NF-YB1-YC12-bHLH144 complex directly activates Wx to regulate grain quality in rice (Oryza sativa L.)

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Introduction

The modulation of transcription of most eukaryotic genes is coordinated through sequence-specific binding of transcription factors to the promoter section situated upstream of the gene (Gelinas et al., 1985; Mantovani, 1998). CCAAT box, which is found between 80 and 300 bp from the transcription start site and may function in either direction, has been found to function as positive regulatory cis-acting elements in various species including human (Martyn et al., 2016), mouse (Bernadt and Rizzino, 2003), yeast (McNabb et al., 1995) and filamentous fungus (Steidl et al., 1999). A diverse of transcription factors with different levels of specificity has been revealed to bind to different CCAAT boxes, (Dorn et al., 1987; Raymondjean et al., 1988), and each is thought to play a discrete role in DNA replication or gene expression (Santoro et al., 1988). The binding of CCAAT box by NF-Ys (Nuclear Factor Y), which also named as HAP (Heme Activator Protein) or CBF (CCAAT box-Binding Factor), is anticipated to be a significant mechanism required for transcription activation (Romier et al., 2003). NF-Y family is comprised by three subunits NF-YA, NF-YB and NF-YC. Protein structure analysis indicated that NF-YA harbours two conserved α-helix domains A1 and A2. The 20-amino acids (AAs)-long A1 domain locates in the N-terminal side and is responsible for protein–protein interaction with NF-YB and NF-YC, whereas the C-terminal locates A2 domain determines the binding specificity with CCAAT boxes (Laloum et al., 2013; Petroni et al., 2012). Like histone H2B and H2A, typical NF-YB and NF-YC subunits have a highly conserved histone fold domains (also called histone-like domains) containing three α-helices (α1, α2 and α3), and another α-helix domain in the C-terminal (αC; Frontini et al., 2004; Laloum et al., 2013). These domains confer the NF-YB and NF-YC subunits with both protein–protein and protein–DNA binding abilities. It has been demonstrated that NF-Ys work as heterotrimer complexes to bind with the CCAAT-containing regulatory elements. The complex forms in a sequential order by NF-YB bind with NF-YC, then the dimer complex bind with NF-YA. NF-YB and NF-YC could not bind with NF-YA without being in a dimer format (Petroni et al., 2012). For the NF-Y complex in mammals, it has been clear that NF-YB-YC dimer binds to the sugar-phosphate backbone of DNA flanking the CCAAT box in a manner similar to the H2A-H2B-DNA assembly. Such nonspecific DNA binding is supposed to stabilize the NF-Y complex with DNA. Meanwhile, the NF-YA part specifically binds to the CCAAT box by inserting into the minor groove of DNA to drive gene transcription (Oldfield et al., 2014).

In contrast to the conditions in mammals and yeasts that each NF-Y subunit is encoded by a single gene, NF-Y subunits in the plant kingdom contain multiple family members, which greatly expand the diversity of potential combinations of trimeric complexes (Laloum et al., 2013). For example, the 10 NF-YAs, NF-YBs and NF-YCs in Arabidopsis could theoretically result in 1000 potential combinations, hence to extensively involve in diverse biological processes (Swain et al., 2016; Zhao et al., 2016). In other species such as soybean, maize and Medicago, NF-Ys were also implicated in legume-rhzobobia symbiosis, Arbuscular mucorhizal symbiosis and nitrogen starvation response (Zanetti et al., 2016). Rice (Oryza sativa L.) is one of the world’s most important food crops as well as a model species for monocot molecular biology investigation (Itoh et al., 2005). In rice, various NF-Y subunits are involved in specific developmental processes or responses to developmental signals, including seed nutrient accumulation, flowering time regulation, ABA response and chloroplast development (Lee et al., 2015; Li et al., 2015; Romier et al., 2003; Siefers et al., 2009; Sinha et al., 1995; Xu

Summary

Identification of seed development regulatory genes is the key for the genetic improvement in rice grain quality. NF-Ys are the important transcription factors, but their roles in rice grain quality control and the underlying molecular mechanism remain largely unknown. Here, we report the functional characterization a rice NF-Y heterotrimer complex NF-YB1-YC12-bHLH144, which is formed by the binding of NF-YB1 to NF-YC12 and then bHLH144 in a sequential order. Knockout of each of the complex genes resulted in alteration of grain qualities in all the mutants as well as reduced grain size in crnf-yb1 and crnf-yc12. RNA-seq analysis identified 1496 genes that were commonly regulated by NF-YB1 and NF-YC12, including the key granule-bound starch synthase gene Wx. NF-YC12 and bHLH144 maintain NF-YB1 stability from the degradation mediated by ubiquitin265 proteasome, while NF-YB1 directly binds to the ‘G-box’ domain of Wx promoter and activates Wx transcription, hence to regulate rice grain quality. Finally, we revealed a novel grain quality regulatory pathway controlled by NF-YB1-YC12-bHLH144 complex, which has great potential for rice genetic improvement.
et al., 2016; Yamamoto et al., 2009). Rice genome contains 10 NF-YA, 11 NF-YB and 12 NF-YC genes, among which NF-YB are the most well-documented subunits (Gusmaroli et al., 2001; Petroni et al., 2012; Thirumurugan et al., 2008). NF-YB2/HAP3A, NF-YB3/HAP3B and NF-YB4/HAP3C were found to be functional redundantly involved in the chloroplast biogenesis. Simultaneous suppressing of the three genes led to pale green leaves and degeneration of chloroplast formation (Miyoshi et al., 2003). OsNF-YB7/LEC1 is essential to rice vegetative and reproductive growth. lec1 mutants were lethal, but the LEC1 overexpression lines displayed erected leaves and defected panicles and spikelet (Ito et al., 2011; Zhang and Xue, 2013). NF-YB11/DTH2/IGHDH is a master regulator of rice heading date, plant height and yield. Under long-day condition, NF-YB1 suppresses the transcription of Ehd1 and HDA3a to delay the rice heading date (Dai et al., 2012; Wei et al., 2010; Yan et al., 2011). NF-YB11 is also implicated in chloroplast biogenesis and carbon assimilation (Adachi et al., 2017; Feng et al., 2014).

OsNF-YB1 and OsNF-YB9 are endosperm-specific genes that play a prominent role in preservation of endosperm cell development (Sun et al., 2014). OsNF-YB1 and OsNF-YB9 have been found to be co-expressed with starch and storage protein synthesis related genes, suggesting their roles in accumulation of seed reserves (Yang et al., 2016). Recently, it has been demonstrated that NF-YB1 might impose multiple effects on grain cell proliferation, assimilate loading to endosperm and grain filling, and eventually regulate various aspects of seed development in rice (Bai et al., 2015; Sun et al., 2014; Xu et al., 2016).

Despite that much understanding of NF-YB1 function have been achieved so far, the detailed molecular mechanism underlying the NF-YB1-regulated seed development remains largely unknown, particularly its counterparts in forms of protein complex as well as the regulatory roles in the seed nutrient synthesis. In the current study, we demonstrated that NF-YB1 interacts with NF-YC12 and bHLH144 in a sequential order to form a heterotrimer complex. NF-YC12 and bHLH144 maintain NF-YB1 protein stability against ubiquitin26S proteasome-mediated degradation, while stable NF-YB1 activates the transcription of the key granule-bound starch synthase gene Wx by directly binding to the ‘G-box’ domain of its promoter, hence to regulate the synthesis of amylose in rice.

**Results**

**NF-YB1 is a seed-specific gene and its protein locates in nucleus and cytoplasm**

As indicated in the RiceGE (http://signal.salk.edu/cgi-bin/RiceGE5) database, NF-YB1 (LOC_Os02g49410) was specifically expressed in seeds. This 187 amino acids-long protein contains a histone-like domain (HLD) at position 33-92 (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; Marchlerbauer et al., 2017). In consensus with other NF-YBs, 4 α-helices structures were identified in NF-YB1 protein, among which α1 and α2 were covered by HLD domain (Figure 1a). The qRT-PCR result confirmed that NF-YB1 highly expressed in seeds, particularly in 7 DAP (Days After Pollination) seeds, in which the grains start to fill with starch and other nutrients (Figure 1b). mRNA in situ hybridization result showed that NF-YB1 majorly accumulated in embryo and aleurone layer, but not in the endosperm of the 7 DAP seeds (Figure 1c). As shown in Figure S1a-c, NF-YB1 protein accumulated in nuclear and cytoplasm, which is consistent with the previous reports (E et al., 2018; Xu et al., 2016).

**Knock-down of NF-YB1 reduced grain size and altered grain quality**

Using CRISPR/Cas9 technique, we generated knock-out/down lines of NF-YB1. Sanger sequencing of the CRISPR/Cas9 target sites detected various types of deletion in the crnf-yb1-4, crnf-yb1-7 and crnf-yb1-9 respectively, which were believed to knock-out the NF-YB1 by missing the start codon or shifting the open reading frame (Figure S2). The homozygous T1 mutants from the three representative lines were employed for a detailed phenotypic characterization. Not surprisingly, no visible phenotypes, such as plant height, flowering date and seed setting, were observed in the crnf-yb1 lines (Figure 1e and Table S1). However, crnf-yb1-4, crnf-yb1-7 and crnf-yb1-9 showed significant reduction in grain length and width, which consequently resulted in a significant reduction in grain size ranging from 82.02% to 89.41% (Figure 1f, g and i, Table S1). In addition, we observed higher chalkiness in crnf-yb1 mutants (Figure 1h), as both Percentage of Grain With Chalkiness (PGWC) and Degree of Endosperm Chalkiness (DEC) were significantly increased when compared with WT (P < 0.05; Figure 1j and k). A followed-up phenotyping of the T2 plants of these three lines revealed consistent reduction in grain size in the mutants; hence we inferred that these phenotypes were ascribed to the knock-out of OsNF-YB1 (Figure S3).

We determined the total starch, amylose, crude protein, crude fiber and lipid contents in the brown rice of WT and crnf-yb1s. The mutants showed significant reduction in starch, amylose, lipid contents as well as elevated crude protein contents when compared to the WT (P < 0.05; Table 1). Rapid Visco Analyzer (RVA) was employed to analyse the pasting properties of the starch of crnf-yb1-4, crnf-yb1-7 and WT. We did not include the crnf-yb1-9 sample due to the unavailability of enough seeds. crnf-yb1 mutants exhibited higher values of viscosity parameters including peak viscosity (PV), hot viscosity (HV) and final viscosity (FV; Figure 1l). Furthermore, we analysed the thermal properties of the mutants and WT using differential scanning calorimetry (DSC). In this assay, onset temperature is used to indicate the starting temperature for melting of the amylopectin crystallites, while gelatinization enthalpy reflects the required heat energies for amylopectin melting. Interestingly, we found the onset, peak and end gelatinization temperatures as well as the gelatinization enthalpy were all significantly decreased in crnf-yb1s (Table S2). The results above implied that NF-YB1 may also affect the starch pasting and alkaline gelatinization properties.

We further generated NF-YB1 overexpression lines, multiple independent lines showed substantial enhanced expression level, but no significant phenotypes were observed in seed size (Figure S4). This phenomenon indicates that NF-YB1 may work as a complex with other proteins; elevating the level of NF-YB1 alone imposes no effect on the protein complex activity.

**NF-YB1, NF-YC12 and bHLH144 forms a heterotrimer complex in a sequential order**

To find out the NF-YC counterparts of NF-YB1, we conducted a bacterial-two-hybrid assay between NF-YB1 and other five seed-specific NF-YC proteins, namely NF-YC8 (LOC_Os01g01290), NF-YC9 (LOC_Os01g24460), NF-YC10 (LOC_Os01g39850), NF-YC11 (LOC_Os05g23910) and NF-YC12 (LOC_Os10g11580). NF-YB1 could physically bind with NF-YC11 and NF-YC12 in Escherichia coli, but not or very weakly interact with the other three NF-YCs (Figure 2a). The interaction intensity of NF-YB1-NF-
YC12 (63.7%) almost doubled that of the positive control (33.8%), suggesting a very strong interaction (Figure 2b). Given that both proteins contain a conserved histone-like domain (HLD) in the middle of the protein, we constructed different truncated versions of NF-YB1 and NF-YC12 to test their interactions. As shown in Figure 2c, for both NF-YB1 and NF-YC12, the absence of 5' upstream regions of HLD did not affect the protein–protein interaction. However, the binding was compromised when either HLD domain or its 3' downstream regions were truncated out. Hence, the indication is that the HLD domain is necessary, but insufficient for the NF-YB1-NF-YC12 interaction, some unknown elements in the 3' regions are essential for their bindings. We also conducted in vitro GST pull-down assays to validate the interaction of NF-YB1 and NF-YC12. Indeed, HIS-NF-YB1 was pulled down with GST-NF-YC12, whereas GST tag alone was not able to pull-down HIS-NF-YB1 (Figure 2d).

Figure 1 Spatial-expression pattern of NF-YB1 and phenotypical characterization of crnf-yb1s and WT. (a) Schematic presentation of the NF-YB1 gene structures and conserved protein domains. Boxes: exons; blue box: coding sequences; red box: conserved HLD; line: intron; α1, α2, α13 and αC are four conserved α-helix structures. (b) qRT-PCR analysis of NF-YB1 transcription abundances in various tissues and stages. The expression level of callus was set as 1. (c) mRNA in situ hybridization analysis of NF-YB1 on 7 DAP seeds. E: embryo; En: endosperm; Al: aluereone layer. Scale bar = 1 mm. (d) Negative CK of (c) using sense probe for hybridization. (e) Plant morphology at grain-filling stage. (f-k) comparison of the seed length (f), width (g), chalkiness (h), 1000-grain-weight (i), percentage of Grain With Chalkiness (j) and Degree of Endosperm Chalkiness (k) of crnf-yb1s and WT. Data are shown as Means ± SD of at least three biological replicates. *: P ≤ 0.05 by the Student’s t test. (l) Pasting properties of seeds analysed by Rapid Visco Analyzer (RVA).
system, NF-YB1 was fused with GAL4 DNA binding domain, while NF-YC12 was driven under a Met25 promoter which is conditionally expressed in the absence of methionine. Given that NF-YA is the mostly identified components of NF-Y complex in previous studies, we firstly checked the interactions between NF-YB1-YC12 heterodimer and three seed-specific NF-YAs, namely NF-YA1 (LOC_Os03g07880), NF-YA5 (LOC_Os07g41720) and NF-YA8 (LOC_Os10g25850). Unfortunately, all the three tested NF-YAs displayed autoactivations in the Y3H experimental system (Figure S5). Thus, we moved to screen a seed-derived prey library using NF-YB1-YC12 as the bait, and finally detected an interactive YB1-YC12 heterodimer and three seed-specific NF-YAs, namely NF-YA8 (LOC_Os10g25850). crbhlh144s, the mutants showed almost identical morphology with the WT in vegetative growth (Figure S2). Similar to crnf-yb1s, crnf-yc12s and crbhlh144s showed almost identical morphology with the WT in vegetative growth (Figure S2). However, there were around 20% reduction in the 1000-grain-weight in crnf-yc12s, when compared with the WT (Figure 3b, c and Table S1). In contrast, BHLH144 may not be functionally related to grain size control as the three mutant lines displayed similar size as the WT (P > 0.05; Figure 3d, f and Table S1). cmyc12s and crbhlh144s all had higher PGWC and DEC (Figure 3g, h and i). The grain quality assay of cmyc12s and cmyc144s obtained similar results as cmyc1b1s. The mutants showed significant reduction in starch, amylose, lipid contents as well as elevated crude protein contents when compared with the WT (P < 0.05), which exactly phenocopied cmyc1b1s (Table 1).

**NF-YB1 and NF-YC12 co-regulate transcription of genes involves in starch biosynthesis**

RNA-sequencing experiments were performed on the 7 DAP seeds of cmyc1b1-4, cmyc12-11 along with the WT to investigate the potential downstream genes. In cmyc1b1-4, 574 up-regulated genes and 1618 down-regulated genes were identified (log2 Ratio ≥1; FDR <0.001). We also found 480 up-regulated and 1493 down-regulated genes in cmyc12-11. By merging the data together, we finally identified 310 and 1186 DEGs that were commonly up-regulated and down-regulated respectively in both cmyc1b1 and cmyc12 plants (Table S4, Figure S6). KEGG pathway enrichment analysis of the common DEGs revealed that many metabolism pathways such as starch and sucrose metabolism, carbon metabolism, photosynthesis were overrepresented, which is consistent with the observed phenotype in grain nutrient accumulation and quality (Figure S7).

**Table 1** Nutrition content assays of cmyc1b1s, cmyc12s and crbhlh144s

|         | Starch (%) | Amylose (%) | Protein (%) | Crude fiber (%) | Lipid (%) |
|---------|------------|-------------|-------------|----------------|-----------|
| WT-1    | 69.61 ± 0.103 | 18.61 ± 1.260 | 10.43 ± 0.003 | 1.81 ± 0.001 | 2.53 ± 0.001 |
| cmyc1b1-4 | 57.76 ± 0.801* | 15.15 ± 0.294** | 12.90 ± 0.010** | 1.74 ± 0.001* | 2.14 ± 0.007* |
| cmyc1b1-7 | 60.39 ± 3.164** | 16.85 ± 1.514** | 13.63 ± 0.003** | 1.59 ± 0.002* | 2.11 ± 0.005** |
| cmyc1b1-9 | 58.16 ± 1.874* | 15.81 ± 0.592* | 14.50 ± 0.001** | 1.61 ± 0.002* | 2.16 ± 0.001** |
| cmyc12-7 | 56.30 ± 19.765* | 14.21 ± 0.007* | 11.58 ± 0.062* | 1.65 ± 0.001** | 2.06 ± 0.008* |
| cmyc12-11 | 56.30 ± 19.765* | 14.21 ± 0.007* | 11.58 ± 0.062* | 1.65 ± 0.001** | 2.06 ± 0.008* |
| cmyc12-14 | 55.13 ± 1.182** | 13.96 ± 0.363* | 12.49 ± 0.909* | 1.52 ± 0.001** | 2.69 ± 0.002* |
| WT-2    | 68.93 ± 1.724 | 19.20 ± 1.221 | 9.93 ± 0.003 | 1.95 ± 0.002 | 2.77 ± 0.003 |
| crbhlh144-2 | 54.38 ± 1.463* | 18.27 ± 0.470* | 11.11 ± 0.003* | 1.72 ± 0.002* | 2.32 ± 0.002* |
| crbhlh144-3 | 54.96 ± 2.492** | 17.88 ± 0.549* | 11.03 ± 0.001* | 1.74 ± 0.002* | 2.45 ± 0.003* |
| crbhlh144-4 | 58.66 ± 4.505* | 18.59 ± 0.436 | 11.76 ± 0.001* | 1.65 ± 0.001* | 2.31 ± 0.003* |

Brown seeds were used for the assays. These data are presented as the Means ± SD of at least three biological replicates. The asterisks represent significant difference between the WT and mutants as determined by the Student’s t test, the single asterisk indicates P < 0.05, double asterisks indicate P < 0.01. WT-1 is the control for cmyc1b1s and cmyc12s, WT-2 is the control for crbhlh144s.

We generated mutants of the NF-YC12 and BHLH1144 using CRISPR/Cas9 technique. In T1, generation, three representative homozygous mutant lines of NF-YC12 (cmyc12-7, cmyc12-11 and cmyc12-14) and three of BHLH1144 (cmyc144-2, cmyc144-3 and cmyc144-4), which were confirmed with shifted open reading frames, were morphologically characterized along with the WT (Figure S2). Similar to cmyc1b1s, cmyc12s and cmyc144s showed almost identical morphology with the WT in vegetative growth (Figure 3a and e; Table S1). However, there were around 20% reduction in the 1000-grain-weight in cmyc12s, when compared with the WT (Figure 3b, c and Table S1). In contrast, BHLH144 may not be functionally related to grain size control as the three mutant lines displayed similar size as the WT (P > 0.05; Figure 3d, f and Table S1). cmyc12s and cmyc144s all had higher PGWC and DEC (Figure 3g, h and i). The grain quality assay of cmyc12s and cmyc144s obtained similar results as cmyc1b1s. The mutants showed significant reduction in starch, amylose, lipid contents as well as elevated crude protein contents when compared with the WT (P < 0.05), which exactly phenocopied cmyc1b1s (Table 1).
The Differentially Expressed Genes (DEGs) covered a large number of starch synthesis genes, and other nutrient synthesis genes. We performed qRT-PCR analysis of the DEGs as well as some reported rice seed development regulator genes in crnf-yc12, crnf-yc12 and crbhlh144 with multiple biological replicates (Figure 4). A number of the starch synthesis genes, such as AGPL1 (ADP-glucose pyrophosphorylase large subunit 1; LOC_Os03g52460), AGPL3 (LOC_Os05g50380) and Wx (LOC_Os06g04200) were commonly down-regulated in the mutants of all the three genes, indicating that these DEGs are under common pathways regulated by the NF-YB1-YC12-bHLH144 complex. Meanwhile, AGPS1 (ADP-glucose pyrophosphorylase small subunit 1) (LOC_Os09g12660), AGPS2b (ADP-glucose pyrophosphorylase small subunit 2b) (LOC_Os08g25734),
SSIIa (Starch Synthesase II a) (LOC_Os06g12450) were only differentially expressed in one or two of the three mutants, suggesting that NF-YB1, NF-YC12 and bHLH144 have their independent regulatory pathways in seed development. Additionally, the expression of chalkiness controlling genes FLO2 (Floury endosperm 2) (LOC_Os04g55230) and FLO4 (Floury endosperm 2) (LOC_Os05g33570), grain nutrient synthesis regulators RSR1 (Rice Starch Regulator 1) (LOC_Os05g03040) and bZIP58 (LOC_Os07g08420) were also differentially expressed in the mutants (Figure 4).

NF-YB1 binds to the G-box of Wx promoter to activate its transcription

Wx has been reported as a master regulator of amylose biosynthesis (Tian et al., 2009; Wang et al., 1995). The reduced amylose content as well as down-regulated Wx level in the mutants implied that Wx is a direct target gene of them. The hypothesis was firstly tested by yeast-one-hybrid (Y1H) experiment. The results showed that NF-YB1, but not NF-YC12 and bHLH144 activated the LacZ expression (Figure 5a). To verify the
NF-YB1-YC12-bHLH144 regulates grain quality

(a) (b) (c) (d) (e) (f) (g) (h) (i) (j)
NF-YB1-mediated activation on Wx, we also performed luciferase (LUC) transient transcriptional activity assay in protoplasts. Strong activation of LUC were detected in pro35S:NF-YB1:tNOS, though the other two proteins also showed weak but significant activations of LUC when compared with the negative control, suggesting that NF-YB1 activates the Wx transcription in vivo (Figure 5b).

Subsequently, we conducted electrophoresis mobility shift assay (EMSA) to test the binding in vitro. In the 1 kb Wx promoter region, we found four G-box, one GCN box, but no typical CCAAT box elements by using the online tool PlantCARE (Plant Cis-Acting Regulatory Elements; Lescot et al., 2002; Figure 5c). As a result, the shift speed of only probe 1, which represented the closest (549 bp) G-box to transcription starting site (TSS), was retarded by HIS-NF-YB1 protein (Figure S8), and the shifted band signal was substantially weakened with the application of unlabeled, competitive probe 1, suggesting binding is highly specific (Figure 5d). It also appeared that probes with mutated G-box lost the binding ability with NF-YB1 (Figure 5e), therefore the G-box in probe 1 is a core binding site for NF-YB1. Such a binding pattern of NF-YB1 on Wx promoter was further confirmed by our ChIP-qPCR experiment. We found significant enrichment of P1 region, which covered the probe 1 in EMSA assay, but no signal in P2 and P3 regions, which represented the negative binding regions in EMSA assay (Figure 5f). Hence, we concluded that NF-YB1 binds to the closest G-box to the TSS in Wx promoter in vivo.

As a master regulator of amylose biosynthesis, Wx has been used as a target site of genome editing for the breeding of sticky rice varieties with very low amylose content (Zhang et al., 2018). We introduced wx mutations into Ningjin 7, an elite japonica cultivar and Huazhan, an elite indica restore line for hybrid rice breeding in China. Homozygous mutation of Wx in both backgrounds resulted in complete chalkiness in the endosperm, demonstrating the great effect of Wx on rice grain quality control (Figure S9).

**NF-YC12 and bHLH144 enhances NF-YB1 stability**

We performed EMSA assay to investigate the effect of NF-YC12 and bHLH144 on NF-YB1 binding ability to Wx promoter (Figure 5g). In contrast to the strong binding ability of HIS-NF-YB1, neither GST-NF-YC12 nor HIS-FLAG-bHLH144 could bind to the Wx promoter probe 1. The additions of GST-NF-YC12 or HIS-FLAG-bHLH144 or both also did not alter the binding intensity of HIS-NF-YB1 to the Wx promoter, implying that the interaction with NF-YC12 or bHLH144 does not affect the NF-YB1 DNA binding ability.

Previous studies reported that complex components may affect the protein stability of each other. We were intrigued to conduct a cell-free degradation assay of HIS-NF-YB1 by incubating the protein with total protein extracts from 10 DAGs (Days After Germination) WT seedlings (Figure 5h). As a result, the half-life of HIS-NF-YB1 is around 15 min. GST-NF-YC12 dramatically stabilized HIS-NF-YB1 with a half-life of 30 min. The best stability of HIS-NF-YB1 was achieved in a status of NF-YB1-YC12-bHLH144 heterotrimer complex, as the HIS-NF-YB1 half-life reached 40 min. Moreover, the application of MG132, an inhibitor of 26S proteasome degradation system, significantly suppressed the HIS-NF-YB1 degradation. Therefore, the above results suggested that NF-YB1 is under the 26S proteasome-mediated degradation, while the presence of NF-YC12 and bHLH144 helps NF-YB1 to maintain a stable protein status, and possibly enhances the NF-YB1-imposed transactivation of Wx.

Taken together, the current study demonstrated a novel transcriptional regulation mechanism of NF-Ys in rice starch synthesis (Figure 6). During the rice seed development, NF-YC12 and bHLH144 sequentially bind with the key regulator NF-YB1 to prevent its degradation by the ubiquitin/26S proteasome pathway. Stable NF-YB1 activates the transcription of the key granule-bound starch synthase gene Wx by directly binding to the ‘G-box’ domain of its promoter, hence to regulate the starch synthesis and grain quality.

**Discussion**

**Comprehensive effects of NF-YB1 on seed development**

During the process of our NF-YB1 project, at least four independent labs reported their works on NF-YB1 with similar biological functions (Bai et al., 2015; E et al., 2018; Sun et al., 2014; Xu et al., 2016). In consistence with this study, RNAi or CRISPR/Cas9 knock-out lines of NF-YB1 had smaller seeds and chalky...
endosperms, when compared with the WT. However, divergent mechanisms were proposed to support its function in rice endosperm development. Sun et al. (2014) found that repression of OsNF-YB1 resulted in differential expression of the genes in cell cycle pathway, and reduced endosperm cell numbers integrated with the development of abnormal seeds in OsNF-YB1 RNAi plants (Sun et al., 2014). Bai et al. (2015) emphasized the key role of NF-YB1 in modulating the expression of sucrose transporters in aleurone to enhance sugar loading to the endosperm (Bai et al., 2015). As described in a very recent publication, by forming a heterotrimeric complex with NF-YC and OsERF115, NF-YB1 promote the binding of OsERF115 to GCC box of the downstream gene promoters, hence to regulate rice grain filling and endosperm development (Xu et al., 2016). In this study, we revealed that NF-YB1 regulates various aspects of grain quality and nutrient synthesis, in addition to the grain size and chalkiness. cmsf-yb1 plants showed significantly lower starch, amylose, lipid contents, elevated crude protein contents as well as altered starch pasting and alkaline gelatinization properties, when compared with the WT. In line with this observation, our RNA-seq and qRT-PCR experiments identified a list of DEGs which are functionally related to starch synthesis and seed development. Notably, we found that the key amylose synthesis gene Wx was over twofold down-regulated (Figure 4). Wx has been well-known as a determinant gene controlling rice amylose content, gel consistency and gelatinization temperature (Tian et al., 2009; Wan et al., 2007; Wang et al., 1995). Suppression of Wx in indica or japonica backgrounds led to significantly lower amylose content, smaller seed size accompanied with changes in the content of glucose, sucrose and other cell-wall polysaccharides (Figure S9; Chen et al., 2006; Zhang et al., 2012). Moreover, we provided multiple lines of evidence to demonstrate that NF-YB1 transactivated the Wx expression in yeast, in vitro and in vivo (Figure 5), indicating Wx is a direct target of NF-YB1 transcription factor. To the best of our knowledge, NF-YB1 is the first reported direct regulator of Wx. Together with the previous reports, we speculate that NF-YB1 is a master regulator imposing comprehensive effects on seed development via controlling cell proliferation, assimilate transportation and nutrient biosynthesis.

**NF-YB directly activates Wx via binding to the G-box in promoter**

Previously, it is believed that NF-YA drives the target gene transcription by specifically binding with the conserved CCAAT box on the promoter, whereas NF-YB and NF-YC possesses no transactivation activities (Liu and Howell, 2010; Zhao et al., 2016). However, the function of each NF-Y subunit may not be strictly conserved in plants. A recent publication reported a systematical analysis of the transactivation activity of rice NF-Y subunits, and found that NF-YB1, NF-YB9, NF-YC11 and NF-YC12 showed no transactivation activity, whereas NF-YC8, NF-YC9 and NF-YC10 did (E et al., 2018). In the case of NF-YB1, a controversial case also reported that it directly attached to the CCAAT box of several sucrose transporter gene promoters and activated their transcription in vivo, suggesting the functional overlapping of NF-YB and NF-YA in rice (Bai et al., 2015).

For the non-canonical NF-Y complexes in which NF-YA is absent, the binding cis-elements of the target genes may be determined by the non-NF-Y subunits (Kumimoto et al., 2010; Yamamoto et al., 2009). One well-documented example is the Arabidopsis NF-YB9-C2-bZIP67 complex, which could directly bind to the conserved bZIP binding site ABA-responsive elements through bZIP67 (Yamamoto et al., 2009). Using ChiP-seq technique, Xu et al. (2016) revealed that NF-YB1 binding sites were enriched with conserved ERF binding elements GCC box. However, the binding is indirect, and the recruitment of NF-YB1 to GCC sites was possibly mediated by its interactive protein OsERF115 (Xu et al., 2016). In the current study, for the NF-YB1-YC12-bHLH144 complex, it is NF-YB1, instead of the non-NF-Y member bHLH144, binds to and activates its downstream gene Wx (Figure 5a and c). Moreover, EMSA result demonstrated that NF-YB1 directly and specifically binds to the G-box of Wx promoter, in an independent manner of the presence of NF-YC12 and/or bHLH144, indicating a novel mechanism of NF-YB-mediated gene transcriptional regulation. The G-box has been known as a ubiquitous DNA element in various plant gene promoters, and functionally involves in responses to phytohormones and environmental stimuli (Menkens and Al, 1995). Although nearly all the identified GBFs (G-box Binding Factors) are bZIPs, a few bHLHs including PIF3 (Phytohormone Interacting Factor 3) and TFHP-1 (Transcription Factor bHLH Protein-1) were found to be able to bind with G-boxes (Kawaoaka et al., 1994; Ni et al., 1998). We have excluded the involvement of bHLH144 in the binding of NF-YB1 with G-box on Wx, yet it remains to be explored that whether other bZIPs or bHLHs potentially participated in this event or not.

**NF-YB1-YC12-bHLH144 works as a complex**

In yeast and human, NF-Y heterotrimeric complex is comprised by a NF-YB, a NF-YC and a NF-YA subunit. Such typical NF-Y heterotrimers have also been identified in plants. For examples, Sato et al. (2014) reported the identification of Arabidopsis NF-YA1-B6-C10 complex and their functions in heat and drought responses (Sato et al., 2014). Additionally, several recent studies have reported that NF-YB-YC interacted with other proteins, instead of NF-YAs. For examples, CONSTANS (CO), which is a key flowering time regulator in Arabidopsis, was found to form complexes with interacting with multiple NF-YC subunits (C1, C3, C4 and C9) and NF-YB subunits (B2 and B3; Kumimoto et al., 2010). Other proteins, such as bZIP28, bZIP67, RGA (Repressor of GA) and RGL2 (RGA-Like 2) were also found to interact with NF-Ys to involve in biological processes in Arabidopsis (Hou et al., 2014; Huang et al., 2015; Liu and Howell, 2010; Yamamoto et al., 2009).

Here, we revealed the sequential interactions of NF-YB1, NF-YC12 and bHLH144 by Y3H, in vitro pull-down assay and BiFC in planta, suggesting that NF-YB1, NF-YC12 and bHLH144 work as a heterotrimeric complex. This hypothesis is supported by several other indirect evidence: (i) the three transcription factors are co-expressed with a seed-specific expression pattern, and co-localized in nuclear; (ii) NF-YB1 and NF-YC12 shared a large number of DEGs; and (iii) nfyb1, nfy-c12 and bhlh144 mutants phenocopied each other in seed development, though bhlh144 did not show significant differences in grain size. Such a finding is consistent with the previous report that NF-YB and NF-YC could only interact with other proteins, like NF-YA, by forming a heterodimer (Petroni et al., 2012). Nevertheless, few literatures also described that NF-YB1 could directly bind to OsMADS18 and OsERF115 without the assistance of other NF-YCs (Masiero et al., 2002; Xu et al., 2016). It should be noted that these reported interactions were only verified using Y2H experiments which is highly risky to yield potential false positive result. Although no rice NF-YAs interacting with NF-YB1-YC12 dimer were detected in our Y3H screening, we could not exclude the participation of NF-
YAs, especially seed-specific NF-YAs, in forming the complex. Given the autoactivities of the NF-YAs in yeast, alternative methods such as in vitro pull-down or Co-IP will be employed to test this hypothesis in our future work.

A cytoplasm-nuclear shuttling model has been proposed to explain the effect of NF-YC on NF-YB. In this model, NF-YB is originally located in cytoplasm, where it assembled with NF-YC and transported to nuclear to form heterotrimer with the third member, either NF-YAs or other proteins (Hackenberg et al., 2012; Zhao et al., 2016). Indeed, Xu et al. (2016) demonstrated that NF-YB1 was specifically targeted from cytoplasm to nuclear by interacting with NF-YC12 and other NF-YCs (Xu et al., 2016). Based on our results, it seemed that NF-YC12 and bHLH144 imposed no effect on the DNA binding capacity of NF-YB1 to the Wx promoter (Figure 5g). However, our cell-free degradation assay found that NF-YB1 was under the degradation of ubiquitin/26S proteasome pathway, while binding with NF-YC12 and bHLH144 greatly enhanced the NF-YB1 stability in vitro, hence maintained the NF-YB1 activity.

**Experimental procedures**

**Plant growth conditions and phenotypical characterizations**

Nipponbare (*Oryza sativa, ssp. japonica*) and all the transgenic plants used in this study were grown in the experimental field and greenhouse of China National Rice Research Institute. The thousand-grain-weight, seed length width and chalkiness of WT and mutant lines were examined by a seed phenotyping system (Wangsheng, Hangzhou, China).

Total starch and amylose contents of brown seedswere measured with a starch assay kits Megazyme K-TSTA and K-AMYl (Megazyme, Ireland, UK). The content is expressed as the percentage of total sample weight on an oven-dry basis. The total amylose, crude fiber, lipid and protein contents in the grains were measured following the previous report (Kang et al., 2005). RVA analysis was done on a Rapid Visco Analyzer (RVA Techmaster, Newport Scientific, Narrabeen, Australia) as described by Sun et al., 2017. DSC assay was conducted on a differential scanning calorimeter DSC1 STARe system (Mettler Toledo, Switzerland) by measuring following the previous report (Kang et al., 2005). These data are presented as mean ± SD. The relative expression level of the tested genes was normalized to ubiquitin and calculated by the 2−ΔΔCT method. The mRNA in situ hybridization were conducted as described by Zhang et al. (2010).

**RNA isolation, qRT-PCR and mRNA in situ hybridization**

The RNA of all the tissues except developing seeds was extracted by Trizol (Invitrogen, Carlsbad, CA). The extraction of developing seed RNA was conducted using a modified SDS-Trizol method (Qiu et al., 2016). RNA reverse transcription and qRT-PCR were performed with technical triplicates as described by Hou et al., 2015. These data are presented as mean ± SD. The relative expression level of the tested genes was normalized to ubiquitin and calculated by the 2−ΔΔCT method. The mRNA in situ hybridization were conducted as described by Zhang et al. (2010).

**Vector construction and plant transformation**

The CRISPR/Cas9 system was adopted from a previous report (Ma et al., 2015). Annealed double strand oligos of the qDNA sequences were ligated into the pYlgRNA-OSuJ3 using Bsal site (Thermo, Waltham, MA). qDNA sequences are shown in Table S3. For the overexpression construct of NF-YB1, the CDS was ligated into pU1301 to be driven by a maize ubiquitin promoter (Zhang et al., 2010).

The rice variety ‘Nipponbare’ was used as the recipient. *Agrobacterium* strain EHA105 was used for transformation. *Agrobacterium*-mediated transformation was conducted as described by Hiei et al., 1994.

**Bacterial-two-hybrid assay**

Fragments of NF-YB1 and NF-YC8-12 were cloned into pBT and pTRG vectors using the enzyme sites as indicated in Table S3. The CRISPR/Cas9 system was adopted from a previous report (Ma et al., 2015). Annealed double strand oligos of the gDNA members, either NF-YAs or other proteins (Hackenberg et al., 2012; Zhao et al., 2016). Indeed, Xu et al. (2016) demonstrated that NF-YB1 was specifically targeted from cytoplasm to nuclear by interacting with NF-YC12 and other NF-YCs (Xu et al., 2016). Based on our results, it seemed that NF-YC12 and bHLH144 imposed no effect on the DNA binding capacity of NF-YB1 to the Wx promoter (Figure 5g). However, our cell-free degradation assay found that NF-YB1 was under the degradation of ubiquitin/26S proteasome pathway, while binding with NF-YC12 and bHLH144 greatly enhanced the NF-YB1 stability in vitro, hence maintained the NF-YB1 activity.

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144 were cloned into MCS3 site for C-terminal fusions (Gookin and Assmann, 2014). In addition, full CDS except stop codon of NF-YC12 and bHLH144 were cloned into pCAMBIA1305-mcherry. Constructs were electroporated into Agrobacterium strain EHA105 and subsequently infiltrated into the leaf epidermal cells of 3-week-old Nicotiana benthamiana. Confocal microscopy was performed using a Zeiss LSM710 confocal laser scanning microscopy (Carl Zeiss AG, Jena, Germany) at 72 h after infiltration.

**Yeast-one-hybrid assay**

The Clontech™ one-Hybrid System (Clontech, Dalian, China) was used in this study. The CDS of potential transactivators were fused with GAL4 AD domain in pB42AD (Clontech, Dalian, China), and the promoter region of Wx were cloned into pLacl2u (Clontech, Dalian, China). Yeast strain EGY48 was transformed with indicated plasmids and grew on SD/-Ura/-Trp plates, and then strike on SD/-Ura/-Trp plates containing 2% galactose, 1% raffinose, 1 × BU salts and 80 mg/L X-Gal (Clontech, Dalian, China). The interaction was confirmed by the visualization of blue colonies on the medium. NF-YB1-pB42AD and SUT4-pLacl2u were used as CK (+) (Bai et al., 2015). The empty vectors pLacl2u and pB42AD were used as negative control.

**Luciferase transient transcripational activity assay**

The CDS of NF-YB1, NF-YC12 and bHLH144 were, respectively, cloned into ‘None’ vector as effectors and the promoter region of Wx was cloned into 190LUV vector as reporter (Zong et al., 2016). Protoplast preparation and transformation were conducted according to the method of (Xie and Yang, 2013). Luciferase® Reporter Assay System (Promega, Madison, WI) was used to measure the luciferase activity according to manufacturer instruction. The relative luciferase activity was calculated as the ratio between rLUS1 and rLUS2.

**Electrophoresis mobility shift assay**

Electrophoresis mobility shift assay probes in a length of 59 nt were commercially synthesized by Tsinke Biological Technology (Hangzhou, China) and labeled with an EMSA Probe Biotin Labeling Kit (Cat No. GS008, Beyotime, Shanghai, China). DNA binding was performed in a 10 µL reaction volume containing EMSA/Gel-shift binding buffer (Beyotime, Shanghai, China), 2 nmol biotin-labeled probe, 5 nmol purified recombinant protein. Non-labeled DNA oligos were used as competitor. Recombinant protein was pre-incubated with the EMSA/Gel-shift binding buffer for 20 min at 25 °C prior to the addition of the biotin-labeled probe and further incubated at 25 °C for 20 min. A 6% (W/V) polyacrylamide gel was pre-run for 30 min, and then the binding reaction is subjected to gel electrophoresis. The DNA probes were then transferred to a charged nylon membrane (Beyotime, Shanghai, China), detected by streptavidin-HRP (Beyotime, Shanghai, China), and finally visualized using the enhanced chemiluminescence (Pierce, Waltham, MA).

**Chromatin immuno-precipitation (ChIP) and ChIP-PCR**

Chromatin immuno-precipitation was performed as described previously (Hou et al., 2015). Briefly, chromatin was isolated from 2 to 4 g leaves of proUbi:NF-YB1-FLAG plants, then fragmented to 200–700 bp by sonication. The DNA/protein complex was immune-precipitated with ChIP-grade antibody against FLAG (F1804, Sigma-Aldrich, St. Louis, MO). After reverse cross-linking and protease K treatment, the immune-precipitated DNA was purified. The immune-precipitated and input DNA was as template for quantitative PCR using gene specific primers (Table S3). The quantitative PCR results were analysed by following a method of Magna Chip™ HiSens kit (Millipore, MA). All the quantitative ChIP-PCR was performed in three biological replicates.

**Cell-free degradation assay**

The experiment was conducted by following Lv et al., 2014. Briefly, total protein of 10 days-after-germination rice seedlings were extracted in degradation buffer (25 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM MgCl2, 4 mM PMSF, 5 mM DTT, and 10 mM ATP) and quantified using a Quabit system (Invitrogen, Carlsbad, CA). Purified recombinant proteins were incubated with 200 µg extracted total proteins in 20 µL degradation buffer at 28 °C. Reactions were terminated at indicated time points, and the protein abundance was visualized by immune detection against anti-HIS. The immune signals were quantified using Quantity Tools of Image Lab software (Bio-Rad, Hercules, CA). The half-life of HIS-NF-YB1 was calculated based on the degradation curves deduced from the tested time points. The protein intensities were quantified using ImageJ software with triplicates. The dissociation-one phase exponential decay curve was plotted on a semilog graph using Graphpad Prism (5.0) software as previously described by (Lv et al., 2014).

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**Author contributions**

J.Zhang. planned and designed the research; B.B., Y.H., G.J., Y.W. J.Zhao, Z.L. and X.W. performed experiments; B.B., Y.H., Y.W., X.T. W.Y. and J.Zhang. analysed data; and B.B., and J.Zhang. wrote the manuscript. B.B. and Y.H. contributed equally.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Subcellular localization of NF-YB1.

Figure S2 Sanger sequencing of the mutated sites in homozygous mutants of crnf-yb1, crnf-yb12 and cbribhlh144.

Figure S3 Grain weights of crnf-yb1s T2 lines.

Figure S4 Expression level and grain size of NF-YB1 over-expression lines.

Figure S5 Y3H assay of the NF-YB1-YC12 complex with three seed-specific NF-YAS.

Figure S6 Venn diagram showing the number of co-regulated DEGs by NF-YB1 and NF-YC12 as revealed by RNA-seqs.

Figure S7 KEGG pathway enrichment analysis of DEGs co-regulated by NF-YB1 and NF-YC12.

Figure S8 EMSA assay showing the binding of NF-YB1 to the Wx promoter.

Figure S9 Seed phenotype and genotype of wx mutants in the background of Ningjin 7 and Huazhang.

Table S1 Major agronomic traits of crnf-yb1s, crnf-yb12s and cbribhlh144s.

Table S2 Differential scanning calorimetry assay of crnf-yb1s.

Table S3 Sequences of primers used in this study.

Table S4 DEGs regulated by NF-YB1 and/or NF-YC12.