Functional conservation of rice OsNF-YB/YC and *Arabidopsis* AtNF-YB/YC proteins in the regulation of flowering time

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Key message: Rice Os*NF-YB* and Os*NF-YC* complement the late flowering phenotype of *Arabidopsis nf-yb* double and *nf-yc* triple mutants, respectively. In addition, OsNF-YB and OsNF-YC interact with AtNF-YC and AtNF-YB, respectively.

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Abbreviations: *CO*, *CONSTANS*; NF-Y, NUCLEAR FACTOR Y transcription factors; *FT*, *FLOWERING LOCUS T*; GFP, green fluorescence protein; GST, glutathione *S*-transferase; HAP, heme-activated protein; LD, long-day; ORFs, open reading frames; SD, short-day

Abstract

Plant NUCLEAR FACTOR Y (NF-Y) transcription factors play important roles in plant development and abiotic stress. In *Arabidopsis thaliana*, two *NF-YB* (At*NF-YB2* and At*NF-YB3*) and five *NF-YC* (At*NF-YC1*, At*NF-YC2*, At*NF-YC3*, At*NF-YC4*, and At*NF-YC9*) genes regulate photoperiodic flowering by interacting with other AtNF-Y subunit proteins. Three rice *NF-YB* (Os*NF-YB8*, Os*NF-YB10*, and Os*NF-YB11*) and five rice Os*NF-YC* (Os*NF-YC1*, Os*NF-YC2*, Os*NF-YC4*, Os*NF-YC6*, and Os*NF-YC7*) genes are clustered with two At*NF-YB* and five At*NF-YC* genes, respectively. To investigate the functional conservation of these *NF-YB* and *NF-YC* genes in rice and *Arabidopsis*, we analyzed the flowering phenotypes of transgenic plants overexpressing the respective Os*NF-YB* and Os*NF-YC* genes in *Arabidopsis* mutants. Overexpression of Os*NF-YB8*/*10*/*11* and Os*NF-YC2* complemented the late flowering phenotype of *Arabidopsis nf-yb2 nf-yb3* and *nf-yc3 nf-yc4 nf-yc9* mutants, respectively. The rescued phenotype of *35S::*Os*NF-YC2 nf-yc3 nf-yc4 nf-yc9* plants was attributed to the upregulation of *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*). *In vitro* and *in planta* protein–protein analyses revealed that OsNF-YB8/10/11 and OsNF-YC1/2/4/6/7 interact with AtNF-YC3/4/9 and AtNF-YB2/3, respectively. Our data indicate that some Os*NF-YB* and Os*NF-YC* genes are functional equivalents of At*NF-YB2*/*3* and At*NF-YC3*/*4*/*9* genes, respectively, and suggest functional conservation of *Arabidopsis* and rice *NF-Y* genes in the control of flowering time.

Keywords: AtNF-YB/YC, Flowering time, NUCLEAR FACTOR Y transcription factors, OsNF-YB/YC, Protein–protein interaction

Introduction

NUCLEAR FACTOR Y transcription factors [NF-Y, also known as heme-activated protein (HAP) in yeast] as important regulators control numerous genes in all eukaryotes (Edwards et al. 1998; Maity and de Crombrugghe 1998; Laloum et al.). The NF-Y transcription factors form a heterotrimeric complex composed of three unique subunits called NF-YA, NF-YB, and NF-YC (McNabb et al. 1995; Maity and de Crombrugghe 1998; Mantovani 1999). In filamentous fungi and mammals, the NF-YB and NF-YC proteins assemble as a heterodimer in the cytoplasm, which is translocated into the nucleus where it interacts with NF-YA and thereby forms active heterotrimeric complexes (Frontini et al. 2004; Steidl et al. 2004; Goda et al. 2005; Tuncher et al. 2005). The active complex then directly binds to highly conserved CCAAT motifs within the promoter of target genes. *In silico* analyses have revealed that approximately 30% of eukaryotic promoters have the predicted binding sites of NF-Y transcription factors (Bucher 1990; Testa et al. 2005). This suggests that the regulation of gene expression by NF-Y transcription factor complex is widespread in many growth and developmental processes.

NF-Y subunits identified in plants play an important role in various aspects of developmental and stress-induced responses (Meinke et al. 1994; Li et al. 2008; Yamamoto et al. 2009; Petroni et al. 2012; Fornari et al. 2013; Laloum et al. 2013; Mu et al. 2013; Zhang et al. 2015). Many studies have revealed that they act as floral regulators in the modulation of flowering time. Individual homologs of NF-YB and NF-YC subunits are found as the interacting partners of CONSTANS (CO), an important regulator in the photoperiod pathway, in tomato and *Arabidopsis thaliana* (Ben-Naim et al. 2006; Wenkel et al. 2006). The At*NF-YB2* and At*NF-YB3* genes promote the onset of flowering through the induction of the *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* genes (At*NF-Y3*, At*NF-Y4*, and At*NF-Y9*) also have functional redundancy in the activation of photoperiodic flowering through the activation of *FT* under LD conditions in *Arabidopsis* (Kumimoto et al. 2010). In addition, recent biochemical studies of *Arabidopsis* have shown that AtNF-YB proteins physically interact with AtNF-YC proteins to form the entire heterotrimeric complex, prior to binding to the consensus CCAAT motif of their target genes (Calvenzani et al. 2012; Hackenberg et al. 2012b). The three AtNF-YC proteins (AtNF-YC3, AtNF-YC4, and AtNF-YC9) are also known to interact with the AtNF-YB2, AtNF-YB3 and CO proteins (Kumimoto et al. 2010), suggesting that the AtNF-YC subunits make at least six possible complexes with AtNF-YB2 or AtNF-YB3 and CO to fine-tune the timing of flowering under a variety of environmental conditions (Wenkel et al. 2006; Kumimoto et al. 2010).

Monocot *NF-YB* and *NF-YC* genes homologous to *AtNF-YB* and *AtNF-YC*, respectively, have been shown to regulate flowering time. In rice, Os*NF-YB11*/Os*HAP3H*/*Ghd8*/*DTH8* has been revealed to regulate flowering time under LD and short-day (SD) conditions by changes in *Early heading date 1* (*Ehd1*), *RICE FLOWERING LOCUS T 1* (*RFT1*), and *Heading date 3a* (*Hd3a*) expression (Wei et al. 2010; Yan et al. 2011). Recently, our group reported that Os*NF-YC2* and Os*NF-YC4* inhibits flowering via decreased expression of *Ehd1*, *RFT1* and *Hd3a* under LD conditions in rice, whereas Os*NF-YC6* promotes flowering via increased their expression under same conditions (Kim et al. 2015). Also, OsNF-YC2/4/6 proteins physically interacted with OsNF-YB8/10/11 proteins. In addition,

overexpression of *Brachypodium distachyon NF-YB3* (Bd*NF-YB3*) and Bd*NF-YB6* in *Arabidopsis nf-yb2 nf-yb3* double mutant results in flowering phenotypes similar to wild-type plants; BdNF-YB3 and BdNF-YB6 are found to physically interact with AtNF-YC3, AtNF-YC4, and AtNF-YC9 by using yeast-two hybrid analysis (Cao et al. 2011). Furthermore, *Ghd7*/*OsI*, a rice CCT domain-containing protein, regulates flowering under LD conditions (Xue et al. 2008). However, the protein–protein interactions between OsNF-Y proteins and Heading date 1 (Hd1), an *Arabidopsis* CO homolog, have not yet been characterized. In wheat (*Triticum aestivum*), the CCT domain of flowering promoter CO2, a rice Hd1 homolog, and flowering repressor VRN2 (ZCCT1 and 2) physically interact with TaNF-YB, and these CCT proteins compete with TaNF-YA for interaction with TaNF-YB (Li et al. 2011). These data suggest the functional conservation of monocot NF-Y transcription factors through a conserved molecular mechanism in the control of flowering time. However, our current knowledge about the role of OsNF-Y transcription factors in the regulation of flowering remains limited.

At*NF-YB2/3* and At*NF-YC1/2/3/4/9* are known to control flowering time in *Arabidopsis* (Chen et al. 2007; Kumimoto et al. 2008; Kumimoto et al. 2010; Hackenberg et al. 2012a). According to phylogenetic tree analysis, Os*NF-YB8*/*10*/11, Os*NF-YC1/4*, Os*NF-YC2* and Os*NF-YC6/7* are clustered with At*NF-YB2/3*, At*NF-YC2*, At*NF-YC1/4*, and At*NF-YC3/9*, respectively (Thirumurugan et al. 2008; Petroni et al. 2012; Laloum et al. 2013). Thus, we investigated the effects of the overexpression of Os*NF-YB* and Os*NF-YC* genes in Arabidopsis *nf-yb* double mutants and *nf-yc* triple mutants, respectively, to understand the functional conservation of these genes. In addition, we examined the protein–protein interactions between OsNF-YB and AtNF-YC or OsNF-YC and AtNF-YB.

Materials and methods

Plant materials and growth conditions

Wild-type or transgenic *Arabidopsis* plants [ecotype Columbia (Col-0)] were grown in soil or Murashige and Skoog (MS) medium at 23 °C under LD conditions (16:8 h light:dark photoperiod) at a light intensity of 120 μmol m^{-2} s^{-1}. *nf-yb2 nf-yb3* and *nf-yc3 nf-yc4 nf-yc9* mutants (Cai et al. 2007; Kumimoto et al. 2008; Kumimoto et al. 2010) were used for plant transformation to investigate the function 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*.

Plasmid construction

For the transgenic approach, full-length open reading frames (ORFs) for 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 amplified from total RNA of wild-type rice seedlings (*Oryza sativa* L., cv. Hwaan; a gift from National Institute of Agricultural Biotechnology, Suwon, South Korea) by reverse transcription (RT)-polymerase chain reaction (PCR) using gene-specific primer sets (Supplementary Table S1). The amplicons were digested with several restriction enzymes and the resulting restriction fragments were subcloned into pCHF3, a plant transformation vector harboring the *CaMV 35S* promoter. For *in vitro* protein expression, the ORFs of At*NF-YB2*, At*NF-YB3*, At*NF-YC3*, At*NF-YC4*, At*NF-YC9*, 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) or pET21a-d(+) vector (GE Healthcare). Oligonucleotide sequences used for cloning are provided in Supplementary Table S1. For subcellular localization analysis, the ORFs of At*NF-YB2*, At*NF-YB3*, At*NF-YC3*, At*NF-YC4*, and At*NF-YC9* were cloned into pCAMBIA1300 or pCAMBIA2300 vectors. Oligonucleotide sequences used for cloning are provided in Supplementary Table S1. For bimolecular fluorescence complementation (BiFC) analysis, the ORFs of At*NF-YB2*, At*NF-YB3*, At*NF-YC3*, At*NF-YC4*, At*NF-YC9*, 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 pSPYNE-*35S* or pSPYCE-35S vectors (Walter et al. 2004). Oligonucleotide sequences used for cloning are provided in Supplementary Table S1. The resulting recombinant plasmid was sequenced to verify the absence of PCR errors during amplification.

Generation of transgenic plants and measurement of flowering time

Transgenic plants were generated using the floral dip method with minor modifications (Weigel and Glazebrook 2002). Kanamycin-resistant transgenic seedlings whose genotype was confirmed via PCR were transferred to the soil. At least 20–30 T_1 seedlings were analyzed for each construct. To measure flowering time, the total number of rosette and cauline leaves of at least 6 independent transgenic lines (average 5 individual plants per independent transgenic line) was counted in the T_2 generation (Lee et

al. 2012). At least 10 individual plants were used for measuring flowering time in the T_3 or T_4 generations. To determine the significant difference in flowering time of transgenic plants compared with that of *Arabidopsis* double or triple mutants, the data was analyzed using SPSS software version 12.0 (Sbaihat et al. 2015).

Expression analysis

Total RNA was isolated from *Arabidopsis* (Col-0) whole seedlings using the Plant RNA Purification Reagent (Invitrogen, Carlsbad, CA, USA). RNA quality was determined with a Nanodrop ND-2000 spectrophotometer (Nanodrop Technologies), and only high quality RNA samples ($A_{260}/A_{230} > 2.0$ and $A_{260}/A_{280} > 1.8$) were used for subsequent experiments. To remove possible DNA contamination, RNA samples were treated with DNaseI for 60 min at 37 °C. RNA (1 µg) was used for cDNA synthesis, in accordance with the manufacturer's instructions (Roche Applied Science, Madison, WI, USA). Gene expression levels were analyzed via RT–PCR or real time (RT)–quantitative polymerase chain reaction (qPCR) methods, as previously described (Lee et al. 2005; Hong et al. 2010). RT–qPCR analysis was carried out in 384-well plates with a LightCycler 480 (Roche Applied Science) using LightCycler 480 SYBR green master (Roche Applied Science) to monitor the PCR amplification. *PP2AA3* (AT1G13320) was used as a reference gene, according to "The eleven golden rules for quantitative RT-PCR" (Udvardi et al. 2008; Hong et al. 2010). All RT–qPCR experiments were carried out in two biological replicates (independently harvested samples) with three technical replicates, each with similar results. Oligonucleotide sequences used for the expression analysis are provided in Supplementary Table S2.

***In vitro* protein–protein interaction analysis**

Glutathione *S*-transferase (GST) fusion recombinant proteins were mixed with the *Escherichia coli* lysates, and the mixtures were gently rotated for 2 h at 4 °C. Subsequently, they were washed three times with the washing buffer and eluted with 10 mM reduced glutathione in 100 mM NaCl and 20 mM Tris-HCl (pH 7.2). Finally, the eluted protein samples were analyzed by 12.5% SDS-PAGE and visualized by western blot analysis. The detailed procedure has been previously reported (Jang et al. 2009).

Subcellular localization and BiFC analyses

For transient expression in tobacco (*Nicotiana benthamiana*) leaves, the *Agrobacterium tumefaciens* strain C58C1 harboring the various combinations of constructs was infiltrated into the abaxial sides of 3-week-old tobacco plants. Subsequently, epidermal cells of infiltrated tobacco leaves were examined for fluorescence using a confocal microscope (LSM 510 META, Carl Zeiss, Germany). The detailed procedure has been previously reported (Jang et al. 2009).

Results and Discussion

Overexpression of Os*NF-YB8/10/11* rescues the late flowering phenotype of *Arabidopsis nf-yb2 nf-yb3* double mutants

At*NF-YB2* and At*NF-YB3* regulate flowering time in *Arabidopsis* (Chen et al. 2007; Kumimoto et al. 2008) and three rice *NF-YB* genes (Os*NF-YB8*, Os*NF-YB10*, and Os*NF-YB11*) are clustered with At*NF-YB2* and At*NF-YB3* (Thirumurugan et al. 2008; Petroni et al. 2012; Laloum et al. 2013). To assess the possible functional conservation of biological activities between rice and *Arabidopsis* homologues, we expressed the respective rice Os*NF-Y*B genes (Os*NF-YB8*, Os*NF-YB10*, and Os*NF-YB11*) under the control of the *35S* promoter in *nf-yb2 nf-yb3* double mutants. For analysis of flowering time, we selected six independent transgenic lines showing a Mendelian inheritance (3:1 ratio) of kanamycin resistance in the T2 generation. In this experiment, we measured flowering time of individual plants in six independent lines overexpressing the respective Os*NF-YB8*, Os*NF-YB10*, and Os*NF-YB11* under LD conditions. Os*NF-YB8/10/11* transgenes were able to rescue the late flowering phenotype of *nf-yb2 nf-yb3* mutants, although the effect of Os*NF-YB11* on the complementation of the *nf-yb2 nf-yb3* mutation was very weak (Figs. 1a–d, Supplementary Table S3, Supplementary Fig. S1). Some *35S::*Os*NF-YB8 nf-yb2 nf-yb3* and *35S::*Os*NF-YB10 nf-yb2 nf-yb3* transgenic lines flowered earlier than wild-type plants showing 12.8 leaves [line 7 (11.3 leaves) in *35S::*Os*NF-YB8 nf-yb2 nf-yb3*, and lines 17 (9.5 leaves) and 18 (8.5 leaves) in *35S::*Os*NF-YB10 nf-yb2 nf-yb3*]. The degree of rescue was correlated with the expression levels of Os*NF-YB8* and Os*NF-YB10* in *35S::*Os*NF-YB8 nf-yb2 nf-yb3* and *35S::*Os*NF-YB10 nf-yb2 nf-yb3* plants, respectively (Supplementary Fig. S2). Because the degree of overexpression of Os*NF-YB11* in transgenic plants was similar to that of Os*NF-YB8* or Os*NF-YB10* in transgenic plants (Supplementary Fig. S2), the comparatively weak rescue seen in *35S::*Os*NF-YB11 nf-yb2 nf-yb3* plants cannot be explained by the differential expression of transgenes among transgenic plants. This result suggests the stronger effect of Os*NF-YB8* and Os*NF-YB10* in rescuing the late flowering phenotype of *nf-yb2 nf-yb3* mutants, compared with that of Os*NF-YB11*. This notion was supported by the findings that OsNF-YB8 and OsNF-YB10 are closer to AtNF-YB2/3 in the phylogenetic tree (Thirumurugan et al. 2008; Petroni et al. 2012; Laloum et al. 2013). These results suggest that Os*NF-YB8/10/11* genes are functional equivalents of At*NF-YB2/3* genes in rice.

Overexpression of Os*NF-YC2* rescues the late flowering phenotype of *Arabidopsis nf-yc3 nf-yc4 nf-yc9* triple mutants

At*NF-YC1*, At*NF-YC2*, At*NF-YC3*, At*NF-YC4*, and At*NF-YC9* are known to regulate flowering time in *Arabidopsis* (Kumimoto et al. 2010; Hackenberg et al. 2012a), and Os*NF-YC1/4*, Os*NF-YC2*, and Os*NF-YC6/7* are clustered with At*NF-YC2*, At*NF-YC1/4*, and At*NF-YC3/9*, respectively (Thirumurugan et al. 2008; Petroni et al. 2012; Laloum et al. 2013). Therefore, to assess the possible functional conservation between rice and *Arabidopsis* homologues, we overexpressed five rice Os*NF-YC* genes (Os*NF-YC1*, Os*NF-YC2*, Os*NF-YC4*, Os*NF-YC6*, and Os*NF-YC7*) under the *35S* promoter in

nf-yc3 nf-yc4 nf-yc9 triple mutants. To measure flowering time, we selected at least seven independent transgenic lines showing a single transgene insertion in the T2 generation. We also analyzed flowering time of individual plants in transgenic lines overexpressing the respective Os*NF-YC1*, Os*NF-YC2*, Os*NF-YC4*, Os*NF-YC6*, and Os*NF-YC7* genes under LD conditions. The Os*NF-YC2* gene was able to rescue the late flowering phenotype of *nf-yc3 nf-yc4 nf-yc9* mutants, but Os*NF-YC1/4/6/7* genes were not able to significantly rescue this phenotype of these mutants (Figs. 2a, b; Supplementary Table S3; Supplementary Fig. S3). Furthermore, some *35S::*Os*NF-YC2 nf-yc3 nf-yc4 nf-yc9* transgenic lines flowered earlier than wild-type plants showing 9.1 leaves [lines 6 (8.6 leaves), 17 (7.7 leaves), 19 (8.0 leaves), 21 (9.0 leaves), and 22 (9.0 leaves)]. The degree of rescue was correlated with the expression levels of Os*NF-YC2* in *35S::*Os*NF-YC2 nf-yc3 nf-yc4 nf-yc9* plants (Supplementary Fig. S4). However, non-rescued flowering phenotypes seen in *35S::*Os*NF-YC4 nf-yc3 nf-yc4 nf-yc9* or *35S::*Os*NF-YC7 nf-yc3 nf-yc4 nf-yc9* plants cannot be explained by the differential expression of transgenes among transgenic plants (Supplementary Fig. S4), suggesting that Os*NF-YC2* exerts the strongest effect on the complementation of the *nf-yc3 nf-yc4 nf-yc9* mutation. Similarly, Os*NF-YC2* could strongly rescue the late flowering phenotype of *nf-yc3 nf-yc9* double mutants compared to other Os*NF-YC* genes (Supplementary Fig. S5, Supplementary Table S3). Although OsNF-YC2 is closer to AtNF-YC4 than to AtNF-YC3/9 in the phylogenetic tree (Thirumurugan et al. 2008; Petroni et al. 2012; Laloum et al. 2013), these results indicate that Os*NF-YC2* is a functional equivalent of At*NF-YC3/4/9* in rice.

Os*NF-YC2* regulates *FT* and *SOC1* expression in *Arabidopsis nf-yc3 nf-yc4 nf-yc9* triple mutants overexpressing Os*NF-YC2*

Because most *35S::*Os*NF-YC2 nf-yc3 nf-yc4 nf-yc9* plants showed wild-type-like flowering phenotypes (Fig. 2), we examined the expression of flowering time genes in *35S::*Os*NF-YC2 nf-yc3 nf-yc4 nf-yc9* plants under LD conditions. Overexpression of Os*NF-YC2* still rescued the late flowering phenotype of *nf-yc3 nf-yc4 nf-yc9* mutants in the T_3 generation (Fig. 3a), indicating that the phenotypes of *35S::*Os*NF-YC2 nf-yc3 nf-yc4 nf-yc9* plants are stably inherited.

Because At*NF-YC3/4/9* regulate photoperiodic flowering time in *Arabidopsis* (Kumimoto et al. 2010), we checked the expression levels of photoperiod pathway genes [*GIGANTEA* (*GI*), *CO*, *FT*, and *SOC1*] in transgenic plants in the T_4 generation under LD conditions. We found that *FT* and *SOC1* expression was significantly increased compared with *nf-yc3 nf-yc4 nf-yc9* mutants (Figs. 3b, c), indicating that Os*NF-YC2* regulates flowering time via the activation of *FT* and *SOC1*. In contrast, *GI* and *CO* expression remained unaltered in transgenic plants (Supplementary Fig. S6). This was consistent with the previous finding that At*NF-YC3/4/9* controls *FT* expression with CO and other AtNF-Y components under LD conditions (Kumimoto et al. 2010). These results suggest that the Os*NF-YC2* promotes flowering via upregulation of *FT* and *SOC1* expression in *Arabidopsis* plants.

OsNF-YB and OsNF-YC proteins interact with AtNF-YC and AtNF-YB proteins, respectively

AtNF-YC3, AtNF-YC4, and AtNF-YC9 regulate photoperiod-dependent flowering time via protein–

protein interaction with AtNF-YB2, AtNF-YB3, and CO proteins in *Arabidopsis* (Kumimoto et al. 2010). In addition, NF-Y transcription factors control the expression of downstream target genes via direct binding to CCAAT consensus motifs within the promoter in the nucleus (Frontini et al. 2004; Steidl et al. 2004; Goda et al. 2005; Tuncher et al. 2005). Therefore, we initially examined the subcellular localization patterns of AtNF-YB and AtNF-YC proteins in tobacco leaves. The AtNF-YC3/4/9-GFP proteins were localized in both the nucleus and the cytoplasm, whereas the AtNF-YB2/3-GFP proteins were present only in the cytoplasm (Supplementary Fig. S7). In addition, the OsNF-YB11-GFP and OsNF-YC1/2/4/6/7-GFP proteins were found in both nucleus and cytoplasm, whereas the OsNF-YB8-GFP and OsNF-YB10-GFP proteins were present only in the cytoplasm and nucleus, respectively (Kim et al. 2015). Despite the different subcellular localizations of OsNF-YB8 and OsNF-YB10 proteins, the overexpression of either Os*NF-YB8* or Os*NF-YB10* rescued the late flowering phenotype of *nf-yb2 nf-yb3* mutants (Fig. 1). This suggests that the localization patterns of OsNF-YBs/AtNF-YCs protein complexes may be important for their function in the regulation of flowering time. This notion is supported by the observation that NF-Y proteins, which are present only in the cytoplasm, were transported into the nucleus via a "piggy-back" mechanism involving interacting NF-Y proteins (Steidl et al. 2004). This result indicates that the subcellular localization patterns of the AtNF-YB/YC and OsNF-YB/YC proteins overlap *in planta*.

The overlapping localization patterns of the AtNF-YB/YC-GFP proteins with the OsNF-YB/YC-GFP proteins raise the possibility of the protein–protein interaction between OsNF-YB and AtNF-YC proteins, or OsNF-YC and AtNF-YB proteins. We studied protein–protein interactions *in vitro* and *in planta*. For the *in vitro* pull-down assay, At*NF-YBs*/*YCs* and Os*NF-YBs*/*YCs* were fused with GST or GFP and His proteins, respectively. For the BiFC assay in tobacco leaves, the N- or C-terminal yellow fluorescent protein (YFP) fragment was fused with At*NF-YBs*, At*NF-YCs*, Os*NF-YBs*, and Os*NF-YCs* constructs (Walter et al. 2004). The GFP and His-tagged OsNF-YB10 protein significantly interacted with two AtNF-YC proteins (AtNF-YC3 and AtNF-YC9), compared with the other two OsNF-YB proteins (OsNF-YB8 and OsNF-YB11) (Fig. 4a; Supplementary Figs. S8a, b). This suggests that the strong effect of Os*NF-YB10* complementing the *nf-yb2 nf-yb3* mutation (Figs. 1a–d; Supplementary Table S3; Supplementary Fig. S1) may be caused by the significant physical interaction between the OsNF-YB10 and AtNF-YC proteins. YFP signals of three OsNF-YB and three AtNF-YC proteins were also observed in the nucleus and cytoplasm of the transformed tobacco epidermal cells (Fig. 4b, Supplementary Fig. S8c).

In the protein–protein interaction between the OsNF-YC and AtNF-YB proteins, the GFP and His-tagged OsNF-YC2 protein significantly bound to AtNF-YB3 protein, compared with the other two OsNF-YC proteins (OsNF-YC1 and OsNF-YC4) (Fig. 5a; Supplementary Figs. S9a, b). This suggests that the significant interaction between OsNF-YC2 and AtNF-YB proteins may result in the strong effect of Os*NF-YC2* in rescuing the late flowering phenotype of *nf-yc3 nf-yc4 nf-yc9* mutants (Figs. 2a, b; Supplementary Table S3; Supplementary Fig. S3). In addition, YFP signals of OsNF-YC2 and two AtNF-YB proteins were detected in the nucleus and cytoplasm of the tobacco epidermal cells (Fig. 5b). Other OsNF-YC proteins (OsNF-YC1, OsNF-YC4, OsNF-YC6, and OsNF-YC7) interacted with AtNF-YB2 and AtNF-YB3 proteins *in vitro* and *in planta* (Supplementary Fig. S9c). These results

indicate that OsNF-YB and OsNF-YC proteins could physically interact with AtNF-YC and AtNF-YB proteins, respectively, *in vitro* and *in planta*.

Collectively, our results suggest that the functions of some Os*NF-YB* and Os*NF-YC* genes involved in the control of flowering time are conserved in *Arabidopsis*. In addition, the present study indicated that OsNF-YB and OsNF-YC proteins interact with AtNF-YC and AtNF-YB proteins, suggesting that the functional NF-Y complexes generated by the interaction between OsNF-YB and AtNF-YC, or OsNF-YC and AtNF-YB proteins can effectively bind the genomic regions of downstream targets, which could rescue the late flowering phenotype of *Arabidopsis* mutants. However, some Os*NF-YC* genes did not complement the *nf-yc3 nf-yc4 nf-yc9* mutation, although they interacted with AtNF-YB2/3 proteins. This suggests their different function among Os*NF-YC* genes in the regulation of flowering time. This notion is supported by the observation that overexpression of the Os*NF-YC2* or Os*NF-YC4* inhibited flowering in rice, whereas overexpression of the Os*NF-YC6* in rice promoted flowering (Kim et al. 2015). In addition, the overexpression effect of Os*NF-YB11* on the late flowering phenotype of *nf-yb2 nf-yb3* plants was weaker than those of Os*NF-YB8/10*, although the overexpression of Os*NF-YB11*/Os*HAP3H*/*Ghd8*/*DTH8* known as a rice floral repressor in *Arabidopsis* led to flower about 10 days earlier than wild-type plants only under LD conditions (Yan et al. 2011). This discrepancy may be due to the different light conditions, because they transferred the seedlings grown under day-neutral conditions into the LD conditions (Yan et al. 2011).

Considering that some NF-Y components are involved in stress-induced processes such as ABA signaling and drought resistance (Li et al. 2008; Zhang et al. 2015), further investigation is needed on whether some Os*NF-YC* genes act in stress responses.

Author contribution statement

J-KK conceived and designed the research. Y-HH, S-KK, and KCL conducted experiments. Y-HH, S-KK, KCL, YSC, JHL, and J-KK analyzed data. JHL and J-KK wrote the manuscript. All authors read and approved the manuscript.

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Disclosure of Potential Conflicts of Interest

The authors declare that they have no conflict of interest.

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Fig. 1 Flowering phenotypes of transgenic plants overexpressing Os*NF-YB8* and Os*NF-YB10* in *Arabidopsis nf-yb2 nf-yb3* double mutants. **a and b.** Flowering time of *35S::*Os*NF-YB8 nf-yb2 nf-yb3* (a) and *35S::*Os*NF-YB10 nf-yb2 nf-yb3* (b) plants in the T_2 generation under LD conditions. Homozygous and hemizygous transgenic plants were used to measure flowering time. Asterisks indicate significant difference in flowering time of transgenic plants compared with that of double mutants (Student's *t* test, $^{*}P < 0.05$, $^{**}P < 0.01$). Error bars indicate standard deviation (SD). **c and d.** Phenotypes of *35S::*Os*NF-YB8 nf-yb2 nf-yb3* (c) and *35S::*Os*NF-YB10 nf-yb2 nf-yb3* (d) plants in the T_2 generation under LD conditions. Photographs were captured when Col-0 plants flowered. *Scale bars,* 2 cm

Fig. 2 Flowering phenotypes of transgenic plants overexpressing Os*NF-YC2* in *Arabidopsis nf-yc3 nf-yc4 nf-yc9* triple mutants. **a.** Flowering time of *35S::*Os*NF-YC2 nf-yc3 nf-yc4 nf-yc9* plants in the T_2 generation under LD conditions. Homozygous and hemizygous transgenic plants were used to measure flowering time. Asterisks indicate significant differences in flowering time of transgenic plants compared with that of triple mutants (Student's *t* test, $^{*}P < 0.05$, $^{**}P < 0.01$). Error bars indicate SD. **b.** Phenotypes of *35S::*Os*NF-YC2 nf-yc3 nf-yc4 nf-yc9* plants in the T_2 generation under LD conditions. Photographs were captured when Col-0 plants flowered. *Scale bars,* 2 cm

Fig. 3 Expression analysis of flowering time genes in *35S::*Os*NF-YC2 Arabidopsis nf-yc3 nf-yc4 nf-yc9* plants. **a.** Flowering time of *35S::*Os*NF-YC2 nf-yc3 nf-yc4 nf-yc9* plants in the T_3 generation under LD conditions. Homozygous transgenic plants were used to measure flowering time. Asterisks indicate significant differences in flowering time of transgenic plants compared with that of triple mutants (Student's *t* test, $^{**}P < 0.01$). Error bars indicate SD. **b.** Expression levels of *FT* and *SOC1* in 8-day-old *35S::*Os*NF-YC2 nf-yc3 nf-yc4 nf-yc9* plants in the T_4 generation under LD conditions. Samples for RT-qPCR were harvested at Zeitgeber time (ZT) 16. Expression levels of each gene in Col-0 plants were set to one

Fig. 4 Protein–protein interaction between OsNF-YB10 and three AtNF-YC proteins *in vitro* and *in planta*. **a.** *In vitro* pull-down assay. Glutathione *S*-transferase (GST) or GST-tagged AtNF-YC proteins were incubated with green fluorescent protein (GFP) and Histidine (His)-tagged OsNF-YB10 protein. The eluates were separated by 12.5% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and probed with anti-His antibody. About 10% of the total sample in each reaction was loaded as an input control. Ponceau S-stained bands indicated by arrows show the amount and quality of the GST fusion proteins used in this assay. **b.** Bimolecular fluorescence complementation (BiFC) analysis. A BiFC assay was performed using tobacco (*Nicotiana benthamiana*) leaf epidermal cells. At*NF-YC3/4/9* and Os*NF-YB10* genes were fused to the N- and C-terminal yellow fluorescent protein (YFP) fragment genes, respectively. YFP signal between bZIP63-YFP^N and bZIP63-YFP^C was used as a positive control. There were no YFP signals between YFP^N and OsNF-YB10-YFP^C or AtNF-YC3/4/9-YFP^N and YFP^C used as negative controls. (I) indicates YFP fluorescence and (II) indicates bright field images. *Scale bars,* 50 μm

Fig. 5 Protein–protein interaction between OsNF-YC2 and two AtNF-YB2/3 proteins *in vitro* and *in planta*. **a.** *In vitro* pull-down assay. GST or GST-tagged AtNF-YB proteins were incubated with GFP and His-tagged OsNF-YC2 protein. The eluates were separated by 12.5% SDS-PAGE, transferred to PVDF membranes, and probed with anti-His antibody. About 4% of the total sample in each reaction was loaded as an input control. Ponceau S-stained bands indicated by arrows show the amount and quality of the GST fusion proteins used in this assay. **b.** BiFC analysis in tobacco leaf epidermal cells. At*NF-YB2/3* and Os*NF-YC2* genes were fused to the N- and C-terminal YFP fragment genes, respectively. YFP signal between bZIP63-YFP^N and bZIP63-YFP^C shown in Fig. 4b was used as a positive control. There were no YFP signals between YFP^N and OsNF-YC2-YFP^C or AtNF-YB2/3-YFP^N and YFP^C used as negative controls. (I) indicates YFP fluorescence and (II) indicates bright field images. *Scale bars,* 50 μm