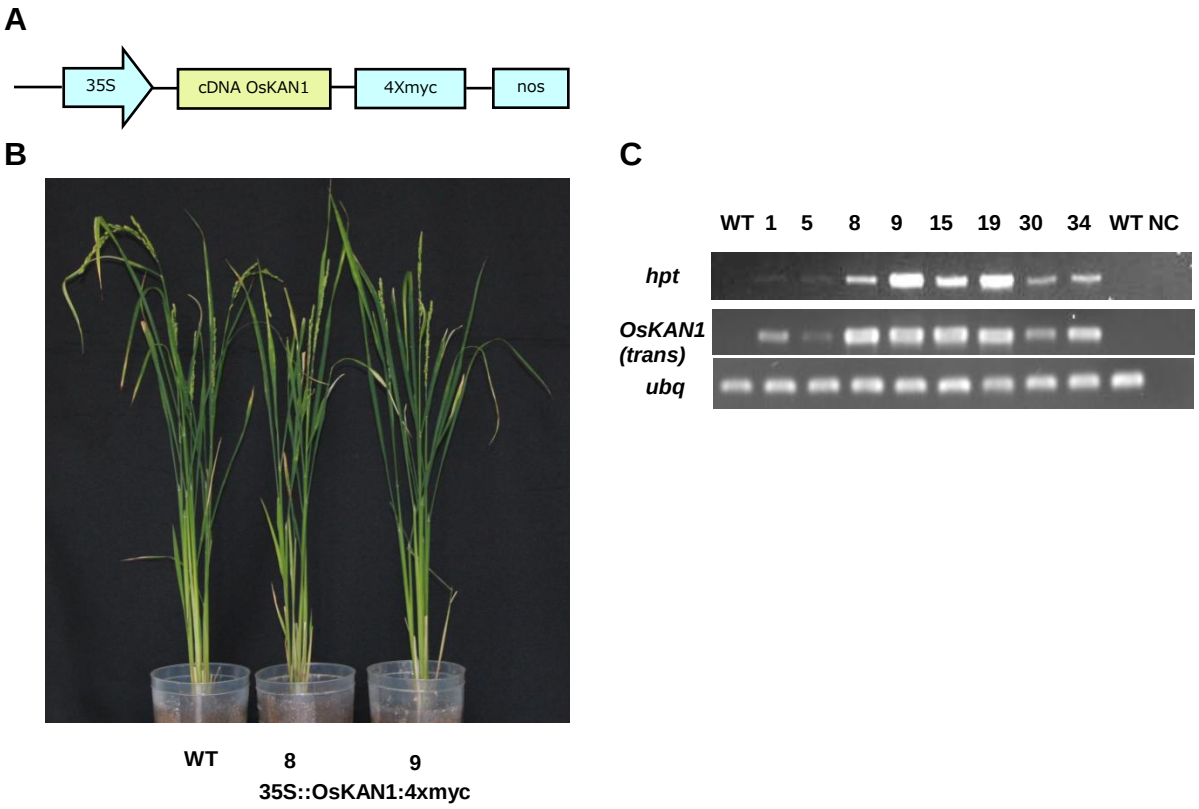


Figure 5-6. Characterization of OsKANADI1-overexpressing plants. (A) *OsKAN1*-overexpression construct was controlled by CaMV 35S promoter and tagged with 4Xmyc. (B) Growth of N8 wild-type and *OsKAN1*-overexpressing plants under SD conditions. (C) Semi quantitative RT-PCR in transgenic plants. The *OsKAN1* transgene was detected in transgenic plants using specific primers. Several lines showed overexpression of *OsKAN1* (lines 8, 9,15,19). Ubiquitin was used as loading control.

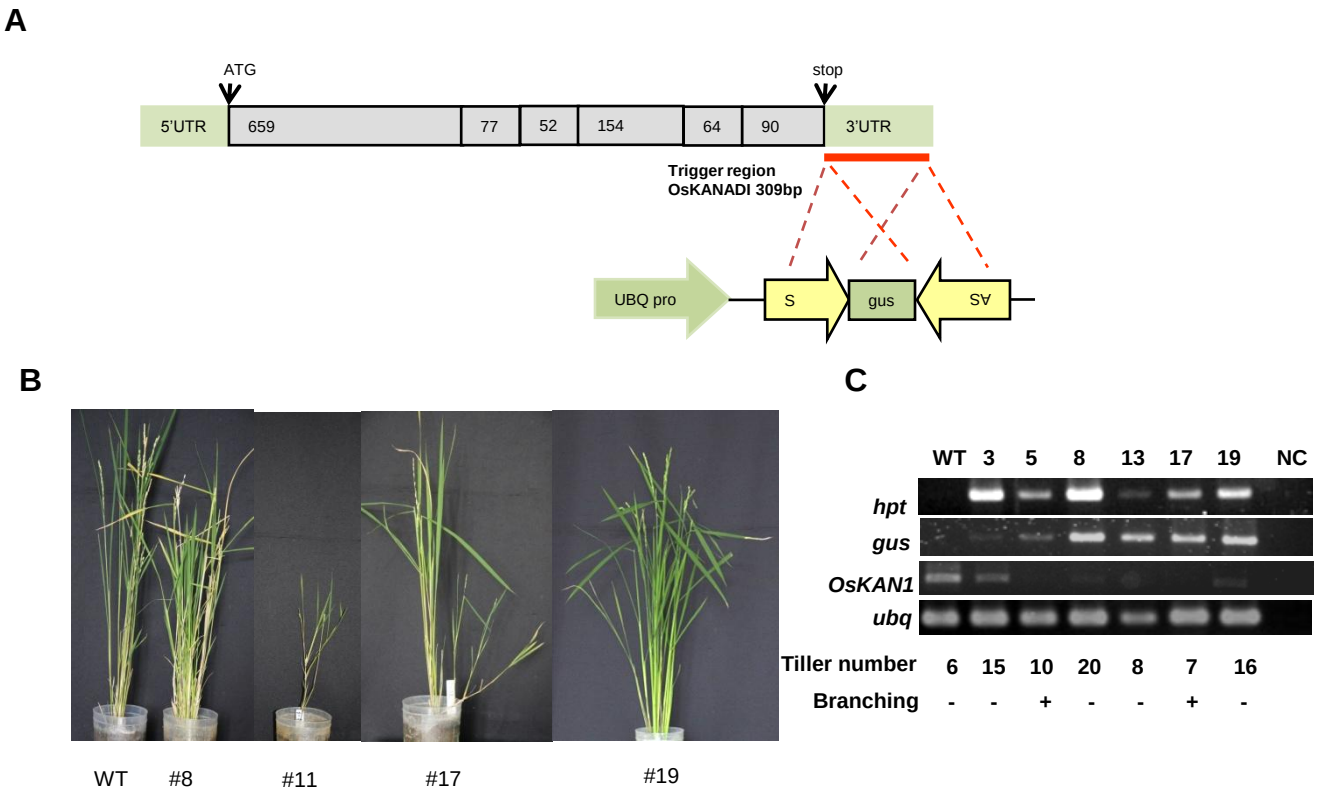


Figure 5-7. Characterization of OsKANADI1-RNAi plants. (A*) OsKAN1-RNAi* construct. Gene specific region (3UTR) was used for *OsKAN1 RNAi* construct. (B) Growth of N8 wild-type and OsKAN1 RNAi plants under SD conditions. (C) Semi quantitative RT-PCR in transgenic plants. The *OsKAN1* transgene was detected in transgenic plants using *gus* primers. Using specific primers, several lines showed supression of *OsKAN1*. Ubiquitin was used as loading control.

```
seed(N8)   1:-----------------MAARGGRWDLPEKAADSTPSGKGVVEMSTDDESDCVVICPPNG 43
BIP116b    1:MAARGGGRRTAASRGVASAHGGAELRTTAARGGSRPSGKGVVEMSTDDESDCVVICPPNG 60
                            *  *           * ************************

seed(N8)  44:KAGHTEVMSGRHDEDSSRGQETPSTIDSHMNGNVQDGVPADQDVLKLVDQQKSSLPSSPI 103
BIP116b   61:KAGHTEVMSGRHDEDSSRGQETPSTIDSHMNGNVQDGVPADQDVLKLVDQQKSSLPSSPI 120
             ************************************************************

seed(N8) 104:NHGIAEQEESNHTVPQPFAPATEREDSGEGDCTPVPHPTSNGEKLSDKSSTSLASMAKKS 163
BIP116b  121:NHGIAEQEESNHTVPQPFAPATEREDSGEGDCTPVPHPTSNGEKLSDKSSTSLASMAKKL 180
             ************************************************************

seed(N8) 164:PSVTPRKPLQADSTSHSHEDDSYSVTSTVTSARTGKIKKTTVPVAPTFICGNRLEKRGEF 223
BIP116b  181:HSSR-----------------------TVTSARTGKIKKTTVPVAPTFICGNRLEKRGEF 217
              *                        *********************************

seed(N8) 224:YTKLEEKRKALEEEKLEAEARKKEEQEEALKQLRKNLVIRAKPMPSFYQEGPPPKVELKK 283
BIP116b  218:YTKLEEKRKALEEEKLEAEARKKEEQEEALKQLRKNLVIRAKPMPSFYQEGPPPKVELKK 277
             ************************************************************

seed(N8) 284:VPPTRAKSPKLTRRKSCSDTPHTPEGKNGSAACCRLHRHSIGNSKEVNSRTQCSPKSAPK 343
BIP116b  278:VPPTRAKSPKLTRRKSCSDTPHTPEGKNGSAACCRLHRHSIGNSKEVNSRTQCSPKSAPK 337
             ************************************************************

seed(N8) 344:TGVAAKPRATKGVMKNVGKPGAANVAVQT                               372
BIP116b  338:TGVAAKPRATKGVMKNVGKPGAANVAVQT                               366
             ****************************
```

Figure 6-1. Alignment of the deduced amino acid sequence of putative seed specific protein annotated in NCBI database (upper line) with its homolog BRI1 KD Interacting Protein (BIP116b) from rice.

Figure 6-2. Alignment of the deduced amino acid sequence of BIP116a with its homolog BIP116b from rice. They belong to a KLEEK domain proteins (red square). It might be has a potential phosphorylation site for Ca^{2+}-dependent protein kinase (grey box), a target binding site of 14-3-3 proteins (RSXpSXP – pS indicates a critical phosphoserine in their target proteins) and additional sequences at C-terminal region (red letters).

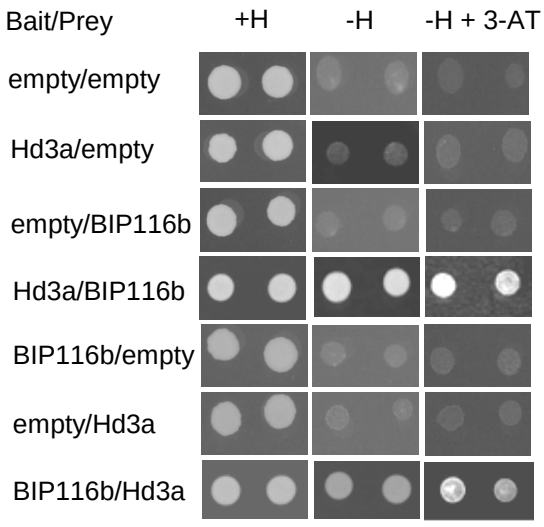


Figure 6-3. Identification of BRI1 Kinase domain interacting protein 116b (BIP116b) as an Hd3a interacting protein in yeast. The swapping experiment using full-length BIP116b, either as bait or prey suggested a strongly interaction with Hd3a. The growth of yeast colonies on the plates lacking histidine (-H) or lacking histidine plus 2.5 mM 3-aminotriazole (3-AT) indicates a positive interaction between Hd3a and GF14c. Two independent clones used as replication.

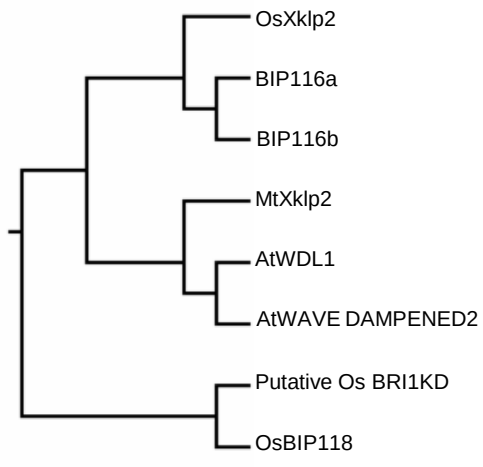


Figure 6-4. A phylogenetic tree of BIP116b family. It was constructed with the ClustalW program using the neighbor-joining method. Accession numbers are as follows : OsXklp2 (gi|108711574), BIP116a (gi|42733494), MtXklp2 (gi|92874845), AtWDL1 (gi|79312782), AtWAVE DAMPENED2 (gi|28453880), Putative Os BRI1KD (gi|50906049), and OsBIP118 (gi|42733498)

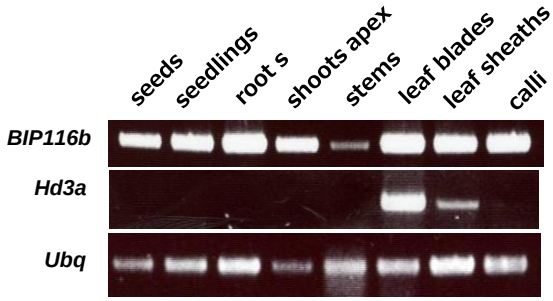


Figure 6-5. Semi-quantitative RT-PCR of *BIP116b* in various organs of wild-type N8 rice.

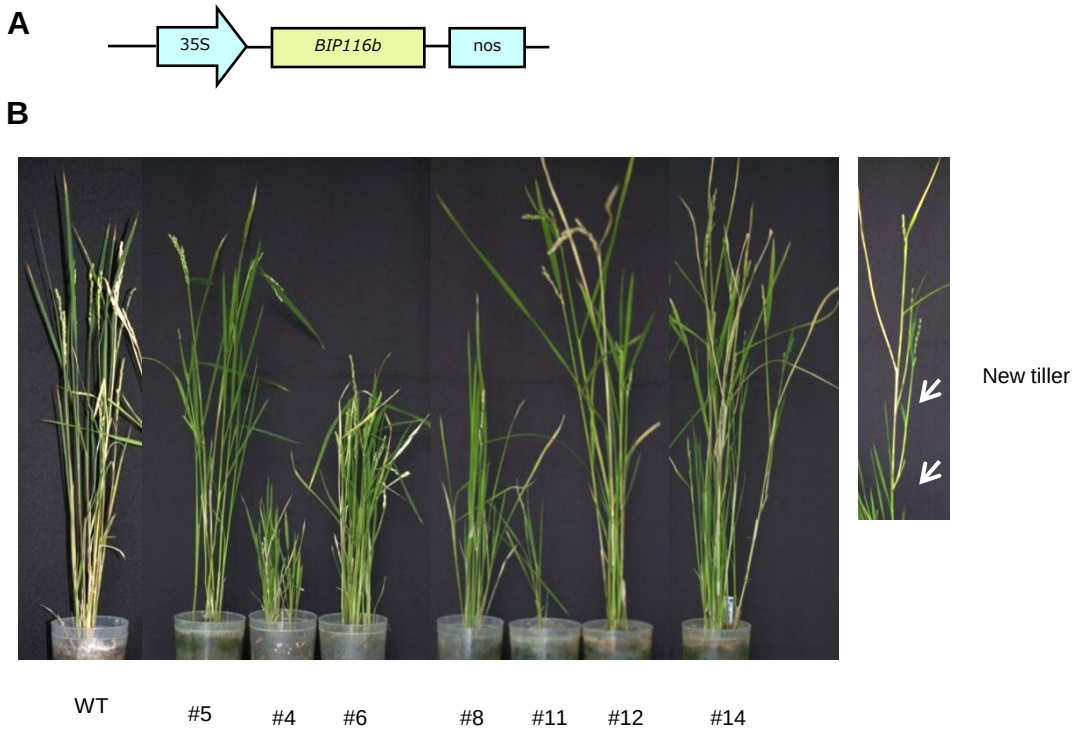


Figure 6-6. Characterization of *BIP116b*-overexpressing plants. (A*) BIP116b*-overexpression construct was controlled by CaMV 35S promoter .(B) Growth of N8 wild-type and BIP116b-overexpressing plants under SD conditions. (C) Semi quantitative RT-PCR in transgenic plants. The *BIP116b* transgene was detected in transgenic plants using specific primers. Several lines showed overexpression of *OsKAN1* (lines 4, 6, 8). Ubiquitin was used as loading control.

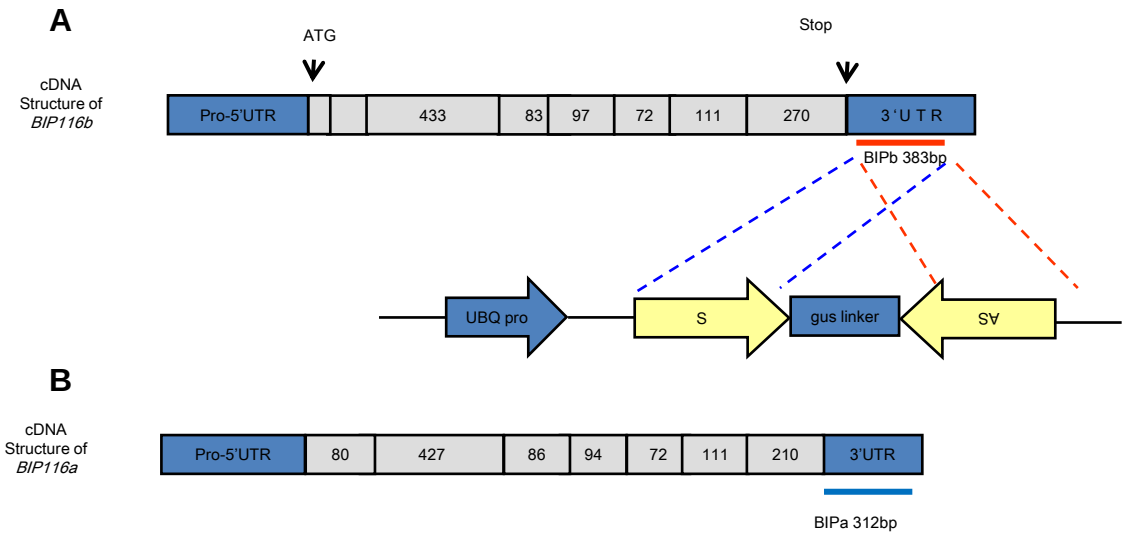


Figure 6-7. RNAi construct for BIP116a and BIP116b transgenic plants (A) cDNA structure of BIP116b (B) cDNA structure of BIP116a. The red and blue underline represent the trigger regions used for RNAi constructs. S indicate the sense and AS indicate the anti sense fragment

Table 6-1. Phenotypic analysis of BIP116b overexpressing plants (T0 generation)

Line	Tiller number	Stem folks	fertility	height
WT	6	-	fertile	normal
#1	24	+	fertile (few seeds)	normal
#4	10	-	sterile	dwarf
#5	11	-	fertile	normal
#6	12	-	fertile (few seeds)	Semi dwarf
#8	21	+	sterile	Semi dwarf
#11	6	+	sterile	dwarf
#12	15	+	fertile (few seeds)	Semi dwarf
#14	20	+	sterile	normal

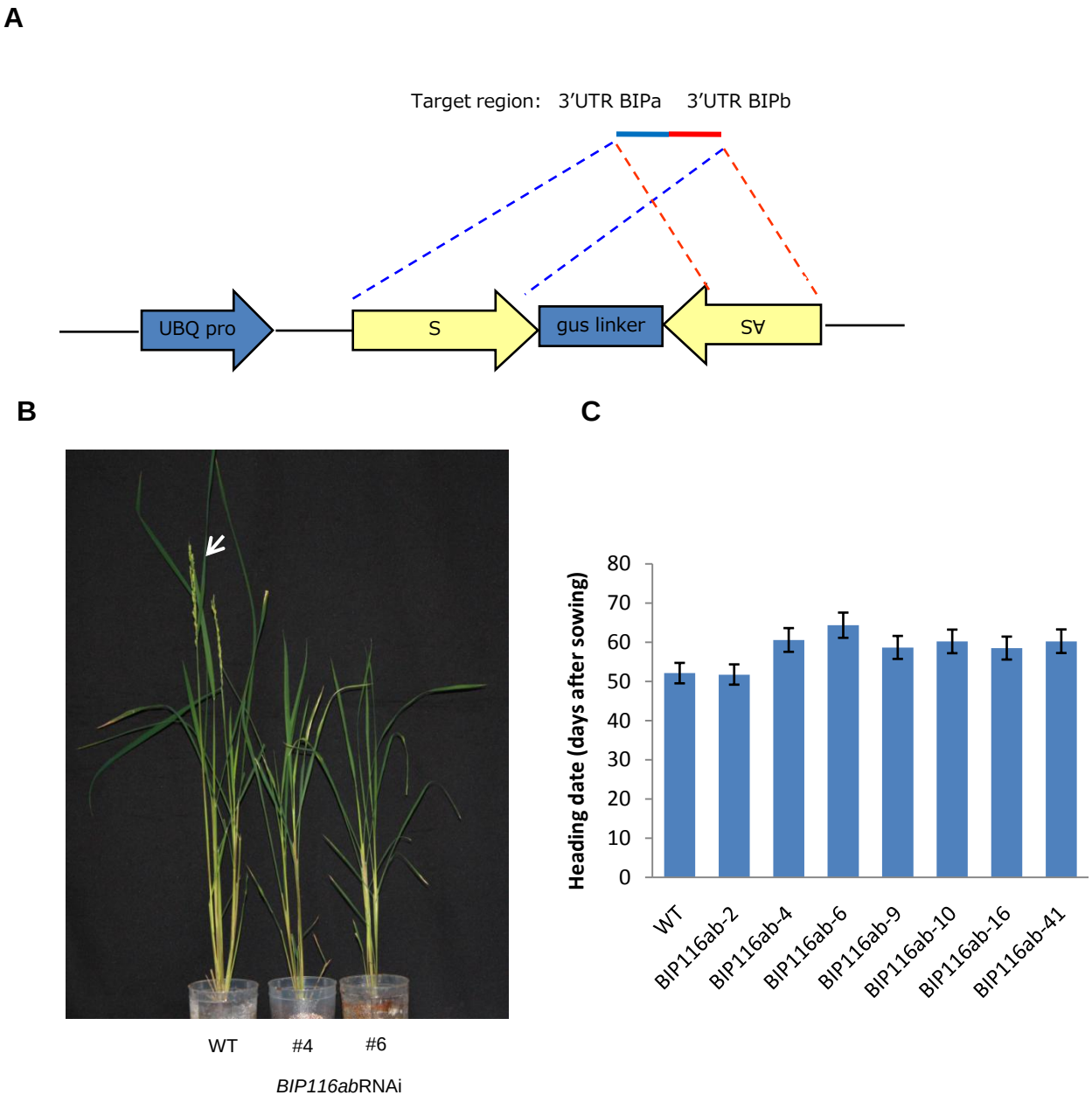
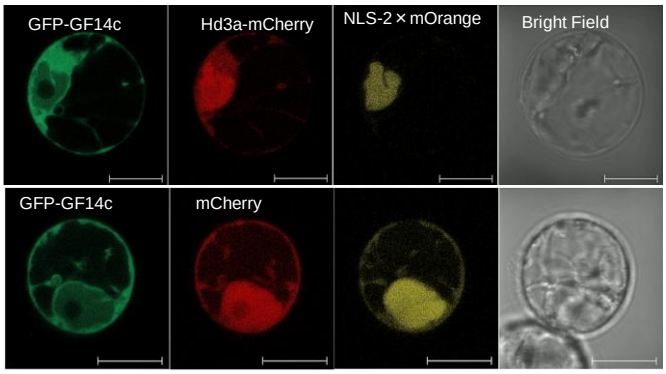
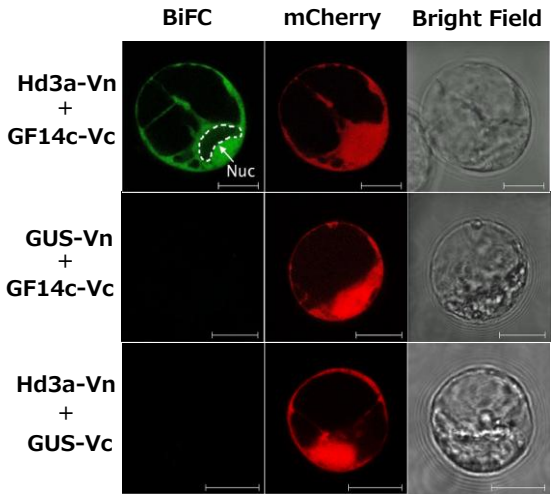


Figure 6-8. Characterization of *BIP116ab-RNAi* plants. (A*) BIP116ab-RNAi* construct. Gene specific regions (3UTR) of *BIP116a* and *BIP116b* were used for *BIP116ab RNAi* construct. (B) Growth of N8 wild-type and BIP116ab RNAi plants under SD conditions.White arrow indicate panicle. (C) Several *BIP116ab-RNAi* lines exhibited delay flowering and reduce height compare to non transgenic control plants.



Supplemental Figure 1. Subcellular localization of Hd3a and GF14c. GF14c is localized mainly in cytosol. Transient expression of GFP-GF14c and Hd3a-mCherry in rice protoplasts was driven by the CaMV 35S and ubiquitin promoter, respectively. NLS-2xmOrange was used as marker for the nucleus. mCherry was used as a marker of the nucleus and cytoplasm. Upper panels: co-expression of GFP-GF14c and Hd3a-mCherry proteins. Lower panels: co-expression of GFP-GF14c and mCherry proteins. Bar=10 μm (This figure provided by Yuka Ogaki).



Supplemental Figure 2. BiFC analysis of the interaction between Hd3a and GF14c in rice protoplasts. GUS-Vn and GUS-Vc were used as negative control. Bars=10 μm (This figure provided by Yuka Ogaki).

Appendix Table 1. Primers sequences used in this study

Primer name	Sequence (5' to 3')
Primers for GF14c full–length construct	
pENTR GFc-F	CAC CAT GTC TCG GGA GGA GA
pENTR GFc-R/P3	TTA CTG GCC CTC GCA GGC GT
pENTR GFc-F (del stop)	TGC GAG GGC CAG AAG GGT GGG CGC
pENTR GFc-R (del stop)	GCG CCC ACC CTT CTG GCC CTC GCA
Primers for yeast two-hybrid	
pBTMHd3a-F	GTCGACATGGCCGGAAGT
pBTMHd3a-R	CTAGACTAGTACCTAGGGGT
Primers for GF14s family expression	
GF14a-F	GGA CAT AGC CAT GAA GGA GC
GF14a-R (3UTR)	CAT CAT AAC TAC CCC CAG CG
GF14b-F	TTT GGC TGA GCT GCC TCC TA
GF14b-R (3UTR)	TCC CGA CAA CAA TCA CAG CC
GF14c-F	TTG CTC CCA CCC ATC CCA TA
GF14c-R (3UTR)	GAC AGC AGC ATG AGG CAT CT
GF14d-F	ACA AGG CTG CTC AGG ACA TTG
GF14d-R (3UTR)	ACA TCA ATG TCC ATT GCC CC
GF14e-F	CTG GAG CTG AGA GGA AGG AT
GF14e-R (3UTR)	GCG CAC GCA TTC GAT CAT GT
GF14f-F	GCT GAG AAC ACT CTT GTG GC
GF14f-R (3UTR)	GCA GGT AGC TGA GTA AAT GG
GF14g-F	CTA CCG GTA CCT TGC AGA GT
GF14g-R (3UTR)	ACC AGC TAT TAG CAC CTC CA
GF14h-F	TCC ATT GGC CAA TTC GGG TG
GF14h-R (3UTR)	GGA CCA AGG TGT TCA GTC AAG C
Primers for screening GF14c transgenic plants	
HPT-F	GAGCCTGACCTATTGCATCTCC
HPT-R	GGCCTCCAGAAGAAGATGTTGG
GFc-F (endo)	AAT GGG AAG ATC GAT CGA TCG ATG GC
GFc-R (endo)	AGA CGG ACC ACT AGC ACC AA
GFc-F (trans)	ATG TCT CGG GAG GAG AAT GTC
GFc-R (trans)-rgwt	CTG TTA TCA ACC ACT TTG TAC AAG AA
GFc-F (trans+endo)	ATG TCT CGG GAG GAG AAT GTC
GFc-R (trans+endo)	TGA GGT CAG AGG TCC AGA GG
UBQ-F	CACAAGAAGGTGAAGCTCGC
UBQ-R	GCCTTCTGGTTGTAGACGTAGG
P2 (Right border)	CCACAGTTTTCGCGATCCAGACTG
P1 (GF14c start)	ATG TCT CGG GAG GAG AAT GTC

Appendix Table 1. Primers sequences used in this study

Primer name	Sequence (5' to 3')
Primers for BIP116b full–length construct	
pENTR BIP116b-F	CACCATGGCGGCGCGCGGGGGCCGGTGGGATCT
pENTR BIP116b-R	TTAGGTTTGTACAGCAACATTCGC
pENTR BIP116b-F (del stop)	GCT GTA CAA ACC AAG GGT GGG CGC G
pENTR BIP116b-R (del stop)	CGC GCC CAC CCT TGG TTT GTA CAG C
Primers for OsKANADI1 full–length construct	
pENTR-OsKAN1-F (Fragment I)	CACCATGGAGCTGTTCCCGGCACA
OsKAN1-R Fragment I)	TCTCTCGTGGCCTCCGAGAA
OsKAN1-F (Fragment II)	CACCATGCGGTGGACGACGTCGCT
OsKAN1-R (Fragment II)	TCATGGCGCTGACAGTCGTC
pENTR OsKAN1-F (del stop)	CTG TCA GCG CCA AAG GGT GGG CCG
pENTR OsKAN1-R (del stop)	CGG CCC ACC CTT TGG CGC TGA CAG
Primers for RNAi constructs	
BIP 116a –F (RNAi)	CAC CGC ATT GCG AAC CCT AA
BIP 116a –R (RNAi)	ACA TGG TCC CAG CAA CAG AG
BIP 116b –F (RNAi)	CCG AAG AGT GCT CCA AAG AC
BIP 116b –R (RNAi)	TGC GAA CGC CGC CAT ACA TC
BIP116ab-F (RNAi)	CAC CGA GGG GAG AGT TTT AC
BIP116ab-R (RNAi)	CAC CGC ATT GCG AAC CCT AA
OsKAN1-F (RNAi)	CAC CAG GAT CCA GCA TGT GT
OsKAN1-R (RNAi)	CCC TCT TCA CCC AAT CTC CG
Primers for screening BIP116b and OsKAN1 transgenic plants	
BIP116b-F1	GAT GTA TGG CGG CGT TCG CA
BIP116b-F2	CGC TGG CAA GTA TGG CGA AG
BIP116b-R1	TGTATGAGGGGTATCGCTGC
BIP116b-R2 (339)	GGCTGAGGAACAGTGTGGTT
OsKAN1-F1	CGG AGA TTG GGT GAA GAG GG
OsKAN1-F2	TGTACCGGACCATCAAGACC
OsKAN1-R1 (281)	GAGCTGGTGATGTTGGTGGT
GUS-F	GTATCAGTGTGCATGGCTGG
GUS-R	CATCAGCACGTTATCGAATCC
BIP116b-F(5UTR)	TTCTGTCTCACTTGTGCGCG
OsKAN-F (5UTR)	CCAAGCAACAAGTTACACCCCC
OsKAN-R (5UTR)	CTCTTCTTGTCCTTGCGATGAC

References

Abe, M., Fujiwara, M., Kurotani, K., Yokoi, S. and Shimamoto, K. (2008) Identification of dynamin as an interactor of rice GIGANTEA by tandem affinity purification (TAP). Plant Cell Physiol. 49: 420-32.

Abe, M., Kobayashi, Y., Yamamoto, S., Daimon, Y., Yamaguchi, A., Ikeda, Y., et al. (2005) FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. Science 309: 1052–1056.

Ahn, J., Miller, S., Winter, V., Banfield, M., Lee, J., Yoo, S., et al. (2006) A divergent external loop confers antagonistic activity on floral regulators FT and TFL1. EMBO J. 25: 605–614.

Aki, T., Shigyo, M., Nakano, R., Yoneyama,T. and Yanagisawa, S. (2008) Nano scale proteomics revealed the presence of regulatory proteins including three FT-Like proteins in phloem and xylem saps from rice. Plant Cell Physiol. 49: 767-90.

Aoki, K., Suzui, N., Fujimaki, S., Dohmae, N., Sakakibara, K.Y., Fujiwara, T., Hayashi, H., Yamaya, T. and Sakakibara, H. (2005) Destination-selective long distance movement of phloem protein. Plant Cell 17: 1801-1814.

Banfield, M. and Brady, R. (2000) The stucture of *Antirrhinum* centroradialis protein (CEN) suggests a role as a kinase regulator. J. Mol. Biol. 297: 1159-1170.

Böhlenius, H., Huang, T., Charbonnel-Campaa, L., Brunner, A.M., Jansson, S., Strauss, S.H., Nilsson, O. (2006a) CO/FT regulatory module controls timing of flowering and seasonal growth cessation in trees. Science 312: 1040-1043.

Böhlenius, H., Eriksson, S., Parcy, F. and Nilsson, O. (2007b) (retraction) The mRNA of the Arabidopsis gene FT moves from leaf to shoot apex and induces flowering. Science. 316: 367

Boss, P.K., Bastow, R.M., Mylne, J.S. and Dean, C. (2004) Multiple pathways in the decision to flower: enabling, promoting, and resetting. Plant Cell. 16 Suppl: S18-31.

Candela, H., Johnston, R., Gerhold, A., Foster, T. and Hake, S. (2008) The milkweed pod1 gene encodes a KANADI protein that is required for abaxial/adaxial patterning in maize leaves. Plant Cell 20: 2073-2087.

Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., et al. (2007). FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis.* Science 316:1030 -1033.

Corbesier, L. and Coupland, G. (2006)The quest for florigen: a review of recent progress. J. Exp. Bot. 57: 3395-3403.

Chardon, F. and Damerval, C. (2005) Phylogenomic analysis of the PEBP gene family in cereals. J. Mol. Evol. 61:579–90.

Chen, F., Li, Q., Sun, L., and He, Z. (2006a) The Rice 14-3-3 Gene family and its involvement in responses to biotic and abiotic stress. DNA Res. 13: 53–63.

Chen, S., Tao, L., Zeng, L., Vega-Sanchez, M.E., Umemura, K. and Wang, G.L. (2006b). A highly efficient transient protoplast system for analyzing defense gene expression and protein-protein interaction in rice. Mol. Plant Pathol. 7: 417-427.

Dai, M., Hu, Y., Zhao, Y., Liu, H., and Zhou, D.X. (2007) A WUSCHEL-LIKE HOMEOBOX gene represses a YABBY gene expression required for rice leaf development. Plant Physiol. 144: 380-390.

Doi, K., Izawa, T., Fuse, T., Yamanouchi, U., Kubo, T., Shimatani, Z., et al. (2004) Ehd1, a B-type response regulator in rice, confers short-day promotion of flowering and controls FT-like gene expression independently of Hd1l. Genes Dev.18: 926–36.

Domagalska, M.A., Schomburg, F.M., Amasino, R.M., Vierstra, R.D., Nagy, F. and Davis, S,J. (2007) Attenuation of brassinosteroid signaling enhances FLC expression and delays flowering. Development. 134: 2841-2850

Emery, J.F., Floyd, S.K., Alvarez, J., Eshed, Y., Hawker, N.P., Izhaki, A., Baum, S.F. and Bowman, J.L. (2003) Radial patterning of Arabidopsis shoots by class III HD-ZIP and KANADI genes. Curr. Biol. 13:1768-1774.

Endo, T., Shimada, T., Fujii, H., Kobayashi, Y., Araki, T. and Omura, M. (2005) Ectopic expression of an FT homolog from citrus confers an early flowering phenotype on trifoliate orange (Poncirus trifoliata L. Raf.). Transgenic Res.14: 703-712.

Eshed, Y., Baum, S.F., Bowman, J.L. (1999) Distinct mechanisms promote polarity establishment in carpels of Arabidopsis. Cell 99:199-209.

Eshed, Y., Baum, S.F., Perea, J.V., Bowman, J.L. (2001) Establishment of polarity in lateral organs of plants. Curr Biol. 11: 1251-1260.

Eshed, Y., Izhaki, A., Baum, S.F., Floyd, S.K., Bowman, J.L. (2004) Asymmetric leaf development and blade expansion in Arabidopsis are mediated by KANADI and YABBY activities. Development 131: 2997-3006.

Ferl, R. (1996) 14-3-3 proteins and signal transduction. Annu. Rev. Plant Physiol. Plant Mol. Biol 47: 49-73.

Fukuda, A., Fujimaki, S., Mori, T., Suzui. N., Ishiyama, K., Hayakawa, T., Yamaya, T., Fujiwara, T., Yoneyama, T. and Hayashi, H. (2005) Differential distribution of protein expressed in companion cells in the sieve element- companion cell complex of rice plants. Plant Cell Physiol. 46: 1779-1786.

Fukuda, A., Okada, Y., Suzui. N., Fujiwara, T., Yoneyama, T. and Hayashi, H. (2004) Cloning and characterization of the gene for a phloem-specific glutathione S-transferase from rice leaves. Physiol. Plantarum 120: 595-602.

Gampala, S., Kim, T., He, J., Tang, W., Deng, Z., Bai, M., et al. (2007). An essential role for 14-3-3 proteins in brassinosteroid signal transduction in Arabidopsis. Dev. Cell 13: 177-189.

Giakountis, A. and Coupland, G. (2008). Phloem transport of flowering signals. Curr Opin Plant Biol. 11: 687-694.

Giavalisco, P., Kapitza, K., Kolasa, A., Buhtz, A. and Kehr, J. (2006) Towards the proteome of Brassica napus phloem sap. Proteomics 6: 896-909.

Hanzawa, Y., Money, T. and Bradley, D. (2005) A single amino acid converts a repressor to an activator of flowering. Proc. Natl Acad. Sci.USA 102: 7748–7753.

Hawker, N.P. and Bowman, J.L. (2004) Roles for Class III HD-Zip and KANADI genes in Arabidopsis root development. Plant Physiol. 2004 135: 2261-2270.

Hayama, R., Izawa, T. and Shimamoto, K. (2002) Isolation of rice genes possibly involved in the photoperiodic control of flowering by a fluorescent differential display method. Plant Cell Physiol. 43: 494-504.

Hayama, R., Yokoi, S., Tamaki, S., Yano, M. and Shimamoto, K. (2003) Adaptation of photoperiodic control pathways produces short-day flowering in rice. Nature 422: 719-722.

Hirabayashi, S., Matsushita, Y., Sato, M., Oh-I, R., Kasahara, M., Abe, H., and Nyunoya, H (2004)Two proton pump interactors identified from a direct phosphorylation screening of a rice cDNA library by using recombinant BRI1 receptor kinase. Plant Biotechnol 21: 35–45.

Hoshikawa, K. The growing rice plant an anatomical monograph. (1989), Nobunkyo, Japan. p 225.

Hosoda, K., Imamura, A., Katoh, E., Hatta, T., Tachiki, M., Yamada, H., Mizuno, T. and Yamazaki, T. (2002) Molecular structure of the GARP family of plant

Myb-related DNA binding motifs of the Arabidopsis response regulators. Plant Cell 14: 2015-2029.

Igarashi, D., Ishida, S., Fukazawa, J., and Takahashi, Y. (2001). 14-3-3 proteins regulate intracellular localization of the bZIP transcriptional activator RSG. Plant Cell, *13*(11), 2483-2497.

Imaizumi, T. and Kay, S. (2006) Photoperiodic control of flowering: not only by coincidence. Trends in Plant Science 11: 550-558.

Ishida, S., Fukazawa, J., Yuasa, T. and Takahashi, Y. (2004) Involvement of 14-3-3 signaling protein binding in the functional regulation of the transcriptional activator REPRESSION OF SHOOT GROWTH by gibberellins. Plant Cell, *16*(10), 2641-2651.

Izawa, T. (2007a) Daylength measurements by rice plants in photoperiodic short-day flowering. Int Rev Cytol. 256:191–222.

Izawa, T., Oikawa, T., Tokutomi, S., Okuno, K. and Shimamoto, K. (2000b) Phytochromes confer the photoperiodic control of flowering in rice (a short-day plant). Plant J. 22: 391-399.

Jaeger, K. and Wigge, P. (2007). FT protein acts as a long-range signal in *Arabidopsis*. Curr. Biol. 17 : 1050 -1054.

Jang, S., Hur, J., Kim, S.J., Han, M.J., Kim, S.R. and An, G. (2004a) Ectopic expression of OsYAB1 causes extra stamens and carpels in rice. Plant Mol Biol. 56: 133-143.

Jang, S., Marchal, V., Panigrahi, K.C., Wenkel, S., Soppe, W., Deng, X.W., Valverde, F. and Coupland, G. (2008b) Arabidopsis COP1 shapes the temporal pattern of CO accumulation conferring a photoperiodic flowering response. EMBO J. 27: 1277-1288.

Kardailsky, I., Shukla, V.K., Ahn, J.H., Dagenais, N., Christensen, S.K., Nguyen, J.T., Chory, J., Harrison, M.J. and Weigel, D. (1999) Activation tagging of the floral inducer FT. Science. 286: 1962-1965.

Kerstetter, R.A., Bollman K., Taylor A., Bomblies, K. and Poethig, R.S. (2001) *KANADI* regulates organ polarity in Arabidopsis. Nature 411:706-709.

Kim, S.L., Lee, S., Kim, H.J., Nam, H.G. and An, G. (2007) OsMADS51 is a short-day flowering promoter that functions upstream of Ehd1, OsMADS14, and Hd3a. Plant Physiol. 145: 1484-1494.

King, R.W., Moritz, T., Evans, L.T., Martin, J., Andersen, C.H., Blundell, C., Kardailsky, I. and Chandler, P.M. (2006) Regulation of flowering in the long-day grass Lolium temulentum by gibberellins and the FLOWERING LOCUS T gene. Plant Physiol. 141: 498-507.

Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M. and Araki, T. (1999) A pair of related genes with antagonistic roles in mediating flowering signals. Science. 286: 1960-1962.

Kojima, S., Takahashi, Y., Kobayashi, Y., Monna, L., Sasaki, T., Araki, T., et al. (2002) Hd3a, a rice ortholog of the Arabidopsis FT gene, promotes transition to flowering downstream of Hd1 under short-day conditions. Plant Cell Physiol. 43: 1096–1105.

Komeda, Y. (2004) Genetic regulation of time to flower in *Arabidopsis thaliana*. Annu. Rev. Plant Biol. 55: 521-535.

Komiya, R., Ikegami, A., Tamaki, S., Yokoi, S. and Shimamoto, K. (2008). Hd3a and RFT1 are essential for flowering in rice. Development 135: 767-774.

Koornneef, M., Alonso-Blanco, C., Peeters, A.J. and Soppe, W. (1998) Genetic control of flowering time in Arabidopsis. Annu Rev Plant Physiol Plant Mol Biol. 49: 345-370.

Kyozuka, J. and Shimamoto, K. (1991) Transformation and regeneration of rice protoplasts. *In* K Lindsey, ed, Plant Tissue Culture Manual B1. Kluwer Academic Publishers, Dordrecht, The Nederland, pp 1-17.

Laubinger, S., Marchal, V., Le Gourrierec, J., Wenkel, S., Adrian, J., Jang, S., Kulajta, C., Braun, H., Coupland, G. and Hoecker, U. (2006) Arabidopsis SPA proteins regulate photoperiodic flowering and interact with the floral inducer CONSTANS to regulate its stability. Development. 133: 3213-3222.

Lifschitz, E., Eviatar, T., Rozman, A., Shalit, A., Goldshmidt, A., Amsellem, Z., et al. (2006) The tomato FT ortholog triggers systemic signals that regulate growth and flowering and substitute for diverse environmental stimuli. Proc. Natl Acad. Sci. USA 103: 6398–6403.

Li, J. and Chory, J. (1997) A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell. 90: 929-38.

Li, C. and Dubcovsky, J. (2008) Wheat FT protein regulates VRN1 transcription through interactions with FDL2. Plant J. 55: 543-554.

Lin, M., Belanger, H., Lee, Y., Varkonyi-Gasic, E., Taoka, K., Miura, E., et al. (2007) FLOWERING LOCUS T protein may act as the long-distance florigenic signal in the Cucurbits. Plant Cell 19: 1488–1506.

Liu, H.L., Xu, Y.Y., Xu, Z.H. and Chong, K. (2007) A rice YABBY gene, OsYABBY4, preferentially expresses in developing vascular tissue. Dev Genes Evol. 217: 629-637.

Liu, L.J., Zhang, Y.C., Li, Q.H., Sang, Y., Mao, J., Lian, H.L., Wang, L. and Yang, H.Q. (2008) COP1-mediated ubiquitination of CONSTANS is implicated in cryptochrome regulation of flowering in Arabidopsis. Plant Cell. 20: 292-306.

Lough, T. and Lucas, W. (2006). Integrative Plant Biology: Role of Phloem Long-Distance Macromolecular Trafficking. Annu. Rev. Plant Biol. 57:203–232.

Luo Z., Yang Z., Zhong B., Li Y., Xie R., Zhao F., Ling Y. and He G. (2007) Genetic analysis and fine mapping of a dynamic rolled leaf gene, RL10(t), in rice (Oryza sativa L.). Genome 50: 811-817.

Mathieu, J., Warthmann, N., Ku¨ ttner, F. and Schmid, M. (2007) Export of FT protein from phloem companion cells is sufficient for floral induction in Arabidopsis. Curr. Biol. 17: 1055–1060.

Matsubara, K., Yamanouchi, U., Wang, Z., Minobe, Y., Izawa, T. and Yano, M. (2008) *Ehd2*, a rice ortholog of the maize *INDETERMINATE1* gene, promotes flowering by up-regulating *Ehd1*. Plant Physiol. 148: 1425-35.

Mayfield, J., Folta, K., Paul, A. and Ferl, R. (2007). The 14-3-3 Proteins mu and upsilon influence transition to flowering and early phytochrome response. Plant Physiol. 145: 1692-1702.

McConnell, J.R. and Barton, M.K. (1998) Leaf polarity and meristem formation in *Arabidopsis*. Development 125: 2935-2942.

Miki, D., Itoh, R. and Shimamoto, K. (2005) RNA silencing of single and multiple members in a gene family of rice. Plant Physiol. 138: 1903-1913.

Monna, L., Lin, H., Kojima, S., Sasaki, T. and Yano, M. (2002) Genetic dissection of a genomic region for a quantitative trait locus, *Hd3*, into two loci, *Hd3a* and *Hd3b*, controlling heading date in rice. Theor Appl Genet. 104:772–778.

Muslin, A. and Xing, H. (2000). 14-3-3 proteins: regulation of subcellular localization by molecular interference. Cell. Signal. 12: 703- 709.

Notaguchi, M., Abe, M., Kimura, T., Daimon, Y., Kobayashi, T., Yamaguchi, A., et al. (2008) Long-distance, graft-transmissible action of *Arabidopsis* FLOWERING LOCUS T protein to promote flowering. Plant Cell Physiol. 49: 1645-1658.

Park S., Kim S., Lee S., Je B., Piao H., Park S., et al. (2008) Rice *Indeterminate 1* (*OsId1*) is necessary for the expression of *Ehd1* (*Early heading date 1*) regardless of photoperiod. Plant J. 56: 1018-1029.

Pnueli, L., Gutfinger, T., Hareven, D., Ben-Naim, O., Ron, N., Adir, N., et al. (2001) Tomato SP-interacting proteins define a conserved signaling system that regulates shoot architecture and flowering. Plant Cell 13: 2687–2702.

Putterill, J., Robson, F, Lee, K., Simon, R. and Coupland, G. (1995) The CONSTANS gene of Arabidopsis promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. Cell. 80: 847-857.

Riechmann, J.L., Heard, J., Martin, G., Reuber, L., Jiang, C., Keddie, J., Adam, L., Pineda, O., Ratcliffe, O.J., Samaha, R.R., Creelman, R., Pilgrim, M., Broun, P., Zhang, J.Z., Ghandehari, D., Sherman, B.K. and Yu, G. (2000). *Arabidopsis* transcription factors: Genome-wide comparative analysis among eukaryotes. Science 290: 2105–2110.

Roberts, M. (2003). 14-3-3 Proteins finds new partners in plant cell signaling. Trends in Plant Science 8: 218-222.

Rosenquist, M., Alsterfjord, M., Larsson, C. and Sommarin, M. (2001) Data mining the Arabidopsis genome reveals fifteen 14-3-3 genes. Expression is demonstrated for two out of five novel genes. Plant Physiol. 127: 142-149.

Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F. and Coupland, G. (2000) Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science 288: 1613-1616.

Schoonheim, P., Veiga, H., da Costa Pereira, D., Friso, G., van Wijk K. and de Boer, A. (2007). A comprehensive analysis of the 14-3-3 interactome in barley leaves using a complementary proteomics and two-hybrid approach. Plant Physiol 143:670–683.

Sehnke, P.C., DeLille, J.M. and Ferl, R.J. (2002a) Consummating signal transduction: the role of 14-3-3 proteins in the completion of signal-induced transitions in protein activity. Plant Cell. 14 Suppl: S339-354.

Sehnke, P.C., Rosenquist, M., Alsterfjord, M., DeLille, J., Sommarin, M., Larsson, C. and Ferl, R.J. (2002b) Evolution and isoform specificity of plant 14-3-3 proteins. Plant Mol Biol. 50: 1011-1018.

Takada, S. and Goto, K. (2003) Terminal Flower2, an Arabidopsis homolog of heterochromatin protein1, counteracts the activation of Flowering Locus T by Constans in the vascular tissues of leaves to regulate flowering time. Plant Cell. 15: 2856-2865.

Takano, M., Inagaki, N., Xie, X., Yuzurihara, N., Hihara, F., Ishizuka, T., Yano, M., Nishimura, M., Miyao, A., Hirochika, H. and Shinomura, T. (2005) Distinct and cooperative functions of phytochromes A, B, and C in the control of deetiolation and flowering in rice. Plant Cell 17: 3311-3325.

Tamaki, S., Matsuo, S., Wong, H., Yokoi, S. and Shimamoto, K. (2007) Hd3a protein is a mobile flowering signal in rice. Science 316: 1033–1036.

Teper-Bamnolker, P. and Samach, A. (2005) The flowering integrator FT regulates SEPALLATA3 and FRUITFULL accumulation in Arabidopsis leaves. Plant Cell. 17: 2661-2675.

Tsuji, H., Tamaki, S., Komiya, R. and Shimamoto, K. (2008) Florigen and photoperiodic control of flowering in rice. Rice 1: 25-35.

Vert, G., Nemhauser, J.L., Geldner, N., Hong, F. and Chory, J. (2005) Molecular mechanisms of steroid hormone signaling in plants. Annu Rev Cell Dev Biol. 21: 177-201.

deVries, S. (2007) 14-3-3 proteins in plant brassinosteroid signaling. Dev. Cell 13: 162-164.

Wigge, P., Kim, M., Jaeger, K., Busch, W., Schmid, M., Lohmann, J., et al. (2005) Integration of spatial and temporal information during floral induction in Arabidopsis. Science 309: 1056–1059.

Wilson, R.N., Heckman, J.W. and Somerville, C.R. (1992) Gibberellin Is Required for Flowering in Arabidopsis thaliana under Short Days. Plant Physiol. 100: 403-408.

Wong, H., Sakamoto, T., Kawasaki, T., Umemura, K., and Shimamoto, K. (2004). Down-regulation of metallothionein, a reactive oxygen scavenger, by the small GTPase OsRac1 in rice. Plant Physiol. 135: 1447–1456.

Wu, C., You, C., Li, C., Long, T., Chen, G., Byrne, M. and Zhang, Q. (2008a). RID1, encoding a Cys2/His2-type zinc finger transcription factor, acts as a master switch from vegetative to floral development in rice. Proc. Natl Acad. Sci. USA 105: 12915-12920.

Wu, G., Lin, W.C., Huang, T., Poethig, R.S., Springer, P.S., Kerstetter, R.A. (2008b) KANADI1 regulates adaxial-abaxial polarity in Arabidopsis by directly repressing the transcription of *ASYMETRIC LEAVES2*. Proc. Natl Acad. Sci USA 105: 16392-16397.

Xue, W., Xing, Y., Weng, X., Zhao, Y., Tang, W., Wang, L., Zhou, H., Yu, S., Xu, C., Li, X. and Zhang, Q. (2008) Natural variation in Ghd7 is an important regulator of heading date and yield potential in rice. Nat Genet. 40: 761-767.

Yamaguchi, A., Kobayashi, Y., Goto, K., Abe, M., Araki, T. (2005) TWIN SISTER OF FT (TSF) acts as a floral pathway integrator redundantly with FT. Plant Cell Physiol. 46: 1175-1189.

Yamaguchi, T., Nagasawa, N., Kawasaki, S., Matsuoka, M., Nagato, Y. and Hirano, H.Y. (2004) The YABBY gene DROOPING LEAF regulates carpel specification and midrib development in Oryza sativa. Plant Cell. 16: 500-509.

Yamamoto, T., Kuboki, Y., Lin, S., Sasaki, T. and Yano, M. (1998) Fine mapping of quantitative trait loci Hd-1, Hd-2 and Hd-3, controlling heading date of rice, as single Mendelian factors. Theor Appl Genet. 97: 37–44.

Yamamuro, C., Ihara, Y., Wu, X., Noguchi, T., Fujioka, S., Takatsuto, S., Ashikari, M., Kitano, H. and Matsuoka, M. (2000) Loss of function of a rice brassinosteroid insensitive1 homolog prevents internode elongation and bending of the lamina joint. Plant Cell 12: 1591-1606.

Yan, L., Fu, D., Li, C., Blechl, A., Tranquilli, G., Bonafede, M., Sanchez, A., Valarik, M., Yasuda, S., Dubcovsky, J. (2006) The wheat and barley vernalization gene VRN3 is an orthologue of FT. Proc Natl Acad Sci USA. 103: 19581-19586.

Yan, S., Yan C.J., Zeng, X.H., Yang, Y.C., Fang, Y.W., Tian C.Y., Sun Y.W., Cheng, Z.K, Gu M.H. (2008) *ROLLED LEAF 9*, encoding a GARP protein, regulates the leaf abaxial cell fate in rice. Plant Mol Biol. 68: 239-250.

Yano, M., Katayose Y., Ashikari, M., Yamanouchi, U., Monna, L., Fuse, T., Baba, T., Yamamoto, K., Umehara, Y., Nagamura, Y. and Sasaki, T. (2000) Hd1, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the Arabidopsis flowering time gene CONSTANS. Plant Cell. 12: 2473-2484.

Yao, Y., Du, Y., Jiang, L. and Liu, J. (2007). Molecular analysis and expression pattern of the 14-3-3 gene family. J. Biochemistry and Molecular Biology 40: 349-357.

Yeung, K., Seitz, T., Li, S., Janoschk, P., McFerrank, B., Kaiser, C., et al. (1999) Suppression of Raf-1 kinase activity and MAP kinase signaling by RKIP. Nature 401: 173-177.

Yu, X., Li, L., Li, L., Guo, M., Chory, J. and Yin, Y. (2008) Modulation of brassinosteroid-regulated gene expression by Jumonji domain-containing proteins ELF6 and REF6 in Arabidopsis. Proc Natl Acad Sci USA. 105: 7618-7623.

Zeevart, J.A. (2006) Florigen coming of age after 70 years. Plant Cell 18: 1783-1789.

Zeevart, J.A. (2008) Leaf-produced floral signals. Curr Opin Plant Biol. 11: 541-547.