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Item Type	Article
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Citation	OsNF-YC2 and OsNF-YC4 proteins inhibit flowering under long-day conditions in rice 2015 Planta
Eprint version	Post-print
DOI	<a href="https://doi.org/10.1007/s00425-015-2426-x">10.1007/s00425-015-2426-x</a>
Publisher	Springer Nature
Journal	Planta
Rights	The final publication is available at Springer via <a href="http://dx.doi.org/10.1007/s00425-015-2426-x">http://dx.doi.org/10.1007/s00425-015-2426-x</a>
Download date	2024-02-22 03:12:46
Link to Item	<a href="http://hdl.handle.net/10754/583374">http://hdl.handle.net/10754/583374</a>

## **OsNF-YC2 and OsNF-YC4 proteins inhibit flowering under long-day conditions in rice**

**Main conclusion:** OsNF-YC2 and OsNF-YC4 proteins regulate the photoperiodic flowering response through the modulation of three flowering-time genes (*Ehd1*, *Hd3a*, and *RFT1*) in rice.

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**Abbreviations:** CO, CONSTANS; Early heading date 1, Ehd1; NF-Y, NUCLEAR FACTOR Y transcription factors; FT, FLOWERING LOCUS T; GFP, green fluorescence protein; GST, glutathione S-transferase; HAP, heme activator protein; Heading date 1, Hd1; Heading date 3a, Hd3a; LD, long-day; ORFs, open reading frames; RICE FLOWERING LOCUS T1, RFT1; SD, short-day; Ubi1, ubiquitin1

## Abstract

Plant NUCLEAR FACTOR Y (NF-Y) transcription factors control numerous developmental processes by forming heterotrimeric complexes, but little is known about their roles in flowering in rice. In this study, it is shown that some subunits of OsNF-YB and OsNF-YC interact with each other, and among them, OsNF-YC2 and OsNF-YC4 proteins regulate the photoperiodic flowering response of rice. Protein interaction studies showed that the physical interactions occurred between the three OsNF-YC proteins (OsNF-YC2, OsNF-YC4 and OsNF-YC6) and three OsNF-YB proteins (OsNF-YB8, OsNF-YB10 and OsNF-YB11). Repression and overexpression of the *OsNF-YC2* and *OsNF-YC4* genes revealed that they act as inhibitors of flowering only under long-day (LD) conditions. Overexpression of *OsNF-YC6*, however, promoted flowering only under LD conditions, suggesting it could function as a flowering promoter. These phenotypes correlated with the changes in the expression of three rice flowering-time genes [*Early heading date 1 (Ehd1)*, *Heading date 3a (Hd3a)* and *RICE FLOWERING LOCUS T1 (RFT1)*]. The diurnal and tissue-specific expression patterns of the subsets of *OsNF-YB* and *OsNF-YC* genes were similar to [those of CCT domain encoding genes](#) such as *OsCO3*, *Heading date 1 (Hd1)* and *Ghd7*. We propose that *OsNF-YC2* and *OsNF-YC4* proteins regulate the photoperiodic flowering response by interacting directly with *OsNF-YB8*, *OsNF-YB10* or *OsNF-YB11* proteins in rice.

**Keywords:** Flowering time, *Oryza sativa* L., OsNF-YB, OsNF-YC, Photoperiodic flowering, Rice

## Introduction

NUCLEAR FACTOR Y transcription factors [NF-Y, also known as heme-activator protein (HAP) and CCAAT box binding factors (CBFs)] regulate diverse genes in all eukaryotes (Edwards et al. 1998; Maity and de Crombrughe 1998; Laloum et al. 2013). Individual NF-Y subunits identified in plant species are also known to play an essential role in the regulation of developmental processes (Meinke et al. 1994; Li et al. 2008; Yamamoto et al. 2009; Petroni et al. 2012; Laloum et al. 2013; Mu et al. 2013). NF-YB and NF-YC subunits are found as CONSTANS (CO)-interacting proteins in tomato and *Arabidopsis thaliana* (Ben-Naim et al. 2006; Wenkel et al. 2006). The *AtNF-YB2* and *AtNF-YB3* genes are known to promote flowering through the activation of *FLOWERING LOCUS T (FT)* under long-day (LD) conditions in *Arabidopsis* (Cai et al. 2007; Chen et al. 2007; Kumimoto et al. 2008). The *AtNF-YC* subunit genes also have functional redundancy in the promotion of flowering through the activation of *FT* under LD condition in *Arabidopsis* (Kumimoto et al. 2010). In addition, recent systematic studies in *Arabidopsis* have revealed that physical interaction between *AtNF-YB* and *AtNF-YC* is required for the formation of the entire heterotrimeric complex, prior to binding to the consensus CCAAT motif, as shown for mammalian cells (Calvenzani et al. 2012; Hackenberg et al. 2012). The *AtNF-YC3*, *AtNF-YC4*, and *AtNF-YC9* proteins are also known to interact with the *AtNF-YB2*, *AtNF-YB3*, and CO subunits (Kumimoto et al. 2010), suggesting that the *AtNF-YC* subunits make heterotrimeric complexes with *AtNF-YB2* or *AtNF-YB3* and CO to provide unique platforms for the fine-tuning of flowering under various environmental conditions (Wenkel et al. 2006; Kumimoto et al. 2010). Contrary to the canonical NF-Y complexes, CO/*AtNF-YB*/*AtNF-YC* trimeric complexes are speculated not to bind the CCAAT motif in the *FT* promoter, because the CO protein does not have the essential histidine residues required for NF-Y complex binding

(Xing et al. 1993; Siefers et al. 2009; Kumimoto et al. 2010). Furthermore, it was proposed that the canonical AtNF-Y complex binds a CCAAT motif within the *FT* distal enhancer and recruits CO to the proximal *FT* promoter elements (Petroni et al. 2012).

Our understanding of the roles of rice (*Oryza sativa*) NF-Y transcription factors in the control of flowering is still very limited. A rice protein, homologous to AtNF-YB2 (OsNF-YB11/OsHAP3H/Ghd8/DTH8), has recently been shown to regulate both flowering and grain yields (Wei et al. 2010; Yan et al. 2011). In addition, *Ghd7/OsI* [encoding a CCT domain-containing protein (Griffiths et al. 2003)] regulates flowering under LD conditions (Xue et al. 2008). Although protein-protein interactions between some subsets of OsNF-YA/OsHAP2 or OsNF-YB/OsHAP3 and OsNF-YC/OsHAP5 were reported in yeast (Thirumurugan et al. 2008), the interactions between OsNF-Y proteins and rice CO homologues such as Heading date 1(Hd1) have not been established. In wheat (*Triticum aestivum*), CO2 (a rice Hd1 homolog) and flowering repressor VRN2 (ZCCT1 and 2) are shown to compete for interactions with TaNF-Y complexes to integrate the vernalization and photoperiodic signals (Li et al. 2011). In addition, the *Brachypodium distachyon* NF-YB3 (BdNF-YB3) and BdNF-YB6 could rescue the late flowering phenotype of *Arabidopsis nf-yb2 nf-yb3* double mutant and also interact with AtNF-YC3, AtNF-YC4, and AtNF-YC9 in yeast (Cao et al. 2011). These data suggest that homologous NF-Y transcription factors form heterotrimeric complexes, which regulate flowering time in monocot plants.

In this study, we investigated the function of rice proteins homologous to AtNF-YC3, AtNF-YC4, and AtNF-YC9. We report here that OsNF-YC proteins (OsNF-YC2, OsNF-YC4, and OsNF-YC6) interact with OsNF-YB proteins (OsNF-YB8, OsNF-YB10 and OsNF-YB11) and the OsNF-YC proteins affect flowering time under LD conditions in rice.

## Materials and methods

### Plant materials and growth conditions

Wild-type rice (*O. sativa* L., cv. Hwaan; a gift from National Institute of Agricultural Biotechnology, Suwon, South Korea) and transgenic rice plants (in the *O. sativa* L., cv. Hwaan background) were grown in a growth chamber at 28°C under either short-day (SD) conditions [10/14 h (light/dark)] or long day (LD) conditions [14.5/9.5 h (light/dark)] with light supplied at an intensity of 100  $\mu\text{mol m}^{-2}\text{s}^{-1}$ , as described in Kim et al. 2008. For growth under SD conditions, rice plants were first grown in LD conditions for 20 days and then transferred to SD conditions until flowering. The flowering time was measured as the days to heading (DTH), which is the number of days from planting in pots to the emergence of the first panicle from the flag leaf sheath, as described previously (Kim et al. 2008).

### Generation of transgenic plants

To generate green fluorescence protein (GFP)-tagged *OsNF-YC* overexpressing plants, the coding sequences of *OsNF-YC2*, *OsNF-YC4* and *OsNF-YC6* were amplified using specific primers and with each cDNA clone as the templates and cloned in pCAMBIA1300 or pCAMBIA2300 binary vectors. Subsequently, the open reading frames (ORFs) of three *OsNF-YC* including GFP sequences were amplified and inserted into the pGA1611 binary vector with the maize *Ubi1* promoter. To generate RNAi transgenic plants, the *OsNF-YC* ORF sequences, for which inverted repeats were made, were amplified using specific primers, cloned into the pDONR201 vector (Invitrogen, Carlsbad, CA, USA), and subsequently recombined into the pANDA vector (a gift from Dr. Ko Shimamoto, NAIST,



Japan). Oligonucleotide sequences used for cloning are provided in Supplementary Table S1. *Agrobacterium*-mediated rice transformation was performed, as described by Hiei and Komari (2008). Regenerated plants were transferred to soil and grown in a greenhouse for 1 month. The transformants were then tested for herbicide resistance by treating leaves with a 0.5% Basta solution. Insertion of the introduced transgenes into the rice genome and their expression were confirmed by polymerase chain reaction (PCR) analysis.

### **RNA expression analysis**

For real-time quantitative polymerase chain reaction (RT-qPCR) analysis, total RNA was extracted from adult leaves using QIAzol Lysis reagent (Qiagen) and first-strand complementary DNA (cDNA) was synthesized from 1 µg of total RNA in accordance with the manufacturer's instructions (Invitrogen). The RT-qPCR analysis was carried out in 384-well plates with a LightCycler 480 (Roche Applied Science, Madison, WI, USA) using SYBR green. RT-qPCR experiments were carried out using LightCycler 480 SYBR Green Master mixture (Roche Applied Science). All RT-qPCR experiments were performed in three technical triplicates. Two or three biological replicates were performed for transgenic plants. Oligonucleotide sequences used for expression analysis are provided in Supplementary Table S1. Changes in gene expression were calculated via the  $\Delta\Delta_{CT}$  method (Ramakers et al. 2003).

### **Protein expression analysis**

Rice leaves from transgenic lines were ground to a powder in liquid nitrogen and the powder was suspended in a buffered solution of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 0.5% Triton X-100, 2 mM phenylmethanesulfonyl fluoride (PMSF) and complete

protease inhibitor cocktail (Roche Applied Science). The protein concentration was determined using the Bradford solution (Bio-Rad, Hercules, CA). Subsequently, proteins were separated by SDS-PAGE onto a 12.5% gel, transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad), and detected by western blot, as described previously (Kim et al. 2008).

### ***In vitro* protein-protein interaction analysis**

For *in vitro* Glutathione S-transferase (GST) pull-down assays, the ORFs of *OsNF-YB8*, *OsNF-YB10*, *OsNF-YB11*, *OsNF-YC1*, *OsNF-YC2*, *OsNF-YC4*, *OsNF-YC6* and *OsNF-YC7* were cloned into the pGEX-5X-1 (GE Healthcare, LC, UK) to express GST fusion proteins in *E. coli*. To generate *in vitro* translated products, the respective ORFs were cloned into the pET21a-d(+) vector (GE Healthcare), and *in vitro* translation products were synthesized using the T7 TNT-coupled Transcription/Translation System (Promega, Madison, USA). GFP-tagged *OsNF-YB* ORFs were cloned into the pET21a-d (+) expression vector (Merck). Oligonucleotide sequences used for cloning are provided in Supplementary Table S1. The detailed procedure has been previously reported (Jang et al. 2009).

### **Subcellular localization and bimolecular fluorescence complementation (BiFC) analyses**

The ORFs of *Ghd7*, *Hdl*, and *OsCO3*, *OsNF-YB8*, *OsNF-YB10*, *OsNF-YB11*, *OsNF-YC1*, *OsNF-YC2*, *OsNF-YC4*, *OsNF-YC6* and *OsNF-YC7* were cloned into pCAMBIA1300 or pCAMBIA2300 for the localization assay and were also cloned into pSPYNE-35S or pSPYCE-35S for the BiFC assay (Walter et al. 2004). Oligonucleotide sequences used for cloning are provided in Supplementary Table S1. *Agrobacterium* strain C58C1 was used for

infiltration of tobacco (*Nicotiana benthamiana*). Subsequently, epidermal cells of infiltrated tobacco leaves were examined for fluorescence using a confocal microscope (LSM 510 META, Carl Zeiss, Germany).

## Results

### **OsNF-YC2, OsNF-YC4, and OsNF-YC6 proteins interact with OsNF-YB8, OsNF-YB10 and OsNF-YB11 proteins**

AtNF-YC3, AtNF-YC4, and AtNF-YC9 are known to regulate flowering time via protein-protein interaction with AtNF-YB2, AtNF-YB3, and CO proteins in *Arabidopsis* (Kumimoto et al. 2010). OsNF-YB11 (rice AtNF-YB2 and AtNF-YB3 homologs) functions in the control of heading date in rice (Wei et al. 2010; Yan et al. 2011). Five OsNF-YC proteins (OsNF-YC1, OsNF-YC2, OsNF-YC4, OsNF-YC6 and OsNF-YC7) and three OsNF-YB proteins (OsNF-YB8, OsNF-YB10 and OsNF-YB11) of rice were identified as AtNF-YC3/4/9 and AtNF-YB2/3 homologous proteins, respectively (Thirumurugan et al. 2008). We tested if these rice proteins are functional homologs to *Arabidopsis* counterparts. GST pull-down assays were performed to examine the interactions between the OsNF-YC proteins and OsNF-YB proteins. OsNF-YB8 and OsNF-YB11 proteins interacted with five OsNF-YC proteins. Compared with the intensity of the 10% input, two OsNF-YB proteins showed more significant interactions with two of the OsNF-YC proteins (OsNF-YC6 and OsNF-YC7) than with the other three OsNF-YC proteins (OsNF-YC1, OsNF-YC2 and OsNF-YC4) (Fig. 1a). [Protein-protein](#) interactions between OsNF-YB10 protein and five OsNF-YC proteins were not determined because it was not possible to produce the OsNF-YB10 protein for this assay.

The OsNF-YB10 protein could be produced in the GFP-tagged form. Thus, experiments were carried out to investigate the protein-protein interactions between five OsNF-YC proteins tagged with GST and three OsNF-YB proteins tagged with GFP. The GFP-tagged OsNF-YB10 protein interacted with four of the OsNF-YC proteins (OsNF-YC2, OsNF-YC4, OsNF-YC6 and OsNF-YC7), but not with OsNF-YC1 (Fig. 1b). The interactions of GFP-

tagged OsNF-YB10 with OsNF-YC6 and OsNF-YC7 proteins were more significant than those with OsNF-YC2 and OsNF-YC4 proteins. This result is similar to the interaction data of the untagged OsNF-YB8 and OsNF-YB11 with OsNF-YC proteins. The GFP-tagged OsNF-YB11 proteins, however, showed somewhat different interaction profiles from those of the untagged proteins. The main difference was that the tagged OsNF-YB11 proteins did not show significant interaction with OsNF-YC1 and OsNF-YC4 proteins. The tagged OsNF-YB11 protein interacted with only three OsNF-YC proteins (OsNF-YC2, OsNF-YC6 and OsNF-YC7) but their interactions were much weaker than the input control. The tagged OsNF-YB8 protein interacted with all five OsNF-YC proteins, but the interaction with the OsNF-YC1 protein was much weaker than with the other four proteins. In general, the tagged OsNF-YB11 proteins showed weaker interactions than the untagged proteins. These data indicate that three OsNF-YB proteins (OsNF-YB8, OsNF-YB10 and OsNF-YB11) could physically interact *in vitro* with at least three OsNF-YC proteins (OsNF-YC2, OsNF-YC6 and OsNF-YC7).

Since NF-Y transcription factors are known to regulate the expression of diverse genes via direct binding to CCAAT motifs within the promoter regions (Frontini et al. 2004; Steidl et al. 2004; Goda et al. 2005; Tuncher et al. 2005), subcellular localization experiments in tobacco leaves were also carried out to investigate whether OsNF-YB and OsNF-YC proteins are nuclear proteins. The OsNF-YB11-GFP protein was present in both the nucleus and the cytoplasm, whereas the OsNF-YB8-GFP protein was found only in the cytoplasm (Supplementary Fig. S1). The OsNF-YB10-GFP protein, however, occurred only in the nucleus. The five OsNF-YC-GFP proteins were localized both in the nucleus and the cytoplasm (Supplementary Fig. S1). This indicates that the subcellular localization patterns of the OsNF-YB and OsNF-YC proteins overlap *in planta*.

The overlapping localization patterns of the OsNF-YB-GFP proteins with the OsNF-

YC-GFP proteins suggest the possibility that the OsNF-YB proteins may interact with the OsNF-YC proteins *in vivo*. A BiFC assay in *Agrobacterium*-infiltrated tobacco leaves therefore was performed using N- or C-terminal yellow fluorescent protein (YFP) fragment fused OsNF-YBs and OsNF-YCs constructs (Walter et al. 2004). YFP signals were observed in the nucleus and cytoplasm of the transformed cells with all combinations of OsNF-YB and OsNF-YC proteins, indicating that these all interact *in planta* (Supplementary Fig. S2). Taken together with the *in vitro* protein-protein interaction data (Fig. 1), these data suggest that OsNF-YB8, OsNF-YB10, and OsNF-YB11 proteins interact with OsNF-YC2, OsNF-YC4 and OsNF-YC6 proteins *in vivo*. Based on this protein-protein interaction data, we decided to characterize the function of only three proteins, OsNF-YC2, OsNF-YC4 and OsNF-YC6 proteins using transgenic approaches.

### **OsNF-YC proteins are LD-specific flowering regulators**

To investigate the effects of the decreased OsNF-YC2, OsNF-YC4 or OsNF-YC6 expression on flowering time in rice, we generated transgenic rice in which the expression of these genes was suppressed by RNA-mediated interference (RNAi). T<sub>1</sub> transgenic plants for each construct were obtained and the flowering time of transgenic plants was examined in the T<sub>2</sub> generation. In this experiment, T<sub>2</sub> lines for the transgenics and their segregated non-transgenic siblings were grown under SD conditions (10 h of light/14 h dark) or LD conditions (14.5 h of light/9.5 h of dark). The endogenous transcript levels of OsNF-YC2, OsNF-YC4 or OsNF-YC6 genes in OsNF-YC2-RNAi, OsNF-YC4-RNAi, or OsNF-YC6-RNAi, respectively, decreased to less than 20% of the corresponding non-transgenic lines (Fig. 2a). However, the transcript levels of other similar OsNF-YC genes were not altered in each transgenic lines, i.e. the expression levels of OsNF-YC1 that occur in the same clade

with *OsNF-YC4* were very similar in transgenic or non-transgenic *OsNF-YC4*-RNAi lines (Supplementary Fig. S3). The respective transgenic RNAi plants showed that the flowering phenotypes changed only under LD conditions (Table 1). For statistical significance, we compared the flowering times of transgenic and non-transgenic segregants for each gene. The flowering time of the transgenic *OsNF-YC2*-RNAi (#7) plants was earlier than that of the non-transgenic lines. The differences of days to heading (DTH) of #7 = 11.4 and 7.5 days earlier than non-transgenic lines in two biological replicate experiments, respectively (Table 1; Fig. 2b). Transgenic *OsNF-YC4*-RNAi (#100 and #136) plants also exhibited earlier flowering time, compared to the non-transgenic lines (difference of DTH of #100 and #136 = 10 and 5.4 days, respectively), but this result was not replicated in the second biological replicate experiment (Table 1; Fig. 2c). Meanwhile, the flowering time of *OsNF-YC6*-RNAi (#33) plants was not altered under the same conditions (Table 1; Fig. 2d). These data suggest that the decreased expression of *OsNF-YC2* leads to early flowering time only under LD conditions.

To further confirm the functional roles of *OsNF-YC2*, *OsNF-YC4* and *OsNF-YC6* in terms of flowering time, we made transgenic plants that overexpressed *OsNF-YC2*, *OsNF-YC4* or *OsNF-YC6* under the maize *ubiquitin1* (*Ubi1*) promoter. T<sub>0</sub> transgenic plants for each construct were obtained and transgenic lines were selected for analysis of flowering time in the T<sub>1</sub> generation. The overexpression of *OsNF-YC2*, *OsNF-YC4* or *OsNF-YC6* transcripts was observed in the respective transgenic lines (Fig. 3a). Western blot analysis further confirmed that GFP-tagged *OsNF-YC2*, *OsNF-YC4* or *OsNF-YC6* proteins were highly expressed in the same transgenic lines (Fig. 3b). Altered flowering phenotypes in the respective transgenic plants were observed only under LD conditions. Transgenic *Ubi1::OsNF-YC2::GFP* plants (#2, #3, #4 and #5) exhibited severely delayed flowering phenotypes, compared to that of the segregated non-transgenic siblings (difference of DTH =

more than 80 days) (Table 2; Fig. 3c). In addition, the transgenic line of *Ubi1::OsNF-YC4:GFP* (#16 and #19) plants also showed a delay in flowering phenotypes (difference of DTH = more than 65 days) (Table 2; Fig. 3d). In contrast, the transgenic *Ubi1::OsNF-YC6:GFP* plants (#2) showed an early flowering phenotype, compared to those of segregated nontransgenic siblings and wild-type rice plants (difference of DTH = more than 7 days) (Table 2; Fig. 3e). The difference of DTH among transgenic lines also appears to correlate well with the degree of expression of the transgenes (Table 2; Fig. 3a, 3b). The flowering phenotypes of some selected transgenic lines (#1 in *Ubi1::OsNF-YC2:GFP*, #9 and #16 in *Ubi1::OsNF-YC4:GFP*, and #2 in *Ubi1::OsNF-YC6:GFP*) were further confirmed in the T<sub>2</sub> generation (Table 2), although the flowering times of segregated nontransgenic T<sub>2</sub> siblings were rather divergent from that of wild-type rice plants. This difference may be resulted from small numbers of nontransgenic siblings. These data, taken together with the transgenic approach using RNAi, suggest that OsNF-YC2 and OsNF-YC4 proteins act as flowering repressors and that the OsNF-YC6 protein may function as a flowering promoter only under LD conditions in rice.

### **Flowering time genes are affected by strong expression of *OsNF-YC2*, *OsNF-YC4* or *OsNF-YC6* genes in transgenic plants**

To elucidate the molecular mechanisms by which *OsNF-YC2*, *OsNF-YC4* or *OsNF-YC6* regulate flowering time in rice, we investigated the expression of some flowering regulators such as *Early heading date 1 (Ehd1)* in segregated lines of the respective transgenic plants at T<sub>1</sub> and T<sub>2</sub> generation (Tables 1, 2). Among the RNAi lines, only *OsNF-YC2*-RNAi plants showed significant acceleration in flowering time compared to non-transgenic controls, however change in the expression of flowering time genes responsible for these phenotypes



was very subtle (data not shown). Some overexpression lines of *OsNF-YC2*, *OsNF-YC4* or *OsNF-YC6* were examined for the expression of flowering time genes by RT-PCR analysis (Supplementary Fig. S4). *Ehd1*, *FTL*, *Heading date 3a (Hd3a)* and *RICE FLOWERING LOCUS T1 (RFT1)* expression levels were noticeably changed. Then, transgenic plants showing strong expression of *OsNF-YC2*, *OsNF-YC4* or *OsNF-YC6* were further examined for the expression of flowering regulators using RT-qPCR analysis (Fig. 4; Supplementary Figs. S5, S6). In transgenic *Ubi1::OsNF-YC2:GFP* (#3 and 1-4) and *Ubi1::OsNF-YC4:GFP* (#19 and 16-12) plants grown under LD conditions, *Ehd1*, *Hd3a*, and *RFT1* expression decreased (Fig. 4a, 4b), whereas the expression of these increased in transgenic *Ubi1::OsNF-YC6:GFP* (#2-1) plants under the same conditions (Fig. 4c). However, the expression level of *Ghd7* (Doi et al. 2004; Itoh et al. 2010) increased significantly only in *Ubi1::OsNF-YC2:GFP* plants, suggesting that the *OsNF-YC2* protein may also promote the expression of the *Ghd7* gene, which then reduces the expression of *Ehd1*, *Hd3a*, and *RFT1*s under LD conditions (Fig. 4a). The expression levels of *OsLFL1* as negative regulators of *Ehd1* (Peng et al. 2007) and *Hd1* as a negative regulator of *Hd3a/RFT1* (Yano et al. 2000; Hayama et al. 2003) under LD conditions were not altered significantly in *Ubi1::OsNF-YC2:GFP*, *Ubi1::OsNF-YC4:GFP*, and *Ubi1::OsNF-YC6:GFP* plants (Supplementary Figs. S4, S5, S6). Other flowering regulators, for example *OsMADS50*, were also not altered (Supplementary Figs. S4, S5, S6) (Komiya et al. 2009; Ryu et al. 2009). Thus, these data suggest that *OsNF-YC4* and *OsNF-YC6* proteins affect *Ehd1*, *Hd3a*, and *RFT1* expression under LD conditions, independently of *Ghd7* and *Hd1*, whereas *OsNF-YC2* protein represses the expression of *Ehd1*, *Hd3a*, and *RFT1* via *Ghd7* or the expression of *Ehd1*, *Hd3a*, and *RFT1* genes directly. Taken together, these data suggest that these three *OsNF-YC* genes function upstream of *Ehd1*, *Hd3a*, and *RFT1* as LD-specific regulators.

## The expression patterns of *OsNF-YB* and *OsNF-YC* genes

The diurnal oscillation patterns of the subsets of *OsNF-YB* and *OsNF-YC* genes, *OsCO3*, *Hdl* and *Ghd7* were compared in rice seedlings grown under LD conditions to examine if there was correlation in the expression patterns. *OsNF-YB11* and *OsNF-YC4* expression showed oscillation patterns with a peak around the end of the light period, which was similar to the pattern observed for *Hdl* expression (Fig. 5a). In contrast, the expression levels of *OsNF-YC2*, *Ghd7* and *OsCO3* exhibited oscillation patterns with a peak at the beginning of the light period (Fig. 5b). However, the transcripts of *OsNF-YB8*, *OsNF-YB10* and *OsNF-YC6* did not show the diurnal oscillation patterns that were observed in *OsCO3*, *Hdl* and *Ghd7* (Fig. 5c). These data indicate that specific subsets of *OsNF-YB* and *OsNF-YC* genes have similar diurnal oscillation patterns to *OsCO3*, *Hdl* and *Ghd7* genes.

The spatial expression patterns of some *OsNF-YB*, *OsNF-YC* and CCT domain-containing genes were also compared in a variety of rice tissues. *OsNF-YB11* and *OsNF-YC4* and *Hdl* expression showed similar expression patterns (Fig. 6a). Abundant expression was observed in leaf sheaths, leaf blades, flag leaves, and roots. *OsNF-YC2*, *Ghd7* and *OsCO3* was mainly expressed in the leaf blades and flag leaves (Fig. 6b). This indicates that tissue-specific expression patterns of some *OsNF-YB* and *OsNF-YC* genes are similar to those of *OsCO3*, *Hdl* and *Ghd7* genes. However, the expression levels of *OsNF-YB8*, *OsNF-YB10* and *OsNF-YC6* were not similar to those of *OsCO3*, *Hdl* and *Ghd7* (Fig. 6c). Taken together, these data suggest that *OsCO3*, *Hdl* and *Ghd7* proteins may interact with specific subsets of *OsNF-YB* and *OsNF-YC* proteins, respectively, in the regulation of photoperiodic flowering in rice.

## Discussion

### OsNF-YC proteins form heterodimeric complexes with OsNF-YB proteins

The rice genome contains multiple gene families of *OsNF-Y* genes (10 *OsNF-YA* genes, 11 *OsNF-YB* genes and 7 *OsNF-YC* genes) (Petroni et al. 2012; Laloum et al. 2013). Overlapping expression patterns of *OsNF-YA*, *OsNF-YB* and *OsNF-YC* genes have been reported (Thirumurugan et al. 2008). Recently, it has been reported that the *OsNF-YB11* (*OsHAP3H/Ghd8/DTH8*) gene is involved in the regulation of flowering time and [other traits such as grain productivity and plant height](#) (Wei et al. 2010; Yan et al. 2011). Thus, an important question is raised as to whether the OsNF-Y subunit interacts with specific members of the other two OsNF-Y subunits to influence flowering time. Several lines of evidence in this study suggest that three OsNF-YC proteins (*OsNF-YC2*, *OsNF-YC4* and *OsNF-YC6*) specifically interact with three OsNF-YB proteins (*OsNF-YB8*, *OsNF-YB10* and *OsNF-YB11*) in the regulation of flowering time. First, specific protein-protein interactions between three OsNF-YC and three OsNF-YB proteins were observed *in vitro* and *in planta* (Fig. 1; Fig. S2). Second, three OsNF-YC proteins were co-localized in the nucleus with three OsNF-YB proteins (Figs. S1, S2). Third, the diurnal oscillation expression and tissue-specific expression patterns of *OsNF-YC2* and *OsNF-YC4* genes were similar to those of *OsNF-YB11*, *Hd1*, *Ghd7* and *OsCO3* genes (Figs. 5, 6). Finally, in the RNAi suppression or overexpression lines of three *OsNF-YC* genes, it was shown that the flowering time in rice was affected (Figs. 2, 3; Tables 1, 2). Our conclusion is supported by three OsNF-YC and three OsNF-YB proteins that are clustered in the same phylogenetic clade (Thirumurugan et al. 2008; Petroni et al. 2012; Laloum et al. 2013) as *AtNF-YC3/-YC4/-YC9* proteins and *AtNF-YB2/-YB3* proteins, which are known to influence flowering (Kumimoto et al. 2008,

2010). However, the possibility that the OsNF-YC subunits interact with other OsNF-YB subunits in other developmental processes, cannot be excluded. This is based both on our finding that each OsNF-YC subunit interacts with three OsNF-YB proteins (Fig. 1; Figs. S1, S2), the expression of some *OsNF-Y* genes was detected in all tissues tested in this study (Fig. 6), and the report of Thirumurugan *et al.* (2008) that OsNF-YB2, which is involved in chloroplast biogenesis (Miyoshi *et al.* 2003), interacts with OsNF-YC subunits in yeast.

### **OsNF-YC2, OsNF-YC4, and OsNF-YC6 proteins act as LD-specific flowering regulators in the regulation of the photoperiod-dependent flowering response in rice**

Since AtNF-YC3, AtNF-YC4 and AtNF-YC9 proteins regulate the photoperiod-dependent flowering through interaction with CO protein (Kumimoto *et al.* 2010), we investigated whether three OsNF-YC proteins regulate the photoperiod-dependent flowering response in rice. The present study provides evidence that OsNF-YC2, OsNF-YC4, and **potentially or possibly** OsNF-YC6 proteins act as LD-specific flowering regulators in rice. Firstly, transgenic approaches using RNAi suppression or overexpression revealed that three *OsNF-YC* genes modulated flowering time only under LD conditions (Figs. 2, 3; Tables 1, 2). Although *OsNF-YC4*-RNAi and *OsNF-YC6*-RNAi plants did not show any significant change in flowering time, *OsNF-YC2*-RNAi plants did show significant acceleration in flowering time when compared to non-transgenic controls. The lack of phenotypic effect in *OsNF-YC4*-RNAi and *OsNF-YC6*-RNAi plants could be due to the functional redundancy of these genes with the homologous *OsNF-YC* genes (Thirumurugan *et al.* 2008; Petroni *et al.* 2012; Laloum *et al.* 2013). Transgenic plants over-expressing the *OsNF-YC* genes showed either delayed or accelerated flowering only under LD conditions (Fig. 3; Table 2). Secondly, the overexpression of three *OsNF-YC* genes affected the expression levels of *Ehd1*, *Hd3a* and

*RFT1* acting within a LD-specific pathway (Komiya et al. 2009) (Fig. 4). These findings indicate that three *OsNF-YC* genes primarily regulate the photoperiod-dependent flowering response under LD conditions in rice.

In this study, it has been demonstrated that three *OsNF-YC* proteins interact with the same *OsNF-YB* proteins (Fig. 1; Fig. S2). Also, the effects of *OsNF-YC2* and *OsNF-YC4* on flowering time were the opposite of *OsNF-YC6* flowering time (Figs. 2, 3; Tables 1, 2), [although the acceleration of flowering by the overexpression of \*OsNF-YC6\* needs further validation](#). This could be explained as follows: *in vivo*, *OsNF-YB* and *OsNF-YC* heterodimeric complexes may interact with flowering repressor proteins such as Hd1 or Ghd7, or alternatively these may interact with flowering promoter proteins and perform the opposite function. However, the possibility cannot be excluded that *OsNF-YC6* affects flowering time via a dominant negative effect when it is ectopically overexpressed, because over-expressed *OsNF-YC6* proteins could promote flowering by interrupting the formation of functional complexes. Further investigation on these possibilities would provide a better understanding of protein-protein interactions between *OsNF-YB* and *OsNF-YC* proteins.

### **Possible interactions between *OsNF-YB* or *OsNF-YC* proteins, and flowering regulators with a CCT domain in rice**

CO protein is known to interact with *AtNF-YC3*, 4 and 9 proteins via the CCT domain (Wenkel et al. 2006; Kumimoto et al. 2010). In wheat, *VRN2* [acting as a flowering repressor (Yan et al. 2004)] and *CO2* [acting as a flowering promoter (Nemoto et al. 2003)] also physically interact with the same subset of eight *TaNF-Y* proteins (Li et al. 2011). In this study, we found a similar subcellular localization of *OsNF-YB*, *OsNF-YC*, Hd1, Ghd7, and *OsCO3* (Fig. S1), and *in vitro* and *in planta* interaction between *OsNF-YB* and *OsNF-YC*

(Fig. S2). In addition, some *OsNF-YB* and *OsNF-YC* genes have similar diurnal and spatial expression patterns with *Hd1*, *Ghd7* and *OsCO3* genes (Figs. 5, 6). This suggests that specific subsets of *OsNF-YB* and *OsNF-YC* proteins regulate the photoperiodic flowering in rice through direct interaction with flowering regulators with the CCT domain. However, the full-length *Hd1*, *Ghd7*, and *OsCO3* (functioning as CCT domain proteins) did not physically interact with subsets of either *OsNF-YB* or *OsNF-YC* proteins *in vitro* (data not shown). This suggests that *Hd1*, *Ghd7*, and *OsCO3* may need appropriate conformational change for the interaction with *OsNF-YB* or *OsNF-YC* proteins *in vivo*, as suggested in a report that the full-length CO does not interact with *AtNF-YB2* (Kumimoto et al. 2010). However, we cannot dismiss the possibility that some unknown factors or phosphorylated form of the CCT domain proteins may be required for formation of the heterotrimeric complex among the CCT domain proteins, *OsNF-YB*, and *OsNF-YC* proteins *in vivo*. This is supported by evidence that a casein kinase I (*Hd16*) controls flowering time in rice through the phosphorylation of *Ghd7* (Hori et al. 2013; Kwon et al. 2015). Finally, the rice flowering regulators with the CCT domain might bind to only complete *OsNF-Y* heterotrimeric complexes (*OsNF-YA*, *OsNF-YB* and *OsNF-YC*) to promote flowering in rice. This is supported by the proposal of Cao et al., (2014) that the canonical *AtNF-Y* complex binds the *FT* distal enhancer and recruits CO to proximal *FT* promoter elements. Further investigation will be needed to identify the *OsNF-Y* protein complexes *in vivo*.

### **Author contribution statement**

SKK designed research, conducted experiments, analyzed data and wrote the manuscript. HYP, YHJ, and KCL conducted experiments and analyzed data. YSC analyzed data. JHL analyzed data and wrote the manuscript. JKK conceived and designed research, analyzed data and wrote the manuscript. All authors read and approved the manuscript.

### **Acknowledgements**

We thank Professors C.D. Han (Gyeongsang National University) and K. Shimamoto (NAIST, Japan) for RNAi vectors. We also thank Prof. C.D. Han for *35S::GFP* transgenic rice plants and technical help. We are grateful to Kyung-Sook Lee for preparing transgenic rice lines and the RGRC-NIAS (Japan) and Rural Development Administration (Republic of Korea) for rice cDNA and seeds. This work was supported by grants from the Next-Generation BioGreen 21 Program (PJ007978), Rural Development Administration, Republic of Korea and a Korea University Grant (to J.-K.Kim) and by the BK 21 program (to S.-K.Kim).

## References

- Ben-Naim O, Eshed R, Parnis A, Teper-Bamnolker P, Shalit A, Coupland G, Samach A, Lifschitz E (2006) The CCAAT binding factor can mediate interactions between CONSTANS-like proteins and DNA. *Plant J* 46:462-476
- Cai X, Ballif J, Endo S, Davis E, Liang M, Chen D, DeWald D, Kreps J, Zhu T, Wu Y (2007) A putative CCAAT-binding transcription factor is a regulator of flowering timing in *Arabidopsis*. *Plant Physiol* 145:98-105
- Calvenzani V, Testoni B, Gusmaroli G, Lorenzo M, Gnesutta N, Petroni K, Mantovani R, Tonelli C (2012) Interactions and CCAAT-binding of *Arabidopsis thaliana* NF-Y subunits. *PLoS One* 7:e42902
- Cao S, Kumimoto RW, Siriwardana CL, Risinger JR, Holt BF, 3rd (2011) Identification and characterization of NF-Y transcription factor families in the monocot model plant *Brachypodium distachyon*. *PLoS One* 6:e21805
- Chen NZ, Zhang XQ, Wei PC, Chen QJ, Ren F, Chen J, Wang XC (2007) AtHAP3b plays a crucial role in the regulation of flowering time in *Arabidopsis* during osmotic stress. *J Biochem Mol Biol* 40:1083-1089
- Doi K, Izawa T, Fuse T, Yamanouchi U, Kubo T, Shimatani Z, Yano M, Yoshimura A (2004) *Ehd1*, a B-type response regulator in rice, confers short-day promotion of flowering and controls *FT-like* gene expression independently of *Hdl*. *Genes Dev* 18:926-936
- Edwards D, Murray JA, Smith AG (1998) Multiple genes encoding the conserved CCAAT-box transcription factor complex are expressed in *Arabidopsis*. *Plant Physiol* 117:1015-1022
- Frontini M, Imbriano C, Manni I, Mantovani R (2004) Cell cycle regulation of NF-YC nuclear localization. *Cell Cycle* 3:217-222
- Goda H, Nagase T, Tanoue S, Sugiyama J, Steidl S, Tuncher A, Kobayashi T, Tsukagoshi N, Brakhage AA, Kato M (2005) Nuclear translocation of the heterotrimeric CCAAT binding factor of *Aspergillus oryzae* is dependent on two redundant localising signals in a single subunit. *Arch Microbiol* 184:93-100
- Griffiths S, Dunford RP, Coupland G, Laurie DA (2003) The evolution of *CONSTANS*-like gene families in barley, rice, and *Arabidopsis*. *Plant Physiol* 131:1855-1867
- Hackenberg D, Wu Y, Voigt A, Adams R, Schramm P, Grimm B (2012) Studies on



- differential nuclear translocation mechanism and assembly of the three subunits of the *Arabidopsis thaliana* transcription factor NF-Y. *Mol Plant* 5:876-888
- Hayama R, Yokoi S, Tamaki S, Yano M, Shimamoto K (2003) Adaptation of photoperiodic control pathways produces short-day flowering in rice. *Nature* 422:719-722
- Hori K, Ogiso-Tanaka E, Matsubara K, Yamanouchi U, Ebana K, Yano M (2013) *Hd16*, a gene for casein kinase I, is involved in the control of rice flowering time by modulating the day-length response. *Plant J* 76:36-46
- Itoh H, Nonoue Y, Yano M, Izawa T (2010) A pair of floral regulators sets critical day length for *Hd3a* florigen expression in rice. *Nat Genet* 42:635-638
- Jang YH, Park HY, Kim SK, Lee JH, Suh MC, Chung YS, Paek KH, Kim JK (2009) Survey of rice proteins interacting with OsFCA and OsFY proteins which are homologous to the *Arabidopsis* flowering time proteins, FCA and FY. *Plant Cell Physiol* 50:1479-1492
- Kim SK, Yun CH, Lee JH, Jang YH, Park HY, Kim JK (2008) *OsCO3*, a *CONSTANS-LIKE* gene, controls flowering by negatively regulating the expression of *FT*-like genes under SD conditions in rice. *Planta* 228:355-365
- Komiya R, Yokoi S, Shimamoto K (2009) A gene network for long-day flowering activates *RFT1* encoding a mobile flowering signal in rice. *Development* 136:3443-3450
- Kumimoto RW, Adam L, Hymus GJ, Repetti PP, Reuber TL, Marion CM, Hempel FD, Ratcliffe OJ (2008) The Nuclear Factor Y subunits NF-YB2 and NF-YB3 play additive roles in the promotion of flowering by inductive long-day photoperiods in *Arabidopsis*. *Planta* 228:709-723
- Kumimoto RW, Zhang Y, Siefers N, Holt BF, 3rd (2010) NF-YC3, NF-YC4 and NF-YC9 are required for *CONSTANS*-mediated, photoperiod-dependent flowering in *Arabidopsis thaliana*. *Plant J* 63:379-391
- Kwon CT, Koo BH, Kim D, Yoo SC, Paek NC (2015) Casein kinases I and 2 $\alpha$  phosphorylate *oryza sativa* pseudo-response regulator 37 (OsPRR37) in photoperiodic flowering in rice. *Mol Cells* 38:81-88
- Laloum T, De Mita S, Gamas P, Baudin M, Niebel A (2013) CCAAT-box binding transcription factors in plants: Y so many? *Trends Plant Sci* 18:157-166
- Li C, Distelfeld A, Comis A, Dubcovsky J (2011) Wheat flowering repressor VRN2 and promoter CO2 compete for interactions with NUCLEAR FACTOR-Y complexes. *Plant J* 67:763-773

- Li WX, Oono Y, Zhu J, He XJ, Wu JM, Iida K, Lu XY, Cui X, Jin H, Zhu JK (2008) The *Arabidopsis* NFYA5 transcription factor is regulated transcriptionally and posttranscriptionally to promote drought resistance. *Plant Cell* 20:2238-2251
- Maity SN, de Crombrughe B (1998) Role of the CCAAT-binding protein CBF/NF-Y in transcription. *Trends Biochem Sci* 23:174-178
- Meinke DW, Franzmann LH, Nickle TC, Yeung EC (1994) Leafy Cotyledon Mutants of *Arabidopsis*. *Plant Cell* 6:1049-1064
- Miyoshi K, Ito Y, Serizawa A, Kurata N (2003) *OsHAP3* genes regulate chloroplast biogenesis in rice. *Plant J* 36:532-540
- Mu J, Tan H, Hong S, Liang Y, Zuo J (2013) *Arabidopsis* Transcription Factor Genes *NFYA1*, 5, 6, and 9 Play Redundant Roles in Male Gametogenesis, Embryogenesis, and Seed Development. *Mol Plant* 6:188-201
- Nemoto Y, Kisaka M, Fuse T, Yano M, Ogihara Y (2003) Characterization and functional analysis of three wheat genes with homology to the *CONSTANS* flowering time gene in transgenic rice. *Plant J* 36:82-93
- Peng LT, Shi ZY, Li L, Shen GZ, Zhang JL (2007) Ectopic expression of OsLFL1 in rice represses *Ehd1* by binding on its promoter. *Biochem Biophys Res Commun* 360:251-256
- Petroni K, Kumimoto RW, Gnesutta N, Calvenzani V, Fornari M, Tonelli C, Holt BF, 3rd, Mantovani R (2012) The promiscuous life of plant NUCLEAR FACTOR Y transcription factors. *Plant Cell* 24:4777-4792
- Ramakers C, Ruijter JM, Deprez RH, Moorman AF (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* 339:62-66
- Ryu CH, Lee S, Cho LH, Kim SL, Lee YS, Choi SC, Jeong HJ, Yi J, Park SJ, Han CD, An G (2009) *OsMADS50* and *OsMADS56* function antagonistically in regulating long day (LD)-dependent flowering in rice. *Plant Cell Environ* 32:1412-1427
- Siefers N, Dang KK, Kumimoto RW, Bynum WE, Tayrose G, Holt BF, 3rd (2009) Tissue-specific expression patterns of *Arabidopsis* NF-Y transcription factors suggest potential for extensive combinatorial complexity. *Plant Physiol* 149:625-641
- Steidl S, Tuncher A, Goda H, Guder C, Papadopoulou N, Kobayashi T, Tsukagoshi N, Kato M, Brakhage AA (2004) A single subunit of a heterotrimeric CCAAT-binding complex carries a nuclear localization signal: piggy back transport of the pre-assembled complex to the nucleus. *J Mol Biol* 342:515-524

- Thirumurugan T, Ito Y, Kubo T, Serizawa A, Kurata N (2008) Identification, characterization and interaction of *HAP* family genes in rice. *Mol Genet Genomics* 279:279-289
- Tuncher A, Sprote P, Gehrke A, Brakhage AA (2005) The CCAAT-binding complex of eukaryotes: evolution of a second NLS in the HapB subunit of the filamentous fungus *Aspergillus nidulans* despite functional conservation at the molecular level between yeast, *A.nidulans* and human. *J Mol Biol* 352:517-533
- Walter M, Chaban C, Schutze K, Batistic O, Weckermann K, Nacke C, Blazevic D, Grefen C, Schumacher K, Oecking C, Harter K, Kudla J (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *Plant J* 40:428-438
- Wei X, Xu J, Guo H, Jiang L, Chen S, Yu C, Zhou Z, Hu P, Zhai H, Wan J (2010) *DTH8* suppresses flowering in rice, influencing plant height and yield potential simultaneously. *Plant Physiol* 153:1747-1758
- Wenkel S, Turck F, Singer K, Gissot L, Le Gourrierec J, Samach A, Coupland G (2006) CONSTANS and the CCAAT box binding complex share a functionally important domain and interact to regulate flowering of *Arabidopsis*. *Plant Cell* 18:2971-2984
- Xing Y, Fikes JD, Guarente L (1993) Mutations in yeast HAP2/HAP3 define a hybrid CCAAT box binding domain. *EMBO J* 12:4647-4655
- Xue W, Xing Y, Weng X, Zhao Y, Tang W, Wang L, Zhou H, Yu S, Xu C, Li X, Zhang Q (2008) Natural variation in *Ghd7* is an important regulator of heading date and yield potential in rice. *Nat Genet* 40:761-767
- Yamamoto A, Kagaya Y, Toyoshima R, Kagaya M, Takeda S, Hattori T (2009) Arabidopsis NF-YB subunits LEC1 and LEC1-LIKE activate transcription by interacting with seed-specific ABRE-binding factors. *Plant J* 58:843-856
- Yan L, Loukoianov A, Blechl A, Tranquilli G, Ramakrishna W, SanMiguel P, Bennetzen JL, Echenique V, Dubcovsky J (2004) The wheat *VRN2* gene is a flowering repressor down-regulated by vernalization. *Science* 303:1640-1644
- Yan WH, Wang P, Chen HX, Zhou HJ, Li QP, Wang CR, Ding ZH, Zhang YS, Yu SB, Xing YZ, Zhang QF (2011) A major QTL, *Ghd8*, plays pleiotropic roles in regulating grain productivity, plant height, and heading date in rice. *Mol Plant* 4:319-330
- Yano M, Katayose Y, Ashikari M, Yamanouchi U, Monna L, Fuse T, Baba T, Yamamoto K, Umehara Y, Nagamura Y, Sasaki T (2000) *Hdl1*, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the Arabidopsis flowering time

gene *CONSTANS*. Plant Cell 12:2473-2484

## Figure Legends

**Fig. 1** Protein-protein interaction between three OsNF-YB proteins and five OsNF-YC proteins *in vitro*. **a** GST pull-down assay between two OsNF-YB proteins and five OsNF-YC proteins. GST or GST-tagged OsNF-YC proteins were incubated with <sup>35</sup>S-labeled OsNF-YB8 or <sup>35</sup>S-labeled OsNF-YB11 proteins. The bands on the gel images indicate the eluted OsNF-YB8 or OsNF-YB11 proteins as visualized by autoradiography. The last lane of each panel shows 10% of the <sup>35</sup>S-labeled proteins added to each pull-down reaction mixture. Coomassie blue-stained bands (CBB) indicated by asterisks show the amount and quality of the GST fusion proteins used in this assay. **b** GST pull-down assay between GST-tagged OsNF-YC proteins, and OsNF-YB proteins tagged with both GFP and His. The eluates were separated by 12.5% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and probed with anti-His antibody ( $\alpha$ -His). About 4% of the total sample in each reaction was loaded as an input control. Ponceau S-stained bands (PonS) indicated by asterisks show the amount and quality of the GST fusion proteins used in this assay.

**Fig. 2** Flowering phenotypes of *OsNF-YC2*-RNAi, *OsNF-YC4*-RNAi and *OsNF-YC6*-RNAi transgenic plants in the T<sub>1</sub> generation. **a** The expression levels of endogenous *OsNF-YC2*, *OsNF-YC4*, and *OsNF-YC6* genes in the segregating lines of the respective transgenic plants. The expression levels of *OsUBQ* gene were used as a normalization control. Error bars indicate the standard deviation of the three technical replicates. *OsNF-YC2*-RNAi (**b**), *OsNF-YC4*-RNAi (**c**) and *OsNF-YC6*-RNAi (**d**) transgenic plants (+) and their segregating non-transgenic controls (-) grown under long-day (LD) conditions were photographed after the heading of the transgenic plants. Arrows indicate panicles showing heading. *Scale bars* 10 cm.

**Fig. 3** Flowering phenotypes of *Ubi1::OsNF-YC2:GFP*, *Ubi1::OsNF-YC4:GFP* and *Ubi1::OsNF-YC6:GFP* transgenic plants in the T<sub>1</sub> generation. **a** The mRNA levels of *OsNF-YC2*, *OsNF-YC4*, and *OsNF-YC6* genes in the segregating lines of the respective transgenic plants. RT-qPCR was performed to examine the expression levels of the *OsNF-YC* genes. The expression levels of *OsUBQ* gene were used as a normalization control. Error bars indicate the standard deviation of the three technical replicates. **b** Protein levels in the segregating lines of the respective transgenic plants. Anti-GFP antibody ( $\alpha$ -GFP) was used for western blot analysis. Ponceau S-stained Rubisco large subunit (*rbcL*) was used as a loading control. *Ubi1::OsNF-YC2:GFP* (**c**), *Ubi1::OsNF-YC4:GFP* (**d**) and *Ubi1::OsNF-YC6:GFP* (**e**) transgenic plants (+) and their segregating non-transgenic controls (-) grown under LD conditions were photographed after the heading of transgenic or non-transgenic plants. Arrows indicate panicles showing heading. Scale bars 10 cm.

**Fig. 4** *Hd3a*, *RFT1*, *Ehd1*, and *Ghd7* expression in *Ubi1::OsNF-YC2:GFP*, *Ubi1::OsNF-YC4:GFP* and *Ubi1::OsNF-YC6:GFP* transgenic plants. Leaves of *Ubi1::OsNF-YC2:GFP* (**a**), *Ubi1::OsNF-YC4:GFP* (**b**) and *Ubi1::OsNF-YC6:GFP* (**c**) transgenic segregants grown under LD conditions were harvested at the initiation of light phase at 41 or 51 days. Two biological replicates were done. RT-qPCR was performed to examine the relative expression levels of the *OsNF-YC* genes and the flowering time genes. The expression levels were normalized with that of *OsUBQ*, respectively. The highest level was set at 1. Transgenic plants (+) and their segregating non-transgenic controls (-) were indicated.

**Fig. 5** Diurnal oscillation patterns of *OsNF-YB8*, *OsNF-YB10*, *OsNF-YB11*, *OsNF-YC2*, *OsNF-YC4*, *OsNF-YC6*, *Hd1*, *Ghd7* and *OsCO3*. **a** Similar oscillation patterns among *OsNF-*

*YC4*, *OsNF-YB11* and *Hd1*. Rice seedlings were harvested every 4 h for 44 h under LD conditions. The expression levels of *OsUBQ* were used as a normalization control. Error bars indicate standard deviation of the three technical replicates. ZT denotes zeitgeber time. **b** Similar oscillation patterns among *OsNF-YC2*, *OsCO3*, and *Ghd7*. **c** Dissimilar patterns of *OsNF-YC6*, *OsNF-YB10* and *OsNF-YB8*.

**Fig. 6** Spatial expression patterns of *OsNF-YB8*, *OsNF-YB10*, *OsNF-YB11*, *OsNF-YC2*, *OsNF-YC4*, *OsNF-YC6*, *Hd1*, *Ghd7* and *OsCO3*. **a** Similar expression patterns among *OsNF-YC4*, *OsNF-YB11* and *Hd1*. Respective samples [culms (C), leaf sheaths (S), leaf blades (B), flag leaves (F), panicles (P) and roots (R)] were harvested from adult rice plants. The expression levels of *OsUBQ* were used as a normalization control. Error bars indicate standard deviation of three technical replicates. **b** Similar expression patterns among *OsNF-YC2*, *OsCO3* and *Ghd7*. **c** Dissimilar expression patterns of *OsNF-YC6*, *OsNF-YB10* and *OsNF-YB8*.