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ORIGINAL ARTICLE

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

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Abstract

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.**

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 Os*NF-YC2* and Os*NF-YC4* genes revealed that they act as inhibitors of flowering only under long-day (LD) conditions. Overexpression of Os*NF-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 Os*NF-YB* and Os*NF-YC* genes were similar to those of CCT domain encoding genes such as Os*CO3*, *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 · NUCLEAR FACTOR Y (NF-Y) · Photoperiodic flowering · Rice · Transcription

Abbreviations

CO	CONSTANS
Ehd1	*Early heading date 1*
NF-Y	NUCLEAR FACTOR Y transcription factor
FT	*FLOWERING LOCUS T*
GFP	Green fluorescence protein
GST	Glutathione *S*-transferase
HAP	Heme activator protein
Hd1(3a)	*Heading date 1*(*3a*)
LD	Long-day
ORFs	Open-reading frames
RFT1	*RICE FLOWERING LOCUS T1*
SD	Short-day
Ubi1	*Ubiquitin1*

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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 Crombrugghe 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 At*NF-YB2* and At*NF-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 At*NF-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 have been 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) 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 (*Oryza 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 μmol m^{-2} 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 Os*NF-YC* overexpressing plants, the coding sequences of Os*NF-YC2*, Os*NF-YC4* and Os*NF-YC6* were amplified using specific primers and with each cDNA clone as the template and cloned in pCAMBIA1300 or pCAMBIA2300

binary vectors. Subsequently, the open-reading frames (ORFs) of three Os*NF-YC* including GFP sequences were amplified and inserted into the pGA1611 binary vector with the maize *Ubi1* promoter. To generate RNAi transgenic plants, the Os*NF-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, Hilden, Germany) 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, USA). 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 Os*NF-YB8*, Os*NF-YB10*, Os*NF-YB11*, Os*NF-YC1*, Os*NF-YC2*, Os*NF-YC4*, Os*NF-YC6* and Os*NF-YC7* were cloned into the pGEX-5X-1 (GE Healthcare, LC, UK) to express GST fusion proteins in *Escherichia 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, Fitchburg, WI, USA). *GFP*-tagged Os*NF-YB* ORFs were cloned into the pET21a-d (+) expression vector (GE Healthcare). 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*, *Hd1*, and Os*CO3*, Os*NF-YB8*, Os*NF-YB10*, Os*NF-YB11*, Os*NF-YC1*, Os*NF-YC2*, Os*NF-YC4*, Os*NF-YC6* and Os*NF-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, Jena, 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

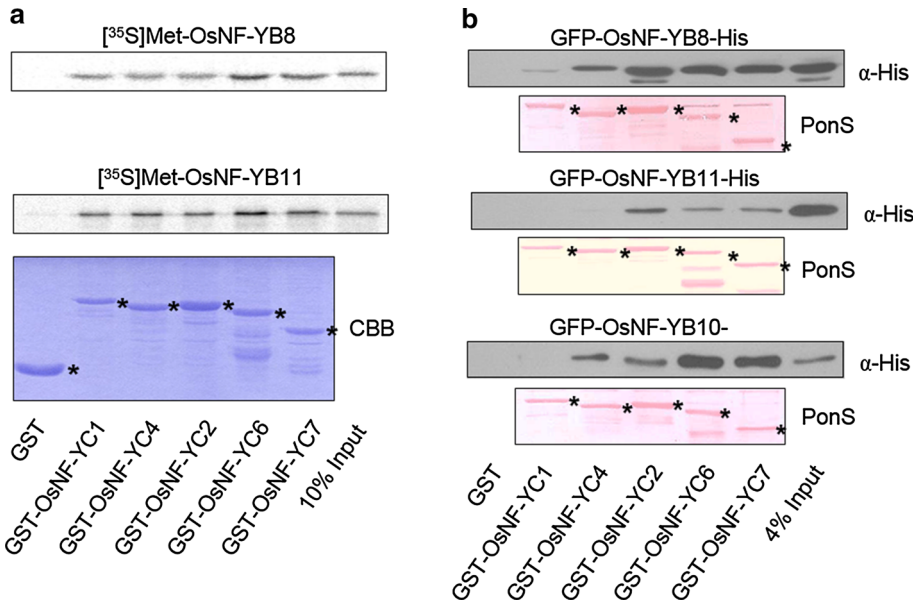

Fig. 1 Protein–protein interaction between three OsNF-YB proteins and five OsNF-YC proteins in vitro. **a** GST pull-down assay between two OsNF-YB proteins and five OsNF-YC proteins. GST or GST-tagged OsNF-YC proteins were incubated with ^{35}S-labeled OsNF-YB8 or ^{35}S-labeled OsNF-YB11 proteins. 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 ^{35}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 (α-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

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).

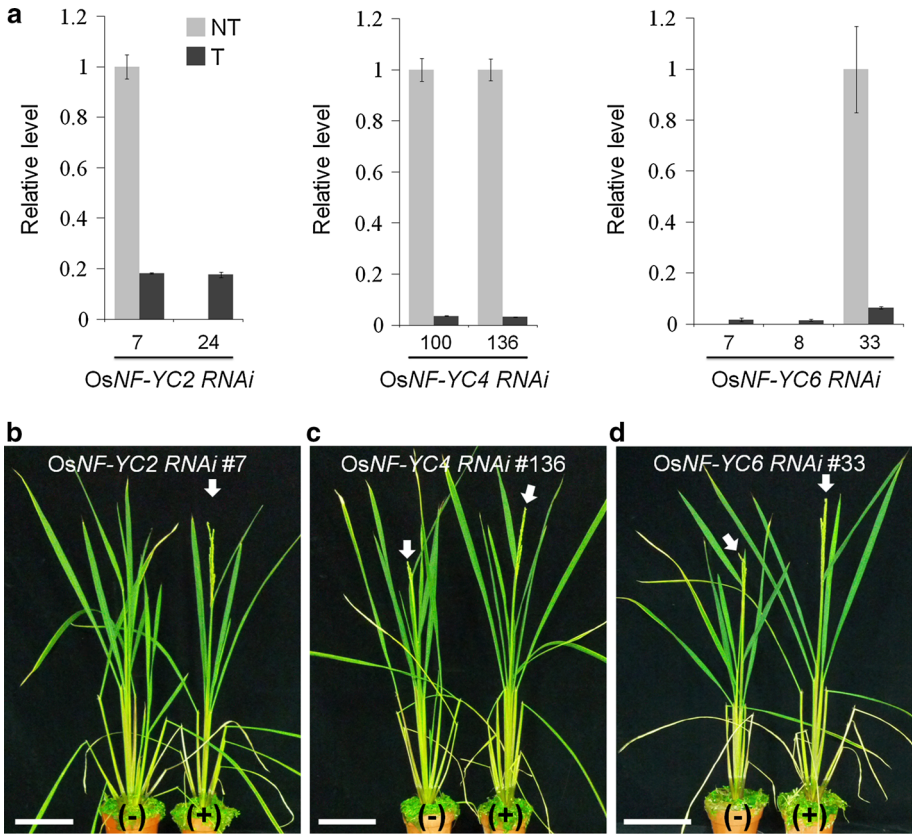

Fig. 2 Flowering phenotypes of Os*NF-YC2*-RNAi, Os*NF-YC4*-RNAi and Os*NF-YC6*-RNAi transgenic plants in the T_1 generation. **a** The expression levels of endogenous Os*NF-YC2*, Os*NF-YC4* and Os*NF-YC6* genes in the segregating lines of the respective transgenic plants. The expression levels of Os*UBQ* gene were used as a normalization control. *Error bars* indicate the standard deviation of the three technical replicates. Os*NF-YC2*-RNAi (**b**), Os*NF-YC4*-RNAi (**c**) and Os*NF-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

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 Os*NF-YBs* and Os*NF-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 planta. 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 Os*NF-YC2*, Os*NF-YC4* or Os*NF-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_1 transgenic plants for each construct were obtained and the flowering time of transgenic plants was examined in the T_2 generation. In this experiment, T_2 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 Os*NF-YC2*, Os*NF-YC4* or Os*NF-YC6* genes in Os*NF-YC2*-RNAi, Os*NF-YC4*-RNAi or Os*NF-YC6*-RNAi, respectively,

Table 1 Flowering time of Os*NF-YC2*-RNAi, Os*NF-YC4*-RNAi and Os*NF-YC6*-RNAi plants

Genotype	No. of days to heading		
	Experiment 1 (SD)	Experiment 2 (LD)	Experiment 3 (LD)
Os*NF-YC2*-RNAi			
line 7(−)	55.3 ± 1.5 (6)	127.2 ± 3.5 (6)	139.3 ± 1.2 (3)
line 7(+)	54 ± 1.4 (7)	115.8 ± 4.7 (4)*	131.8 ± 4.4 (4)*
line 24(+)	54.3 ± 2.3 (7)	112.4 ± 7.8 (5)	133.7 ± 4.5 (3)
Os*NF-YC4*-RNAi			
line 4(+)	55.3 ± 2.4 (7)	106.3 ± 5.0 (4)	119.3 ± 3.8 (3)
line 100(−)	56 ± 1.7 (3)	116 ± 7.2 (6)	126.3 ± 0.6 (3)
line 100(+)	56.3 ± 1.7 (4)	106 ± 2.5 (4)*	126.6 ± 6.5 (5)
line 136(−)	55 ± 2.8 (2)	113.9 ± 6.4 (9)	136 ± 2.8 (2)
line 136(+)	54.4 ± 3.1 (5)	108.5 ± 5.0 (4)*	136.3 ± 1.5 (3)
Os*NF-YC6*-RNAi			
line 7(+)	55.7 ± 1.4 (7)	113.8 ± 7.2 (5)	119 ± 7.2 (3)
line 8(+)	55.1 ± 3.5 (7)	115.6 ± 6.0 (5)	114.7 ± 3.5 (3)
line 33(−)	NA	NA	127 ± 1.0 (3)
line 33(+)	NA	NA	127.3 ± 7.5 (3)
Wild-type	54 ± 1.8 (4)	112.3 ± 6.5 (4)	127.6 ± 6.4 (5)[a]

Wild-type (*O. sativa* L., cv. Hwaan) and transgenic plants for each transgene, and transgenic plants without each transgene (T_1 generation), were grown under LD (14.5 h light/9.5 h dark) and SD (10 h light/14 h dark) conditions. The total number of plants analyzed is given in parenthesis. Plus and minus signs of the lines denote segregants for the transgenic and nontransgenic lines, respectively

NA not available

* Significant difference in flowering time between transgenic and non-transgenic plants (Student's *t* test, $P < 0.05$)

[a] Growth cabinet used for experiment 3 caused delayed days to heading, as compared to that one of experiment 2

decreased to less than 20 % of the corresponding non-transgenic lines (Fig. 2a). However, the transcript levels of other similar Os*NF-YC* genes were not altered in each transgenic lines, i.e., the expression levels of Os*NF-YC1* that occur in the same clade with Os*NF-YC4* were very similar in transgenic or non-transgenic Os*NF-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 Os*NF-YC2*-RNAi (#7) plants was earlier than that of the non-transgenic lines. The differences of days to heading (DTH) of #7 were 11.4 and 7.5 days earlier than non-transgenic lines in two biological replicate experiments, respectively (Table 1; Fig. 2b). Transgenic Os*NF-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 Os*NF-YC6*-RNAi (#33) plants was not altered under the same conditions (Table 1; Fig. 2d). These data suggest that the decreased expression of Os*NF-YC2* leads to early flowering time only under LD conditions.

To further confirm the functional roles of Os*NF-YC2*, Os*NF-YC4* and Os*NF-YC6* in terms of flowering time, we made transgenic plants that overexpressed Os*NF-YC2*, Os*NF-YC4* or Os*NF-YC6* under the maize *ubiquitin1* (*Ubi1*) promoter. T_0 transgenic plants for each construct were obtained and transgenic lines were selected for analysis of flowering time in the T_1 generation. The overexpression of Os*NF-YC2*, Os*NF-YC4* or Os*NF-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::*Os*NF-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::*Os*NF-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

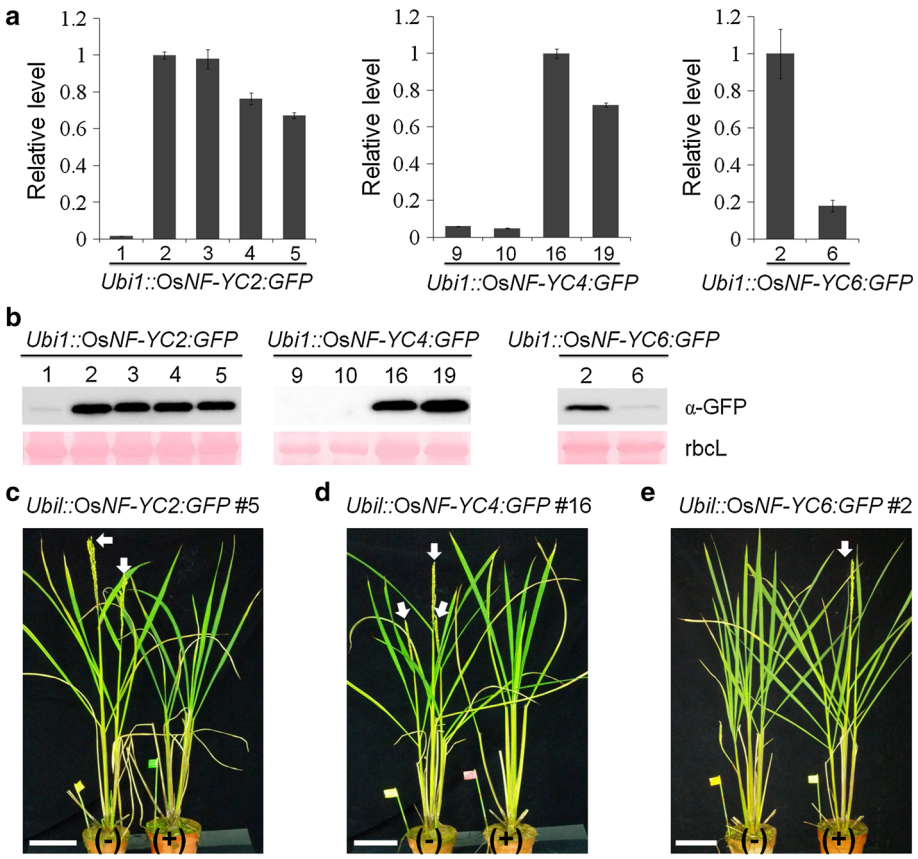

Fig. 3 Flowering phenotypes of *Ubi1::*Os*NF-YC2:GFP*, *Ubi1::*Os*NF-YC4:GFP* and *Ubi1::*Os*NF-YC6:GFP* transgenic plants in the T_1 generation. **a** The mRNA levels of Os*NF-YC2*, Os*NF-YC4* and Os*NF-YC6* genes in the segregating lines of the respective transgenic plants. RT-qPCR was performed to examine the expression levels of the Os*NF-YC* genes. The expression levels of Os*UBQ* 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 (α-GFP) was used for western blot analysis. Ponceau S-stained Rubisco large subunit (rbcL) was used as a loading control. *Ubi1::*Os*NF-YC2:GFP* (**c**), *Ubi1::*Os*NF-YC4:GFP* (**d**) and *Ubi1::*Os*NF-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

*Ubi1::*Os*NF-YC6:GFP* plants (#2) showed an early flowering phenotype, compared to those of segregated non-transgenic 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 appeared to correlate well with the degree of expression of the transgenes (Table 2; Fig. 3a, b). The flowering phenotypes of some selected transgenic lines (#1 in *Ubi1::*Os*NF-YC2:GFP*, #9 and #16 in *Ubi1::*Os*NF-YC4:GFP*, and #2 in *Ubi1::*Os*NF-YC6:GFP*) were further confirmed in the T_2 generation (Table 2), although the flowering times of segregated nontransgenic T_2 siblings were rather divergent from that of wild-type rice plants. This difference may result from the small number of nontransgenic siblings. In addition, we presented RNA and protein expression data of lines #2 and #6 of *Ubi1::OsNF-YC6:GFP* transgenic plants in the T_1 generation (Fig. 3a, b). The flowering times of the two different lines (#2 and #6) correlated with the RNA and protein expression levels. The RNA levels were examined using RT-qPCR analysis which was indicated in the legend of Fig. 3. The difference in flowering time of wild-type rice plants in experiments 2 and 3 was about 17 days. This was not such an extreme difference for the control value because flowering time under LD condition (14.5 h light) was usually more than 110 days in the growth cabinets we have used. 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 Os*NF-YC2*, Os*NF-YC4* or Os*NF-YC6* genes in transgenic plants

To elucidate the molecular mechanisms by which Os*NF-YC2*, Os*NF-YC4* or Os*NF-YC6* regulate flowering time in rice, we investigated the expression of some flowering

Table 2 Flowering time of *Ubi1::*Os*NF-YC2:GFP*, *Ubi1::*Os*NF-YC4:GFP* and *Ubi1::*Os*NF-YC6:GFP* transgenic plants

Genotype	No. of days to heading	
	Experiment 1 (SD)	Experiment 2 (LD)
*Ubi1::*Os*NF-YC2:GFP*		
line 1(−)	58 (1)	114 ± 7.1 (2)
line 1(+)	57.5 ± 1 (4)	138.3 ± 5.5 (3)*
line 2(−)	58 (1)	110 (1)
line 2(+)	58 ± 3 (4)	>190 (3)
line 5(−)	59 (1)	109 (1)
line 5(+)	56 (3)	>190 (2)
*Ubi1::*Os*NF-YC4:GFP*		
line 9(−)	58.5 ± 1.5 (2)	110.5 ± 0.7 (2)
line 9(+)	57.5 ± 1.3 (4)	112.3 ± 0.5 (4)
line 16(−)	58 (3)	118 ± 1.4 (2)
line 16(+)	60 ± 0.8 (5)	183.4 ± 7.9 (5)**
*Ubi1::*Os*NF-YC6:GFP*		
line 1(+)	60 (2)	115 ± 4.2 (2)
line 2(−)	59.3 ± 3.1(3)	117 (1)
line 2(+)	60 ± 2.0 (6)	96.2 ± 2.4 (5)
line 6(+)	63 ± 1.0 (2)	111.5 ± 3.5 (2)
Ubi1::GFP		
line 39(−)	58 (1)	115 (1)
line 39(+)	57.3 ± 1.8 (4)	109.3 ± 1.2 (3)
Wild-type	57 ± 1.2 (5)	112.5 ± 1.9(6)
	Experiment 3 (LD)	
*Ubi1::*Os*NF-YC2:GFP*		
line 3(−)	121.1 ± 1.4 (2)	
line 3(+)	>210 (3)**	
line 4(−)	125 (1)	
line 4(+)	>210 (1)	
line 1–4(−)[a]	134.7 ± 1.5 (3)	
line 1–4(+)[a]	177 ± 3.7 (4)**	
*Ubi1::*Os*NF-YC4:GFP*		
line 10(−)	128.5 ± 6.4(2)	
line10(+)	135.5 ± 4.8(4)	
line 19(−)	124.7 ± 0.6(3)	
line19(+)	>210(3)**	
line 9–3(−)[a]	137.7 ± 4.0 (3)	
line 9–3(+)[a]	138.5 ± 5.8 (4)	
line 16–12(−)[a]	124.5 ± 4.8 (4)	
line 16–12(+)[a]	>210 (4)**	
*Ubi1::*Os*NF-YC6:GFP*		
line 2–1(−)[a]	137 ± 3.3 (4)	
line 2–1(+)[a]	102.3 ± 7.9 (7)**	
line 6–3(+)	128.8 ± 1.9 (4)	

Table 2 continued

Genotype	No. of days to heading	
	Experiment 1 (SD)	Experiment 2 (LD)
Wild-type	129.6 ± 5.2(11)	

Wild-type (*O. sativa* L., cv. Hwaan) and transgenic plants for each transgene, and transgenic plants without each transgene (experiments 1 and 2, T_1 generation; experiment 3, T_1 or T_2 generations) were grown under LD (14.5 h light/9.5 h dark) and SD (10 h light/14 h dark) conditions. The total number of plants analyzed is given in parenthesis. Plus and minus signs of the lines denote segregants for the transgenic and non-transgenic lines, respectively. *NA* not available. > Indicates the days when heading of panicles from plants was not yet observable

* Single (Student's *t* test, $P < 0.05$) and ** double (Student's *t* test, $P < 0.005$) asterisks indicate significant difference in flowering time between each transgenic and non-transgenic plants

[a] Indicate the lines used in the T_2 generation

regulators such as *Early heading date 1* (*Ehd1*) in segregated lines of the respective transgenic plants at T_1 and T_2 generation (Tables 1, 2). Among the RNAi lines, only Os*NF-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 Os*NF-YC2*, Os*NF-YC4* or Os*NF-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 Os*NF-YC2*, Os*NF-YC4* or Os*NF-YC6* were further examined for the expression of flowering regulators using RT-qPCR analysis (Fig. 4; Supplementary Figs. S5, S6). In transgenic *Ubi1::*Os*NF-YC2:GFP* (#3 and 1–4) and *Ubi1::*Os*NF-YC4:GFP* (#19 and 16–12) plants grown under LD conditions, *Ehd1*, *Hd3a*, and *RFT1* expression decreased (Fig. 4a, b), whereas the expression of these increased in transgenic *Ubi1::*Os*NF-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::*Os*NF-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

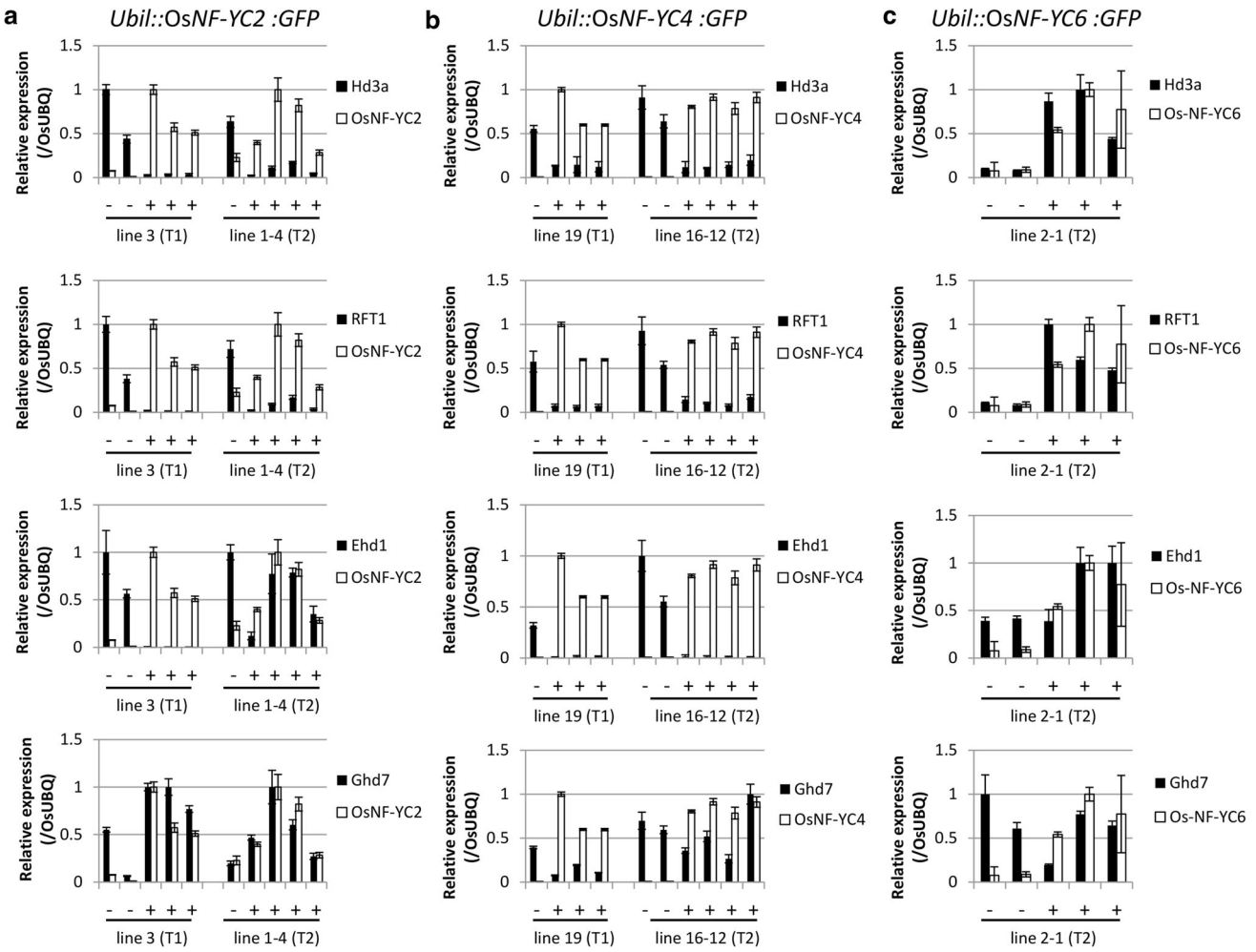

Fig. 4 *Hd3a*, *RFT1*, *Ehd1* and *Ghd7* expression in *Ubi1::*Os*NF-YC2:GFP*, *Ubi1::*Os*NF-YC4:GFP*, and *Ubi1::*Os*NF-YC6:GFP* transgenic plants. Leaves of *Ubi1::*Os*NF-YC2:GFP* (**a**), *Ubi1::*Os*NF-YC4:GFP* (**b**) and *Ubi1::*Os*NF-YC6:GFP* (**c**) transgenic segregants grown under LD conditions 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 Os*NF-YC* genes and the flowering time genes. The expression levels were normalized with that of Os*UBQ*, respectively. The highest level was set at 1. Transgenic plants (+) and their segregating non-transgenic controls (−) were indicated

altered significantly in *Ubi1::*Os*NF-YC2:GFP*, *Ubi1::*Os*NF-YC4:GFP*, and *Ubi1::*Os*NF-YC6:GFP* plants (Supplementary Figs. S4, S5, S6). Other flowering regulators, for example Os*MADS50*, 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 Os*NF-YC* genes function upstream of *Ehd1*, *Hd3a*, and *RFT1* as LD-specific regulators.

The expression patterns of Os*NF-YB* and Os*NF-YC* genes

The diurnal oscillation patterns of the subsets of Os*NF-YB* and Os*NF-YC* genes, Os*CO3*, *Hd1*, and *Ghd7* were compared in rice seedlings grown under LD conditions to examine if there was correlation in the expression patterns. Os*NF-YB11* and Os*NF-YC4* expression showed oscillation patterns with a peak around the end of the light period, which was similar to the pattern observed for *Hd1* expression (Fig. 5a). In contrast, the expression levels of Os*NF-YC2*, *Ghd7*, and Os*CO3* exhibited oscillation patterns with a peak at the beginning of the light period (Fig. 5b). However, the transcripts of Os*NF-YB8*, Os*NF-*

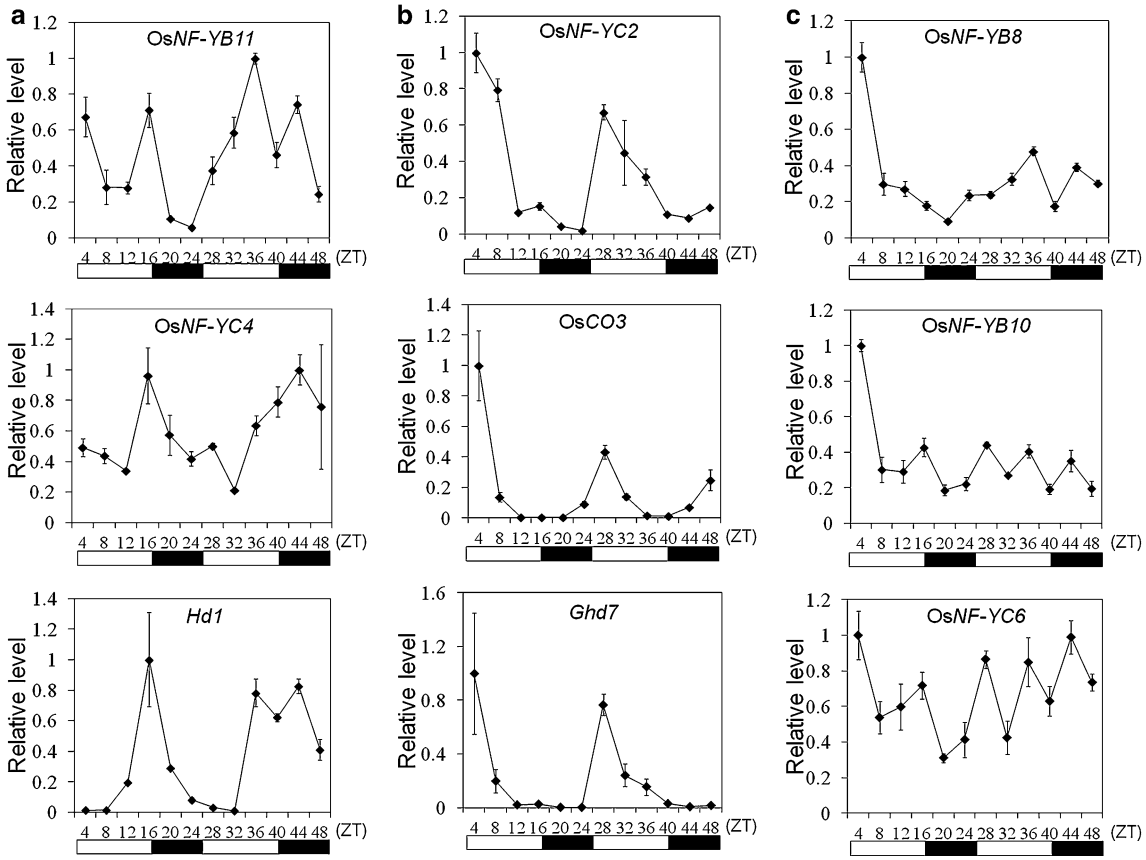

Fig. 5 Diurnal oscillation patterns of Os*NF-YB8*, Os*NF-YB10*, Os*NF-YB11*, Os*NF-YC2*, Os*NF-YC4*, Os*NF-YC6*, *Hd1*, *Ghd7*, and Os*CO3*. **a** Similar oscillation patterns among Os*NF-YC4*, Os*NF-YB11*, and *Hd1*. Rice seedlings were harvested every 4 h for 44 h under LD conditions. The expression levels of Os*UBQ* 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 Os*NF-YC2*, Os*CO3*, and *Ghd7*. **c** Dissimilar patterns of Os*NF-YC6*, Os*NF-YB10*, and Os*NF-YB8*

YB10, and Os*NF-YC6* did not show the diurnal oscillation patterns that were observed in Os*CO3*, *Hd1*, and *Ghd7* (Fig. 5c). These data indicate that specific subsets of Os*NF-YB* and Os*NF-YC* genes have similar diurnal oscillation patterns to Os*CO3*, *Hd1*, and *Ghd7* genes.

The spatial expression patterns of some Os*NF-YB*, Os*NF-YC*, and CCT domain-containing genes were also compared in a variety of rice tissues. Os*NF-YB11* and Os*NF-YC4* and *Hd1* expression showed similar expression patterns (Fig. 6a). Abundant expression was observed in leaf sheaths, leaf blades, flag leaves, and roots. Os*NF-YC2*, *Ghd7*, and Os*CO3* was mainly expressed in the leaf blades and flag leaves (Fig. 6b). This indicates that tissue-specific expression patterns of some Os*NF-YB* and Os*NF-YC* genes are similar to those of Os*CO3*, *Hd1*, and *Ghd7* genes. However, the expression levels of Os*NF-YB8*, Os*NF-YB10*, and Os*NF-YC6* were not similar to those of Os*CO3*, *Hd1*, and *Ghd7* (Fig. 6c). Taken together, these data suggest that OsCO3, Hd1 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 Os*NF-Y* genes (10 Os*NF-YA* genes, 11 Os*NF-YB* genes and 7 Os*NF-YC* genes) (Petroni et al. 2012; Laloum et al. 2013). Overlapping expression patterns of Os*NF-YA*, Os*NF-YB*, and Os*NF-YC* genes have been reported (Thirumurugan et al. 2008). Recently, it has been reported that the Os*NF-YB11* (Os*HAP3H*/*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

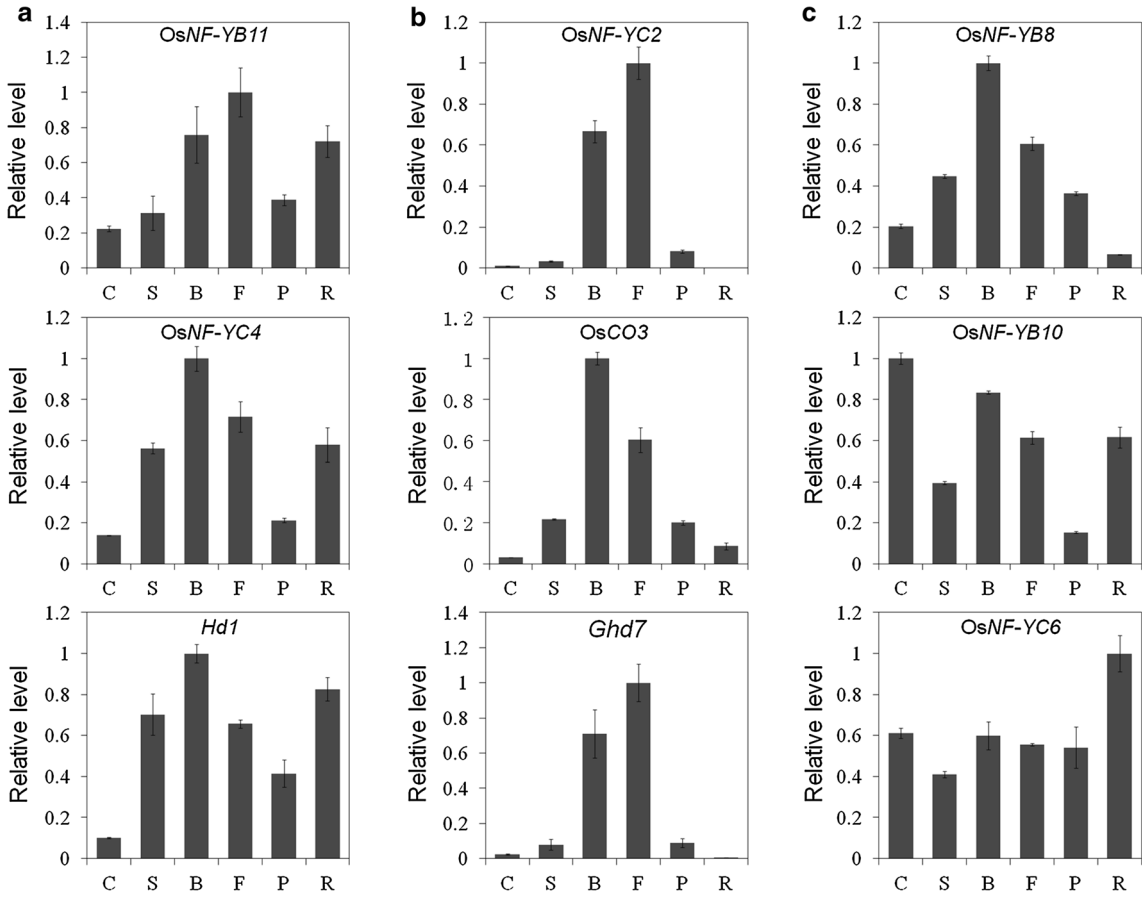

Fig. 6 Spatial expression patterns of Os*NF-YB8*, Os*NF-YB10*, Os*NF-YB11*, Os*NF-YC2*, Os*NF-YC4*, Os*NF-YC6*, *Hd1*, *Ghd7*, and Os*CO3*. **a** Similar expression patterns among Os*NF-YC4*, Os*NF-YB11*, and *Hd1*. Respective samples [culms (C), leaf sheaths (S), leaf blades (B), flag leaves (F), panicles (P), and roots (R)] were harvested from adult rice plants. The expression levels of Os*UBQ* were used as a normalization control. *Error bars* indicate standard deviation of three technical replicates. **b** Similar expression patterns among Os*NF-YC2*, Os*CO3*, and *Ghd7*. **c** Dissimilar expression patterns of Os*NF-YC6*, Os*NF-YB10*, and Os*NF-YB8*

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 Os*NF-YC2* and Os*NF-YC4* genes were similar to those of Os*NF-YB11*, *Hd1*, *Ghd7*, and Os*CO3* genes (Figs. 5, 6). Finally, in the RNAi suppression or overexpression lines of three Os*NF-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 Os*NF-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. First, transgenic approaches using RNAi suppression or overexpression revealed that three Os*NF-YC* genes modulated flowering time only under LD conditions (Figs. 2, 3; Tables 1, 2). Although Os*NF-YC4*-RNAi and Os*NF-YC6*-RNAi plants did not show any significant change in flowering time, Os*NF-YC2*-RNAi plants did show significant acceleration in flowering time when compared to non-transgenic controls. The lack of phenotypic effect in Os*NF-YC4*-RNAi and Os*NF-YC6*-RNAi plants could be due to the functional redundancy of these genes with the homologous Os*NF-YC* genes (Thirumurugan et al. 2008; Petroni et al. 2012; Laloum et al. 2013). Transgenic plants over-expressing the Os*NF-YC* genes showed either delayed or accelerated flowering only under LD conditions (Fig. 3; Table 2). Second, the overexpression of three Os*NF-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 Os*NF-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 Os*NF-YC2* and Os*NF-YC4* on flowering time were the opposite of Os*NF-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 Os*NF-YB* and Os*NF-YC* genes have similar diurnal and spatial expression patterns with *Hd1*, *Ghd7* and Os*CO3* 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.

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