A group of nuclear factor Y transcription factors are likely sub-functionalized in the endosperm development of monocot

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Abstract

Nuclear factor Y (NF-Y), also known as Heme Activator Protein or CCAAT-binding factor, is a heterotrimeric transcription factor that consists of three subunits, NF-YA, NF-YB, and NF-YC. Although NF-Ys play multiple roles in plant development, their functions during endosperm development are not well understood. In this study, we identified eight rice (*Oryza sativa*) NF-Y encoding genes, including *OsNF-YA8*, *OsNF-YB1,9*, and *OsNF-YC8,9,10,11,12*, which predominantly express in the endosperm. Interestingly, their closest homologs could only be found in monocot species and these genes also showed an endosperm-preferential expression pattern, which suggest that the phylogenetically related group of NF-Ys may be involved in the regulation of endosperm development. A systemic analysis of the interactions between rice endosperm-preferential NF-Ys in yeast revealed that NF-YBs and NF-YCs could interact with each other in all the combinations that we tested. OsNF-YA8 is a recently evolved NF-YA in rice. NF-YA does not usually interact with NF-YB monomers in plants; however, OsNF-YA8 could interact with OsNF-YB9. Our results also indicated that the endosperm-preferential OsNF-YBs, as well as the OsNF-YCs, could interact with some endosperm-specific ERFs of rice. Unlike the OsNF-YC8,9,10, OsNF-YB1,9 or OsNF-YC11,12 alone lacks transcriptional activation activity. However, the dimers they formed displayed transcriptional activation activities. Considering that mutated *OsNF-YB1* can severely impair endosperm development in rice, our findings strongly suggest that there is a group of phylogenetically conserved NF-Ys that have differentiated in monocots to regulate endosperm development.

Introduction

Rice (*Oryza sativa*) provides most of the calories consumed by human beings globally. The endosperm is the edible part of the rice plant and other cereals. After double fertilization, the primary rice endosperm nucleus develops into the syncytium, a cell with multiple free nuclei that is formed by cell division but uncoupled with cytokinesis (Wu *et al.*, 2016). Simultaneous cellularization of the free nuclei initiates proliferation of the endosperm cells. After several rounds of mitotic division, the endosperm cells occupy the central vacuole and start to accumulate starch (Wu *et al.*, 2016). Two to five layers of the outermost cells of the endosperm are differentiated into an aleurone layer while the inner cells are differentiated into storage cells (Zhou *et al.*, 2013). The pattern of endosperm development in other monocots is similar to that of rice (Olsen, 2001; Leroux *et al.*, 2014; Zhang *et al.*, 2016*b*).

Several groups of transcription factors, such as the MADS-box genes *OsMADS6* (Zhang *et al.*, 2010), *OsMADS29* (Yin and Xue, 2012) and *OsMADS87* (Chen *et al.*, 2016), the bZIP family gene *RISBZ1* (Yamamoto *et al.*, 2006; Kawakatsu *et al.*, 2009), the DOF family gene *RPBF* (Yamamoto *et al.*, 2006; Kawakatsu *et al.*, 2009), and the WRKY family gene *OsWRKY78* (Zhang *et al.*, 2011), are essential for rice endosperm and seed development. The nuclear factor Y (NF-Y), also known as the Heme Activator Protein or CCAAT-binding factor, is conserved across kingdoms (Petroni *et al.*, 2012; Laloum *et al.*, 2013). The NF-Ys consist of three families: NF-YA, NF-YB, and NF-YC. Owing to possession of the Histone Fold Domains (HFDs), NF-YB and NF-YC can form a heterodimer, which generates a surface for NF-YA accession to form an NF-YA/B/C trimeric complex (Nardone *et al.*, 2016; Gnesutta *et al.*, 2017). NF-YA is able to recognize and bind to CCAAT motifs (Laloum *et al.*, 2013; Gnesutta *et al.*, 2017), whereas NF-YB and NF-YC have the transcriptional activation activities (Coustry *et al.*, 1995, 1996), allowing the complex to act as a transcription factor. The NF-Y complex may interact with other transcription factors to regulate the expression of downstream targets (Yamamoto *et al.*, 2009; Cao *et al.*, 2014; Huang *et al.*, 2015; Xu *et al.*, 2016). Interestingly, there are only one or two encoding genes of each NF-Y

family in mammals and yeast (Dolfini *et al.*, 2012). However, plants have substantially expanded their NF-Y genes (Laloum *et al.*, 2013). As an example, the rice genome encodes 11 NF-YAs, 11 NF-YBs, and 12 NF-YCs (Yang *et al.*, 2016). The expansion of plant NF-Ys increases the number of possible NF-Y complexes and likely contributes to the neo-functionalization or sub-functionalization of the NF-Y complex. For instance, a group of nodule predominantly expressed and phylogenically related NF-Ys can specifically function on nodulation in legumes (Baudin *et al.*, 2015).

Several NF-Ys have been found to be indispensable for seed development. *Leafy Cotyledon 1* (*LEC1*), also known as *NF-YB9* of *Arabidopsis* (*AtNF-YB9*), and its homolog *LEC1-like* (*L1L* or *AtNF-YB6*) are required for embryo maturation (Kwong *et al.*, 2003; Lee *et al.*, 2003). LEC1 and L1L belong to a phylogenically conserved clade of plants (Xie *et al.*, 2008). NF-YB7 of rice (OsNF-YB7) and OsNF-YB9 are the most similar homologs of LEC1 in rice. *OsNF-YB7*/*OsHAP3E* is expressed in the developing embryo and callus (Thirumurugan *et al.*, 2008). The ectopic expression of *OsNF-YB7* results in vegetative and reproductive development defects (Ito *et al.*, 2011; Zhang and Xue, 2013). However, due to the lethality caused by RNA-interference mediated gene silencing of *OsNF-YB7* (Ito *et al.*, 2011; Zhang and Xue, 2013), its role in embryogenesis and seed development remains to be elucidated. *OsNF-YB1*, an endosperm-specifically expressed gene, can coordinate with NF-YC members to regulate cell proliferation, grain filling, and sugar loading of the endosperm (Sun *et al.*, 2014; Bai *et al.*, 2015; Xu *et al.*, 2016). Bai et al. (2015) showed that OsNF-YB1 was able to recognize the CCAAT motifs of some sucrose transporters, while Xu et al. (2016) suggested that OsNF-YB1 likely lacks CCAAT-box binding activity but it could bind to ERF transcription factors in endosperms to regulate gene expression. In addition, OsNF-YB1 is able to interact with OsMADS18, a MADS-box family transcription factor of rice (Masiero *et al.*, 2002). The biological significance of the interaction is yet to be identified.

Some NF-Ys are found to be preferentially expressed in rice, wheat, and maize endosperms (Stephenson *et al.*, 2007; Yang *et al.*, 2016; Zhang *et al.*, 2016*a*). However, the importance of NF-Ys for endosperm development is not well

understood. Here, by comprehensively analyzing the endosperm-preferential NF-Ys in rice, we identified two phylogenically-conserved NF-YB clades and one NF-YC clade, the members of which are exclusively found in monocots. The genes of these groups displayed an endosperm-predominant expression pattern, which strongly suggests that a group of NF-Ys have sub-functionalized endosperm development in monocots. We also analyzed the interactions between different endosperm-preferential NF-Ys in rice and their transcriptional activation activities. The findings will help us shed light on the functions that NF-Ys play during seed development.

Results

Endosperm-preferentially expressed NF-Ys in rice

The rice genome encodes 11 NF-YAs, 11 NF-YBs, and 12 NF-YCs (Yang *et al.*, 2016). A cluster analysis of the expression profiles showed that a group of NF-Ys, including *OsNF-YA8*, *OsNF-YB1*, and *OsNF-YB9*, and *OsNF-YC8* to *OsNF-YC12*, were predominantly expressed in rice endosperms (**Fig. 1 and Fig. 2A**). We further confirmed the expression pattern of these NF-Ys by real-time polymerase chain reaction (PCR). All endosperm-preferential NF-Ys were activated 3 d after fertilization (DAF) to 4 DAF (**Fig. 2B**). There is another rice NF-YB gene, *OsNF-YB7*, which also showed the seed preferentially-expressed pattern (**Fig. 1 and Fig. 2**). However, the microarray data **(Fig. 2A)** and a previous study (Thirumurugan *et al.*, 2008) suggested that its expression is mainly restricted to the embryo rather than the endosperm. The results show that each NF-Y family has members predominantly expressed in rice endosperms, which infers that the NF-Y complex may play important roles in the regulation of endosperm development.

Phylogenetic analysis of endosperm-preferential NF-Y rice genes

To further study gene divergence of the endosperm-preferential NF-Ys, we performed a sequence alignment and phylogenetic analysis of all NF-YAs, NF-YBs, and NF-YCs in rice. Because the sequence outside the conserved domain is very variable, we mainly used the core sequence of the conserved domains for the

analysis. In comparison with other NF-YAs, OsNF-YA8 showed several differences in the core sequence, many of which were located within the A2 domain (**Supplementary Figs. 1A**). Previous studies have proved that the A2 domain is important to NF-YAs for binding of the CCAAT motif (Nardone *et al.*, 2016). Due to the variation in the protein sequence of the A2 domain, we inferred that OsNF-YA8 has possibly evolved a novel ability to recognize other *cis*-elements, in addition to the CCAAT motif. Using the core sequence of OsNF-YA8 as the query to search its close homologs in other species, we failed to identify any homologs of OsNF-YA8 outside of the *Oryza* genus, which indicated that *OsNF-YA8* was newly evolved after the divergence of the *Oryza* genus.

An alignment analysis of the NF-YB family members showed that the core sequence of OsNF-YB1 was distinct from the other NF-YBs of rice (**Supplementary Figs. 1B and E**). Many of the variations are situated within the α2 domain, which is believed to involve the NF-YA and NF-YC interactions of NF-YBs (Petroni *et al.*, 2012; Laloum *et al.*, 2013). The core sequences of OsNF-YB9 and OsNF-YB7, two LEC1 homologs in rice, are very similar (**Supplementary Fig. 1B**), although their expression patterns are distinct (**Fig. 2A**). Previous studies have confirmed that the Asp at position 55 in LEC1 (or 84 in L1L) is diagnostic for LEC1 family members and is essential for gene function in *Arabidopsis* (Gnesutta *et al.*, 2017). Moreover, a crystal structural analysis suggested that His at position 79 of L1L was essential for the CCAAT-binding ability of L1L to compensate for the Lys→Asp substitution at position 84 (Gnesutta *et al.*, 2017). The Asp and His residues are also conserved in OsNF-YB9 and OsNF-YB7 (**Supplementary Fig. 1B**). Due to less conservation out of the core region of OsNF-YB9 and OsNF-YB7, it is still an open question as to whether the functions of OsNF-YB9 and OsNF-YB7 are similar or have been differentiated in different tissues of the seed. Interestingly, the LEC1 homologs in monocots can be divided into two phylogenetic clads. OsNF-YB7 is included in the same group of LEC1 and L1L, while OsNF-YB9 belongs to another clade that consists exclusively of monocot homologs (**Fig. 3A**). Three substitutions were found by comparing the core sequences of the OsNF-YB7-like (OsYB7L) and OsNF-YB9-like (OsYB9L) proteins in different monocot

species (**Supplementary Fig. 2**). Thr at position 33 of OsYB7Ls was substituted by Ala, Val, or Leu, all of which are hydrophobic, in OsYB9Ls. QREQ at position 49-52 of OsYB7Ls is much conserved, however, it is variable in OsYB9Ls. In addition, the Tyr or Phe at position 89 of OsYB7Ls is substituted by Met in OsYB9Ls. Whether the variations contribute to their function differentiations still needs to be elucidated.

In terms of the NF-YC family, OsNF-YC8, OsNF-YC9, OsNF-YC10, OsNF-YC11, and OsNF-YC12 are phylogenetically distinct from the other rice NF-YCs (**Supplementary Figs. 1C and F**). Even the intact nucleotide sequences of *OsNF-YC8*, *OsNF-YC9*, and *OsNF-YC10* are very similar (**Supplementary Fig. 3A**), indicating that these genes were formed by recent gene duplication events. Likewise, OsNF-YC11 and OsNF-Y12 show very high similarities (**Supplementary Fig. 3B**). Because of a very close relationship among OsNF-YC8, OsNF-YC9, and OsNF-YC10, and between OsNF-YC11 and OsNF-YC12, we used the core protein sequence of OsNF-YC8 and OsNF-YC12 to search their homologs in other species. The results showed that only monocots have OsNF-YC8 and OsNF-YC12 homologs (**Fig. 3B**), which suggests that this group of genes were formed after the divergence of dicots and monocots. Together, our results show that the endosperm-preferentially expressed NF-YA, NF-YBs, or NF-YCs have very unique sequence features that separate them from widely-expressed canonical NF-Ys.

Endosperm-preferential expression of NF-Ys in other monocot species

Because it is possible to distinguish the endosperm-preferential NF-Ys from other family members simply by their core sequences in rice, we tested whether the rule could also be applied to NF-Ys of other plant species. Zm00001d017899 and Sb04g029340 are the most similar homologs of OsNF-YB1 in maize and sorghum (**Fig. 3A**), respectively. By searching RNA-seq data deposited in the Expression Atlas database, we found that these genes are predominantly or exclusively expressed in the endosperm (**Fig. 4A**). Likewise, Zm00001d045772 (GRMZM2G167576) and Sb10g010520, the maize and sorghum homologs of *OsNF-YB9* (**Fig. 3A**), are also exclusively expressed in the endosperm (**Fig. 4B**). Sb08g006350, Sb08g003700, and

Sb07g004410 are three homologs of the endosperm-preferentially expressed rice NF-YCs in sorghum (**Fig. 3B**), and we found that the mRNA transcripts of Sb08g006350 and Sb07g004410 were only detectable in the endosperm (**Fig. 4B**). Sb08g003700 has a lack of expression data in the Expression Atlas. However, in another expression experiment of which data were deposited in the MOROKOSHI Sorghum Transcriptome Database, we found that Sb08g003700 was expressed in the endosperm but not in the embryo or other tissues (**Supplementary Fig. 4A**). The maize genome encodes only one homology gene, Zm00001d023466 (GRMZM2G052499), of *OsNF-YC8* or *OsNF-YC12*. Its expression data were not found in the Expression Atlas database, but the microarray-based experiments indicated that Zm00001d023466 was predominantly expressed in the endosperm as well (**Supplementary Fig. 4B**). Taken together, these results clearly indicate that monocots have evolved a group of phylogenically conserved NF-YBs and NF-YCs that are predominantly expressed in the endosperm. This occurred after the divergence of dicots and these genes most likely play roles in the regulation of endosperm development. We also explored the expression pattern of *OsNF-YB7* homologs in other monocot species. Similar to the *OsNF-YB7*, Zm00001d017898 (GRMZM2G011789) and Zm00001d051697 (GRMZM2G124663) of maize and Sb04g029350 of sorghum, were predominantly expressed in the embryo (**Fig. 4, Supplementary Figs. 4C and D**), indicating that these genes may function on seed development, like the role of *LEC1* in *Arabidopsis*.

The homologs of OsNF-YB1 and OsNF-YB7 could be found in barley **(Fig. 3A)**. However, due to relatively poor annotation of the barley genome, we did not find the OsNF-YB9, OsNF-YC8 or OsNF-YC12 homologs, using the protein sequences to search against the protein dataset of barley. Instead, when we used the core protein sequences of these rice NF-Ys to search against the barley genomic DNA sequence, we did discover two genes, which respectively designated as *Hv9BL* and *Hv8CL* hereafter, showing high similarities to OsNF-YB9 and OsNF-YC8/12 in terms of the core sequence of the conserved domains **(Supplementary Fig. 5)**. As expected, the MLOC_75867 (an *OsNF-YB1* homolog), *Hv9BL* and *Hv8CL* were predominantly

expressed in the endosperm, whereas the MLOC_36682 (an *OsNF-YB7* homolog) showed high expression in the embryo but not in the endosperm **(Fig. 4)**.

Sequence similarity and conserved expression pattern of these endosperm-preferential NF-Y groups in different monocot species strongly suggest that the functional differentiation of these genes occurred in a common rice, maize, and sorghum ancestor. Notably, *OsNF-YB1* (LOC_Os02g49410) and *OsNF-YB7* (LOC_Os02g49370) and their corresponding homology genes in maize (Zm00001d017899 and Zm00001d017898) and sorghum (Sb04g029340 and Sb04g029350), are located on adjacent chromosomal regions (**Supplementary Fig. 6**), which further confirms that the duplication event to form *NF-YB1* and *NF-YB7* is very ancient.

Subcellular localization of the endosperm-preferentially expressed OsNF-Ys

To detect the subcellular localization of the endosperm-preferential NF-Ys, we fused the NF-Ys with a Venus-tag and transiently expressed them in tobacco (*Nicotiana benthamiana*) epidermal cells, respectively. The results indicated that NF-YA8 was predominately targeted to the nucleus (**Fig. 5A**), while the other endosperm-preferential OsNF-Ys were expressed in both the cytoplasm and the nucleus (**Figs. 5B-H**). The results are consistent with previous findings that NF-YB and NF-YC can be dimerized in the cytoplasm and then be imported into the nucleus to form a heterotrimer with NF-YA (Laloum *et al.*, 2013).

Interactions between the endosperm-preferential OsNF-Ys

Although the core sequences of endosperm-preferential NF-Ys are somewhat different from the canonical NF-Ys (**Supplementary Fig. 1**), our homology modeling analysis suggested that sequence divergence likely did not change the protein structure of the conserved domains (**Supplementary Fig. 7**), indicating that these endosperm-preferential NF-Ys may interact with different members as the canonical NF-Ys. To test this idea, we performed a yeast two-hybrid assay to detect protein

interactions between different groups of endosperm-preferential NF-Ys of rice. The results showed that OsNF-YA8 was only able to interact with OsNF-YB9 (**Fig. 6A and Supplementary Fig. 8**). Notably, despite the high similarity between OsNF-YB7 and OsNF-YB9, OsNF-YA8 could not interact with OsNF-YB7 in yeast (**Supplementary Fig. 8**). All of the NF-YBs tested, including OsNF-YB1, OsNF-YB9, and OsNF-YB7, were able to interact with all the endosperm-preferential NF-YC family members (**Supplementary Fig. 9**).

Usually, NF-YA does not interact with NF-YB or NF-YC monomer (Hackenberg et al., 2012). To confirm the interaction between OsNF-YA8 and OsNF-YB9, we conducted a split luciferase complementation assay in the tobacco epidermal cells. After the co-filtration of nLUC (the N-terminal of luciferase) and cLUC (the C-terminal of luciferase), we failed to detect luciferase activity (**Fig. 6B**). However, co-expression of nLCU-OsNF-YA8 and cLUC- OsNF-YB9 showed a high luciferase activity (**Fig. 6B**). In addition, neither the combination of nLCU-OsNF-YA8 and cLUC, nor the combination of nLCU and cLUC-OsNF-YB9 could activate luciferase activity (**Fig. 6B**). The results confirmed the interaction between OsNF-YA8 and OsNF-YB9 in plant. Next, we used a bimolecular fluorescence complementation (BiFC) assay to determine the organelle in which the interaction occurs. The fluorescence signal was observed in the nucleus but not in the cytoplasm (**Fig. 6C**), which is consistent with previous findings that NF-YA is a nuclear localized protein and can interact with NF-YB and NF-YC to form a heterotrimer (Laloum *et al.*, 2013).

Endosperm preferential NF-YBs can interact with ERFs

A previous study has indicated that OsNF-YB1 can interact with the ERF protein, OsERF115, to form NF-YB/NF-YC/ERF heterotrimers in the nucleus of rice endosperm (Xu *et al.*, 2016). We performed yeast-two-hybrid assays to test whether the other NF-YBs have the same ability. The result suggested that OsNF-YB9 was able to interact with OsERF114 and OsERF115 (**Fig. 7A**) but not with OsERF74 and OsERF72 (**Supplementary Fig. 10**). Notably, OsNF-YB7 also showed an interaction with OsERF115, and possibly with OsERF114 as well (**Fig. 7A**). We also tested the

interaction between OsERF114/115 and the endosperm-preferential OsNF-YA8 and OsNF-YCs. Neither of OsERF114 nor OsERF115 showed interaction with OsNF-YA8 (**Fig. 7B**). However, OsNF-YC12 could interact with OsERF114 in yeast (**Fig. 7B**). Due to the self-activation activity of OsNF-YC8 (**Fig. 7B**), the interactions between OsERFs and OsNF-YC8 need to be further confirmed. Interestingly, the expression patterns of *OsERF114* and *OsERF115* were very similar to those of the endosperm-preferential NF-Ys (**Supplementary Fig. 11**), indicating that OsERF114 and OsERF115 may coordinate with endosperm-preferential NF-YB and NF-YC members to regulate rice endosperm development.

Transcriptional activation activity

It is largely unknown which NF-Y family displays transcriptional activation activity in plants. We fused the endosperm-preferential NF-Ys of rice with the GAL4 binding domain to detect their transcriptional activation ability in yeast. OsNF-YA8, OsNF-YB1, OsNF-YB9, and OsNF-Y7B did not survive on the selective synthetic dropout medium (**Supplementary Fig. 12**), which suggested that these proteins alone are not capable of transcription activation. In terms of the NF-YC family members, OsNF-YC8, OsNF-YC9, and OsNF-YC10 could survive on the dropout medium like the positive control (the fusion of the GAL4 activation domain with the GAL4 DNA binding domain), while neither OsNF-YC11 nor OsNF-YC12 showed transcriptional activation activity (**Supplementary Fig. 12**). Next, we fused the N-terminal (containing the core domain of NF-YC) of OsNF-YC8 with the C-terminal of OsNF-YC12 (designed as YC8N+YC12C hereafter), and found that this chimeric protein did not show transcriptional activity (**Fig. 8A**). In contrast, transformants with the fusion of the N-terminal of OsNF-YC12 and the C-terminal of OsNF-YC8 (YC12N+YC8C) could survive on the selective medium (**Fig. 8A**), which indicated that the C-terminal of OsNF-YC8 determines the transcriptional activation activity.

Because OsNF-YC11 and OsNF-YC12 alone lacks transcriptional activation activity, we assumed that the transcriptional activation ability of these NF-YC family members may require the assistance of the NF-YBs it interacted with. OsNF-YB1 and

OsNF-YC12 can interact with each other **(Supplemental Fig. 9A)**, but neither of OsNF-YB1 nor OsNF-YC12 showed transcriptional activation activity (**Fig. 8B and Supplementary Fig. 12**). However, when we expressed the fusion of OsNF-YB1 and the GAL4 DNA binding domain, and OsNF-YC12 simultaneously in yeast, the transformants could survive on the selective dropout medium (**Fig. 8B**), suggesting that the dimer OsNF-YC12 and OsNF-YB1 formed has transcriptional activation activity, which strongly supported the hypothesis we conceived.

Phenotypic analysis of the OsNF-Ys knockout mutants

To uncover the biological roles of the endosperm-preferential OsNF-Ys in endosperm development, we made knockout mutants of these genes, taking advantages of the CRISPR/Cas9 constructs expressing the guide RNAs which targeted different regions of the first-half of the endosperm-preferential *OsNF-Ys*. We now have obtained the homozygous mutants of *OsNF-YB1*, *OsNF-YC8*, *OsNF-YC11* and *OsNF-YC12* (**Supplementary Fig. 13**). The mutants did not show any visible defects of vegetative development, which in agreement with their endosperm-preferential expression pattern. Consistent to previous findings, the *osnf-yb1* mutant showed reduced seed size and increased chalkiness of endosperm (**Figs. 9A-C**). However, *osnf-yc8*, *osnf-yc11* and *osnf-yc12* did not display any seed abnormalities, in terms of the seed size and endosperm appearance (**Figs. 9A-C**). Most possibly, it is due to the redundancies among *OsNF-YC8*, *OsNF-YC9* and *OsNF-YC10* and between *OsNF-YC11* and *OsNF-YC12*. Notably, disruption of *OsNF-YB7* seemed not affect the endosperm development of the mutant as well (**Figs. 9A-C**).

Discussion

Functional analyses revealed that plant NF-Ys may have multiple functions that regulate stress response, flowering time, embryo development, and chloroplast biogenesis (see review Petroni et al., 2012; Laloum et al., 2013). Compared to *Arabidopsis*, the function of the rice NF-Ys is much less understood. Although the

expression of some NF-Y genes in the endosperm has been reported (Stephenson *et al.*, 2007; Yang *et al.*, 2016; Zhang *et al.*, 2016*a*), the biological function of plant NF-Ys on endosperm development is largely unknown. To explore the possible roles that NF-Ys play in endosperm development, we conducted a comprehensive phylogenetic analysis of endosperm-preferential NF-Ys. Our results suggest that there are several phylogenetically-conserved groups of NF-YB and NF-YC that are likely to be important for cereal endosperm development (**Fig. 3**). Moreover, these genes in other monocot species were also predominantly expressed in the endosperm (**Fig. 4**), which allows us to assume that the function of this group of genes in endosperms is much conserved in monocots. We could not find any homologs of these endosperm-preferential NF-Ys in dicots, indicating that the genes evolved after the divergence of dicots and monocots. The fate of the endosperm in dicots and monocots is distinct (Zhou *et al.*, 2013). Dicots usually consume the endosperm for embryo growth, while monocots preserve the endosperm as an organ for starch and nutrient accumulation. We believe that the endosperm-preferential NF-Ys of monocots likely contributed to the divergence. Previous studies have showed that LEC1-family NF-YBs are essential for seed maturation and embryo development (Kwong *et al.*, 2003; Lee *et al.*, 2003). Interestingly, here we found that the LEC1-like proteins could be divided into two plant subgroups (**Fig. 3A**). The OsNF-YB7 and its homologs of other monocot species were more phylogenically close to LEC1 (**Fig. 3A**). Resembling the expression pattern of *LEC1*, these genes were also predominantly expressed in the embryo (**Fig. 4 and Supplementary Figs. 4C and D**). OsNF-YB9 and OsYB9Ls consisted of the other clade of LEC1-like proteins (**Fig. 3A**). Distinct from the *OsYB7Ls*, the *OsYB9Ls* of rice, maize, barley (*Hordeum vulgare*), and sorghum were endosperm-preferentially expressed (**Fig. 4 and Supplementary Fig. 4**). The findings suggest that some LEC1-like NF-YBs of monocots are sub-functionalized for endosperm development.

A systematic analysis of the interactions between different seed preferential NF-Ys of rice indicated that OsNF-YA8 could interact with OsNF-YB9 in yeast but not with

other NF-YB and NF-YC members that we tested (**Fig. 6 and Supplementary Fig. 8**). This observation surprised us, because NF-YAs can usually only bind to NF-YB/NF-YC dimers and not to any single subunit (Hackenberg *et al.*, 2012). The function of the interaction on rice seed development needs to be further discussed. Notably, in spite of the high similarities of the core sequence between OsNF-YB9 and OsNF-YB7 (**Supplementary Fig. 1B**), OsNF-YA8 failed to show an interaction with OsNF-YB7 in yeast (**Supplementary Fig. 8**). It is possible that the interaction between the OsNF-YA8 and OsNF-YB9 depends on certain sequences outside of the core domain.

NF-YA family members of *Arabidopsis* have showed a variable CCAAT-binding activity (Calvenzani *et al.*, 2012). A phylogenic analysis suggested that OsNF-YA8 was a recently-evolved gene that could only be found in the *Oryza* genus. Whether OsNF-YA8 is capable of binding to CCAAT-box is questionable. OsNF-YA8 carries many variations within the core domain compared with the canonical NF-YA family members (**Supplementary Fig. 1B**). Several conserved residuals are differentiated in the A2 domain of OsNF-YA8, nevertheless the changes seemed not to alter protein structure (**Supplementary Fig. 7**). The A2 domain is involved in recognition and binding with the CCAAT motif (Gnesutta *et al.*, 2017). It remains to be determined whether the variations allow OsNF-YA8 to recognize *cis*-elements other than CCAAT-box. The CCAAT-binding ability of NF-YA requires NF-YB and NF-YC (Calvenzani *et al.*, 2012; Gnesutta *et al.*, 2017). In addition to NF-YAs, NF-YB and NF-YC dimers can interact with some other transcription factor family members to regulate downstream targets (Yamamoto *et al.*, 2009; Cao *et al.*, 2014; Huang *et al.*, 2015; Xu *et al.*, 2016). A previous study has showed that OsNF-YB1 was able to interact with OsERF115 to modulate grain filling in rice (Xu *et al.*, 2016). In the present study, we found that OsNF-YB9 and OsNF-YB7 of rice could also interact with OsERF114 or OsERF115 (**Fig. 7**). Moreover, The NF-YC family member OsNF-YC12 showed interaction with the OsERF114 as well. Like the OsNF-Ys we studied here, *OsERF114* and *OsERF115* are endosperm-preferentially expressed (**Supplementary Fig. 11**). Therefore, we assumed that the interaction between ERFs and NF-Y complex is very

important for the endosperm development of rice (**Fig. 10**).

Mammal studies have showed that the transcriptional activity of NF-Ys was dependent on the glutamine-rich domains present in the NF-YB and NF-YC subunits, both of which are located outside the core domains (Coustry *et al.*, 1995, 1996). Our understanding of the transcriptional activation activity of plant NF-Ys is much poorer. Here, we found that most of the endosperm-preferential NF-Y monomers in rice were lacking the transcriptional activation activity in yeast, but OsNF-YC8 did show the activity, for which the C-terminal sequence was required (**Fig. 8A and Supplementary Fig. 12**). Interestingly, the dimer of OsNF-YB1/OsNF-YC12 displayed the transcriptional activation activity, though no such activity were observed in yeast by OsNF-YB1 or OsNF-YC12 alone (**Fig. 8B**). In addition, the OsNF-YB/OsNF-YC dimers can recruit other transcription factors, such as OsERF114, to form a more complicated complex to modulate the expression of downstream genes (**Fig. 10**). As an example, rice is likely to form a complex that consists of NF-Ys, Hd1, OsHAPL1, and the general transcription factors to control plant flowering (Zhu *et al.*, 2017). The NF-Y complex may also recruit transcription repressors to regulate gene expression (Li *et al.*, 2011). In this aspect, the NF-Y complex is more like a scaffold of transcriptional machinery. Leyva-González et al. (2012) showed that NF-YA2 of *Arabidopsis* could act like a repressor of a subset of genes that lack CCAAT-box. Therefore, the alternative interpretation of no transcriptional activation by OsNF-YA8, OsNF-YB1/7/9, or OsNF-YC11/12 alone in yeast could because these NF-Ys function as transcriptional repressors rather than activators. These findings suggested that the function of NF-Ys on transcriptional regulation may be very diverse, and the NF-Y complex of plants may require the coordination of multiple subunits for transcriptional activation activity. The role of endosperm-preferential NF-Ys in cereal endosperm development needs to be further discussed.

In summary, our findings indicated that the monocots have evolved a group of endosperm-preferentially expressed and phylogenetically-conserved NF-Ys in each family. Although more genetic evidences are required to support our hypothesis that

endosperm-preferential genes are indispensable for endosperm development, the endosperm defects of the *osnf-yb1* mutant (**Fig. 9**, Bai et al., 2015; Sun et al., 2014; Xu et al., 2016) provide strong evidence to confirm that this group of genes is important for endosperm development.

Materials and Methods

Plant materials and growth conditions

Kitaake (*O. sativa* subsp. *japonica*) rice plants were grown in a greenhouse with regular water and nutrient management. Various tissues, including the leaf blade, leaf sheath, flag leaf, stem, and young panicles at the booting stage were collected for RNA isolation. Before flowering, the plants were moved into a growth chamber that was maintained at a consistent 28 ˚C. The seeds were labeled with a marker pen when flowering. Caryopsis of different ages (0 HAF, 24 HAF, 48 HAF, 72 HAF, 96 HAF, and 120 HAF) were collected and immediately frozen by liquid nitrogen for RNA isolation. The barley variety Morex was planted in the experimental plot in 2016, Chengdu, China. Various barley tissues were collected at the flowering stage. The seeds were labeled on the day of flowering for sampling of different age caryopsis. Twenty-five days after fertilization, endosperms and embryos were carefully separated to avoid contamination of maternal tissues.

Generation of the mutant plants

The targets for CRISPR/Cas9-mediated gene targeting were designed by the web-based tool CRISPR Primer Designer (http:// plantsignal.cn/CRISPR/crispr_primer_designer.html). The targets were then cloned into the vector BGK032. All the constructs were transformed into the Agrobacterium strain EHA105 and used for plant transformation as described previously (Chen *et al.*, 2014). The DNA fragments embracing the targets of the plant transformats were amplified. The PCR products were sent for Sanger sequencing. The T0 homozygous mutants of *osnf-yb1*, *osnf-yb7*, *osnf-yc8*, *osnf-yc11* and *osnf-yc12* were obtained for phenotypic analysis.

RNA extraction and real-time PCR assay

Total RNA from different tissues was isolated using the Plant RNA Kit (OMEGA) following the manufacturer's protocol. The total RNA was treated with an RNA-free DNase set (OMEGA) to remove contamination. Complementary DNA (cDNA) was synthesized using the PrimeScript RT Master Mix (TAKARA) by oligo-dT primers. The standard procedure provided by the manufacturer was used for the reactions. A 2 µL sample of the diluted cDNA was used for real-time PCR in a 20 µL reaction using the AceQ® qPCR SYBR® Green Master Mix (Vazyme). The real-time PCR reactions were performed on the CFX ConnectTM Real-Time System (BioRad). Three independent replicates were set for each sample. The proteasome gene (LOC_Os03g63430) of rice was used as the endogenous control. Quantification of the relative expression was calculated by the ΔΔCt method. Primers used for the real-time PCR reactions can be found in **Supplementary Table 1**.

Expression analysis of the endosperm-preferential NF-Ys in monocots

We used expression data deposited in Genevestigator® for the expression analysis of rice NF-Ys. A hierarchical clustering analysis of the expression of rice NF-Ys was also performed with the similarity search tool provided by Genevestigator®. Expression data of the *OsNF-YB1,7,9-like* and *OsNF-YC8,11-like* genes of maize and sorghum were obtained from the Expression Atlas (https://www.ebi.ac.uk/gxa/home). For those genes with no data in the Expression Atlas, we searched their expression through the Maize eFP Browser (http://bar.utoronto.ca/efp_maize/cgi-bin/efpWeb.cgi) for maize or the MOROKOSHI Sorghum transcriptome database (http://sorghum.riken.jp/morokoshi/Home.html) for sorghum.

Alignment, phylogenetic analysis, and protein structure modeling

The core domains of OsNF-YA8, OsNF-YB1,7,9, and OsNF-YC8,11 were used as queries for Blast to identify their close homologs in other plant species. The protein

and DNA sequences of the endosperm-preferential NF-Ys were obtained from the BioMart of the Phytozome database (www.phytozome.jgi.doe.gov/biomart/martview/). Alignment was conducted by Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/). The phylogenetic neighbor-joining trees were generated by MEGA7.0 (Kumar *et al.*, 2016). The intensive model of the Phyre2 web portal was used for NF-Ys modeling (Kelley *et al.*, 2015).

Yeast two-hybrid assay

The coding sequences of *OsNF-YA8*, *OsNF-YB1,7,9*, *OsNF-YC8,9,10,11,12*, and *OsERF72,74,114,115* were amplified by high-fertility DNA polymerase GXL (Takara) from endosperm cDNA using the primers list in **Supplementary Table 1**. The genes were cloned into either pGBK-T7 or pGAD-T7 using the ClonExpress® II One Step Cloning Kit (Vazyme). The prey and bait plasmids were transformed respectively into the yeast strains Y187 and Y2HGold. After mating of the two strains, co-transformants were selected on SD-L-T plates. Interactions were tested using SD-L-T-H with an optimized content of 3-AT and SD-L-T-H-A medium, simultaneously.

Transcriptional activation assay in yeast

The coding sequences of *OsNF-YA8*, *OsNF-YB1,7,9*, and *OsNF-YC8,9,10,11,12* were cloned into pGBK-T7. Meanwhile, we amplified the GAL4 activation domain from a pGAD-T7 empty vector and fused it with GAL4 DNA-binding domain using the ClonExpress® II One Step Cloning Kit (Vazyme). This construct was used as a positive control of transcriptional activation activity. Meanwhile, we respectively cloned *OsNF-YB1* and *OsNF-YC12* into the multiple cloning site 1 (MCS1) and the MCS2 of the pBridge. All of the constructs were then transformed into yeast strain Y2HGold to investigate the activity of transcriptional activation on the SD-Trp/-His/-Ade medium. The primers used in the experiment are listed in **Supplementary Table 1**.

Sub-cellular localization analysis

The coding sequences of *OsNF-YA8*, *OsNF-YB1,7,9*, and *OsNF-YC8,9,10,11,12* were cloned into the binary vector p1300-LV using the ClonExpress® II One Step Cloning Kit (Vazyme). The constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 and infiltrated into tobacco leaf epidermal cells to transiently express the NF-Y:Venus recombinant fusions. About 48 h after infiltration, the fluorescence signals were examined with a confocal laser scanning microscope (LSM710, Zeiss). Primer information is listed in **Supplementary Table 1**.

BiFC and split luciferase complementation assay

The coding sequence of *OsNF-YA8* and *OsNF-YB9* was, respectively, cloned into pCAMBIA1300S-YN and pCAMBIA2300S-YC to fuse with the N-terminal and C-terminal of yellow fluorescence protein (nYFP and cYFP) and transformed into *Agrobacterium tumefaciens* strain GV3101. In addition, HAL3, which can dimerize in cytoplasm, was cloned into pCAMBIA1300S-YN and pCAMBIA2300S-YC as a positive control (Su *et al.*, 2016). *nYFP-YA8* and *cYFP-YB9*, *nYFP-OsHAL3* and *cYFP-OsHAL3*, and *nYFP-5790* and *cYFP-5790* were co-infiltrated in tobacco leaves for about 48 h. The YFP fluorescence signals were examined with a confocal laser scanning microscope (LSM710, Zeiss).

Similarly, *OsNF-YA8* and *OsNF-YB9* was cloned respectively into binary vectors JW771 and JW772 to fuse with the N-terminal of luciferase (nLUC) and the C-terminal of luciferase (cLUC). *nLUC-OsNF-YA8* and *cLUC-OsNF-YB9* along with the combinations of *cLUC* and *nLUC*, *nLUC-OsNF-YA8* and *cLUC*, and *nLUC* and *cLUC-OsNF-YB9* were transiently expressed in tobacco leaves for about 48 h. The Luciferase Assay System (Promega) was used to detect the interactions. The chemiluminescence signal was detected by the Tanon Imaging System (5200 Multi, Tanon).

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Figure legends

Figure 1. Cluster analysis of the expression of rice NF-Ys in various tissues. A group of rice NF-Ys (boxed by dash lines), including *OsNF-YA8*, *OsNF-YB1*, *OsNF-YB9*, *OsNF-YC8*, *OsNF-YC9*, *OsNF-YC10*, *OsNF-YC11* and *OsNF-YC12*, are predominantly expressed in the endosperm.

Figure 2. Activation of the seed-preferential *OsNF-Ys* after fertilization.

(A) Heat map of the expression of *OsNF-YA8*, *OsNF-YB1*, *OsNF-YB7*, *OsNF-YB9*, *OsNF-YC8*, *OsNF-YC9*, *OsNF-YC10*, *OsNF-YC11* and *OsNF-YC12* at different days after fertilization (DAF). Yellow and green bars indicated ovary (including seed coat, endosperm and embryo) and embryo, respectively. **(B)** Confirmation of the seed-preferential expression pattern and gene activation after fertilization of *OsNF-YA8*, *OsNF-YB7*, *OsNF-YB1*, *OsNF-YB9*, *OsNF-YC8*, *OsNF-YC9*, *OsNF-YC10*, *OsNF-YC11* and *OsNF-YC12* by Real-time PCR. LB, FL, LS, ST, YP and AN indicate leaf blade, flag leaf, leaf sheath, stem, young panicle and anther, respectively; 0 DAF indicate unfertilized ovary; 1 to 5 DAF indicate ovaries of different ages (from 1 to 5 DAF). Three biological replicates are used for analysis; error bars indicate standard deviations.

Figure 3. Neighbor-joining phylogenetic trees of the endosperm-preferential OsNF-YBs and OsNF-YC.

(A) Phylogenetic tree of the OsNF-YB1, OsNF-YB7, OsNF-YB9 and their homologs. **(B)** Phylogenetic tree of the OsNF-YCs and the close homologs of OsNF-YC8 and OsNF-YC12. The locus identities initiating with TRIUR, TRIAE, F775, Sb, GRMZM(ZM), Os, Si, At, MLOC and BRADI indicate that genes are from *Triticum urartu*, *Triticum aestivum*, *Aegilops tauschii*, *Sorghum bicolor*, *Zea mays*, *Oryza sativa*, *Setaria italica*, *Arabidopsis thaliana*, *Hordeum vulgare* and *Brachypodium distachyon*, respectively. The core sequences of the conserved domains of NF-YBs and NF-YCs were used for trees construction.

Figure 4. Gene expression of the *OsNF-YB1 Likes* (A), *OsNF-YB7 Likes* (B), *OsNF-YB9 Likes* (C) and *OsNF-YC8/12* Likes (D) in maize, sorghum and barley.

The green, yellow and blue bars indicate gene expression of the maize, sorghum and barley homologs, respectively. The expression abundance in maize and sorghum are indicated by FPKM (fragments per kilobase of exon per million fragments mapped). Data is obtained from the Expression Atlas. The relative expression in barley is detected by Real-time PCR assay. Y-axes indicate the tissues used for expression analysis. DAF, days after fertilization. Three biological replicates were used for analysis; error bars indicate standard deviations.

Figure 5. Subcellular localization of the endosperm-preferential NF-Ys of rice in tobacco epidermal cells.

The florescence signal of the OsNF-YA8:Venus fusion is predominantly expressed in nucleus **(A)** while these of the rest OsNF-Y:Venus fusions are expressed in cytoplasm, as well as in nucleus **(B-H)**. The NF-Ys are N-terminally fused to a venus tag.

Figure 6. Interactions between the OsNF-YA8 and OsNF-YB9.

(A) Yeast-two-hybrid assay shows interaction between OsNF-YA8 and OsNF-YB9. AD and BD indicate the activation domain and the DNA binding domain of GAL4, respectively; AD-YA8, AD-YB9, BD-YA8 and BD-YB9 indicate AD:OsNF-YA8, AD:OsNF-YB9, BD:OsNF-YA8 and BD:OsNF-YB9 fusions, respectively. Serial dilutions of the yeast cells expressing the indicated proteins were plated on the non-selective medium (SD-L-T) or selective plates (SD-L-T-H and SD-L-T-H-A). **(B)** Split luciferase complementation assay confirms the interaction between OsNF-YA8 and OsNF-YB9. nLUC, cLUC, nLUC-YA8 and cLUC-YB9 indicate the N-terminal of luciferase, the C-terminal of luciferase, the nLUC:OsNF-YA8 fusion and the cLUC:OsNF-YB9 fusion, respectively. Different constructs combinations are transiently co-expressed in the tobacco epidermal cells. **(C)** BiFC assay shows interactions between OsNF-YA8 and OsNF-YB9. OsNF-YA8 and OsNF-YB9 are fused with the N-terminal of YFP (nYFP) and the C-terminal of YFP (cYFP), respectively. OsHAL3, which can dimerize in the

cytoplasm, is used as the positive control, while the gene LOC_Os01g05790 (5790), which cannot form a homo-dimer, is used as the negative control.

Figure 7. Interactions between the seed-preferential OsNF-Ys and ERFs.

(A) OsNF-YB1, OsNF-YB7 and OsNF-YB9 interact with OsERF114 or OsERF115. BD-YB1, BD-YB9 and BD-YB7 indicate that the OsNF-YB1, OsNF-YB9 and OsNF-YB7 are C-terminally in fusion with the DNA binding domain of GAL4 (BD), respectively. AD-ERF114 and AD-ERF115 indicate that the OsERF114 and OsERF115 are C-terminally in fused with the activation domain of GAL4 (AD), respectively. Serial dilutions of the yeast cells expressing the indicated proteins were plated on the non-selective medium (SD-L-T) or selective plates (SD-L-T-H with 2mM 3AT or SD-L-T-H-A). **(B)** OsNF-YC12 interacts with OsERF114 in yeast. BD-YA8, BD-YC8 and BD-YC12 indicate that the OsNF-YA8, OsNF-YC8 and OsNF-YC12 are C-terminally in fusion with the BD, respectively. The yeast grew on the selective triple dropout medium with 5mM 3AT are indicated by stars. High self-activation activity of OsNF-YC8 was observed; the yeast cells expressing the AD and the BD:OsNF-YC12 fusion was survivable on the selective medium.

Figure 8. Transcriptional activation activities of the endosperm-preferential OsNF-Ys.

(A) C-terminal of OsNF-YC8 is important for the transcriptional activation activity. BD and AD indicate the DNA binding domain and activation domain of GAL4, respectively. The serially diluted yeast cells expressing BD:OsNF-YC8 fusions (BD-YC8) and BD:OsNF-YC12 fusions (BD-YC12) could grow on the nonselective medium (SD-T), but only the transformants expressing BD-YC8 survived on the selective medium (SD-T-H-A), like what the BD:AD (a positive control) expressing yeast cells performed. When using the C-terminal of the OsNF-YC12 (amino acid residuals 361-984) to replace the C-terminal of OsNF-YC8 (385-1392), the fusions (BD-YC8N:YC12C) lose transcriptional activation activity. In contrast, the OsNF-YC12 N-terminal (1-360) and

OsNF-YC8 C-terminal (385-1392) fusion (BD-YC12N:YC8C) showed transcriptional activation activity. **(B)** The OsNF-YB1 and OsNF-YC12 dimer shows transcriptional activation activity in yeast. The BD:OsNF-YB1 and BD:OsNF-YC12 do not show transcriptional activation activity; but the yeast cells simultaneously expressing of BD:OsNF-YB1 and OsNF-YC12 (BD-YB1&YC12) could survive on the selective medium (SD-H-T-M).

Figure 9. Seed morphologies of the seed-preferential *OsNF-Ys* mutants generated by CRISPR/Cas9 approach.

(A) Seed morphology of the *osnf-yb1*, *osnf-yb7*, *osnf-yc8*, *osnf-yc11*, *osnf-yc12* and the wild type (Kitaake). **(B-C)** Seed length **(B)** and seed width **(C)** of the *osnf-yb1*, *osnf-yb7*, *osnf-yc8*, *osnf-yc11*, *osnf-yc12* and the wild type (Kitaake). More than 30 well-filled seeds from the main stem were chose for the measurements; error bars indicate standard deviations. **, $p<0.01$; ns, no significance; t-test was used for the statistical analysis.

Figure 10. A hypothetical model showing the endosperm preferential OsNF-Ys function on rice endosperm development.

The OsNF-YBs (OsNF-YB1/9 and possibly OsNF-YB7) and the OsNF-YCs (OsNF-YC8/9/10/11/12) are dimerized in the cytoplasm of endosperm cells and then imported into the nucleus to interact with OsNF-YA8 and OsERF114/115, or possibly with other transcription factors. OsNF-YA8 can recognize the CCAAT-motif while the OsERF114/115 can bind to the GCC-box. The OsERFs coordinate with the OsNF-Y complex to regulate the downstream genes involving endosperm development.

Supplementary materials

Supplementary Figure 1. Amino acid sequence alignment and phylogenetically analysis of the conserved domains of rice NF-Ys.

Supplementary Figure 2. Sequence logos of the conserved domains of OsNF-YB7 likes (OsYB7Ls) and OsYB9Ls.

Supplementary Figure 3. Multiple sequence alignment of the endosperm-preferential OsNF-YCs.

Supplementary Figure 4. Expression of some *OsNF-YC8 like* and *OsNF-YB7 like* genes in sorghum and maize.

Supplementary Figure 5. Multiple sequence alignments of the OsNF-YB9 and HvB9L (A) and of the OsNF-YC8, OsNF-YC12 and HvC8L (B).

Supplementary Figure 6. Diagram showing the physical linkage of the *OsNF-YB7 like* and the *OsNF-YB1 like* genes in rice, maize and sorghum.

Supplementary Figure 7. Predicted structures of the NF-Y conserved regions.

Supplementary Figure 8. OsNF-YA8 interacts with OsNF-YB9, but not with other endosperm-preferential OsNF-Ys.

Supplementary Figure 9. OsNF-YB1, OsNF-YB7 and OsNF-YB9 interact with all the endosperm-preferential OsNF-YCs.

Supplementary Figure 10. OsNF-YB1, OsNF-YB7 and OsYB9 do not show interaction with OsERF72 or OsERF74.

Supplementary Figure 11. *OsERF114* and *OsERF115* are activated after fertilization.

Supplementary Figure 12. OsNF-YC8, OsNF-YC9 and OsNF-YC10 exhibit transcriptional activation activities.

Supplementary Figure 13. The mutations in the mutants of endosperm-preferential OsNF-Ys.

Supplementary Table 1. The primers used in the present study.

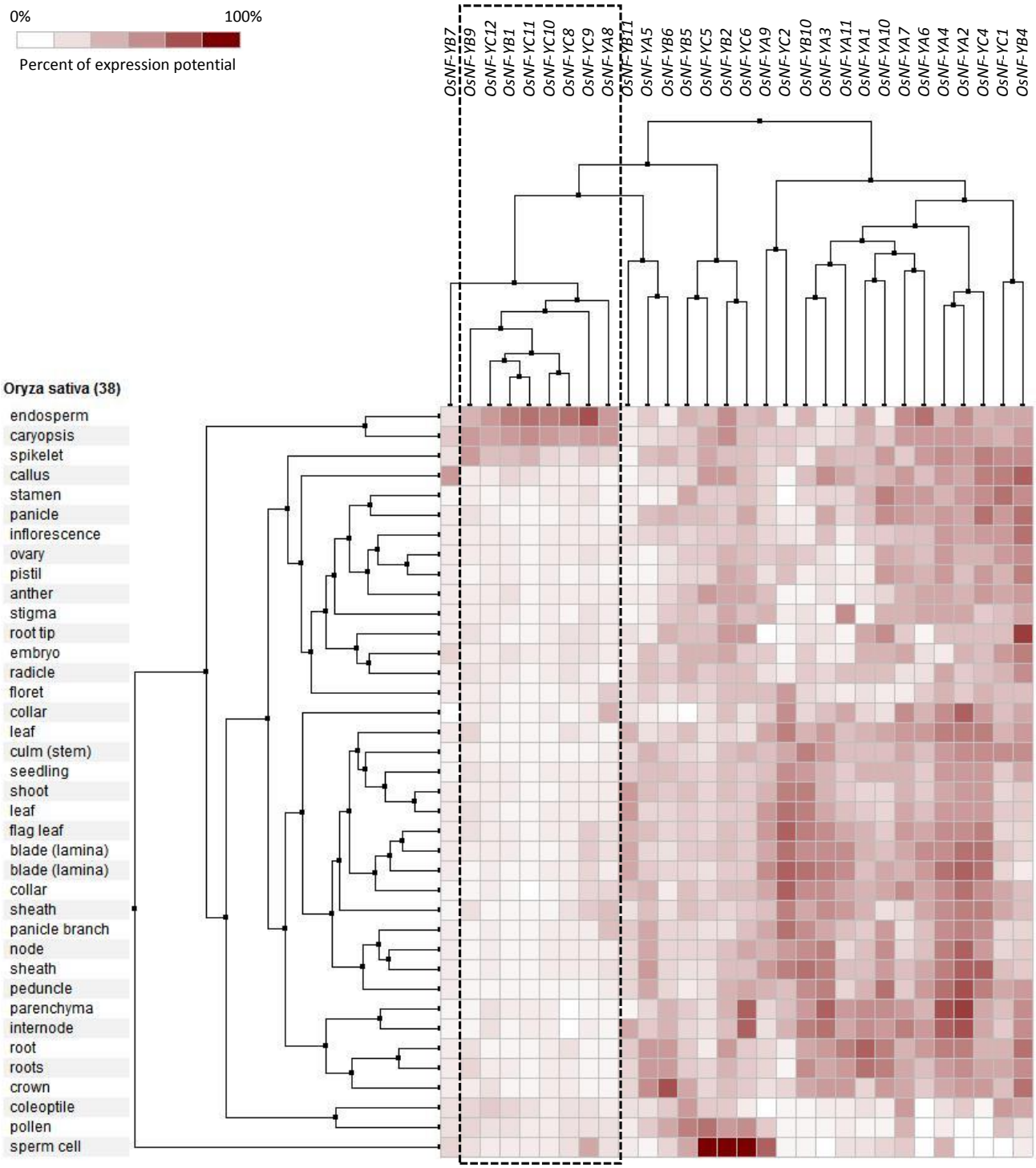


Figure 1. Cluster analysis of the expression of rice NF-Ys in various tissues. A group of rice NF-Ys (boxed by dash lines), including *OsNF-YA8*, *OsNF-YB1*, *OsNF-YB9*, *OsNF-YC8*, *OsNF-YC9*, *OsNF-YC10*, *OsNF-YC11* and *OsNF-YC12* are predominantly expressed in the endosperm.

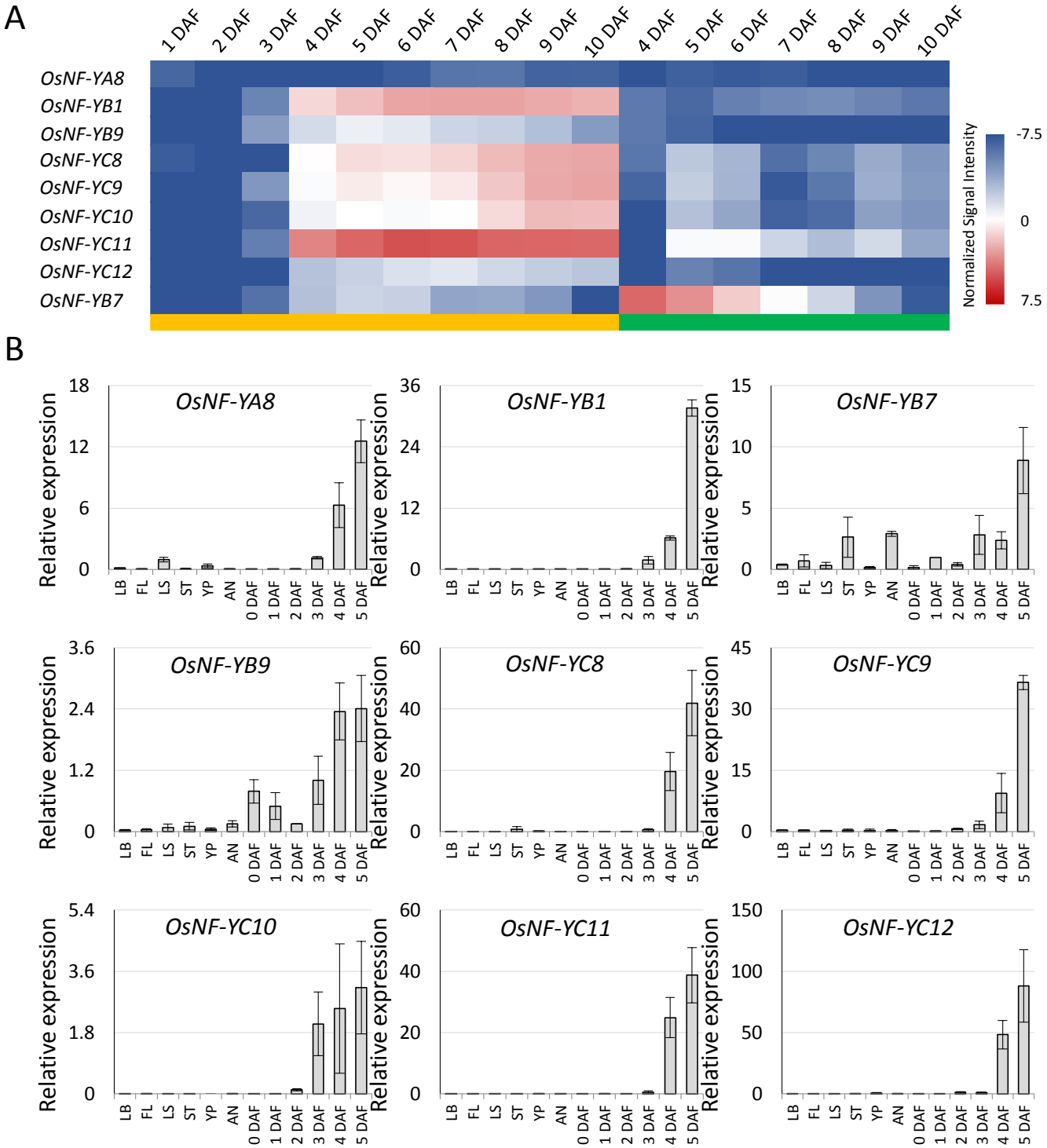


Figure 2. Activation of the seed-preferential *OsNF-Ys* after fertilization. (A) Heat map of the expression of *OsNF-YA8*, *OsNF-YB1*, *OsNF-YB7*, *OsNF-YB9*, *OsNF-YC8*, *OsNF-YC9*, *OsNF-YC10*, *OsNF-YC11* and *OsNF-YC12* at different days after fertilization (DAF). Yellow and green bars indicated ovary (including seed coat, endosperm and embryo) and embryo, respectively. **(B)** Confirmation of the seed-preferential expression pattern and gene activation after fertilization of *OsNF-YA8*, *OsNF-YB7*, *OsNF-YB1*, *OsNF-YB9*, *OsNF-YC8*, *OsNF-YC9*, *OsNF-YC10*, *OsNF-YC11* and *OsNF-YC12* by Real-time PCR. LB, FL, LS, ST, YP and AN indicate leaf blade, flag leaf, leaf sheath, stem, young panicle and anther, respectively; 0 DAF indicate unfertilized ovary; 1 to 5 DAF indicate ovaries of different ages (from 1 to 5 DAF). Three biological replicates are used for analysis; error bars indicate standard deviations.

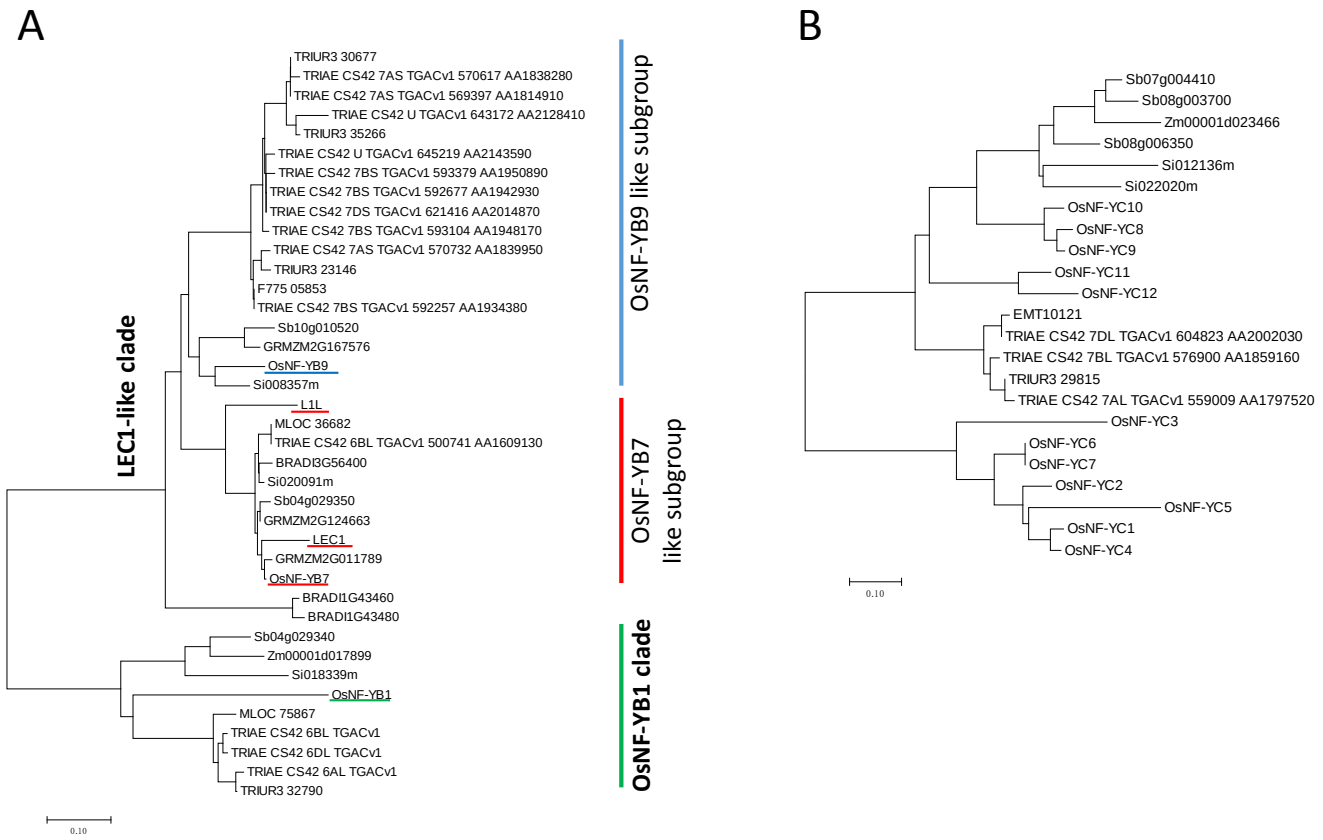


Figure 3. Neighbor-joining phylogenetic trees of the endosperm-preferential OsNF-YBs and OsNF-YC. (A) Phylogenetic tree of the OsNF-YB1, OsNF-YB7, OsNF-YB9 and their homologs. **(B)** Phylogenetic tree of the OsNF-YCs and the close homologs of OsNF-YC8 and OsNF-YC12. The locus identities initiating with TRIUR, TRIAE, F775, Sb, GRMZM(ZM), Os, Si, At, MLOC and BRADI indicate that genes are from *Triticum urartu*, *Triticum aestivum*, *Aegilops tauschii*, *Sorghum bicolor*, *Zea mays*, *Oryza sativa*, *Setaria italica*, *Arabidopsis thaliana*, *Hordeum vulgare* and *Brachypodium distachyon*, respectively. The core sequences of the conserved domains of NF-YBs and NF-YCs were used for trees construction.

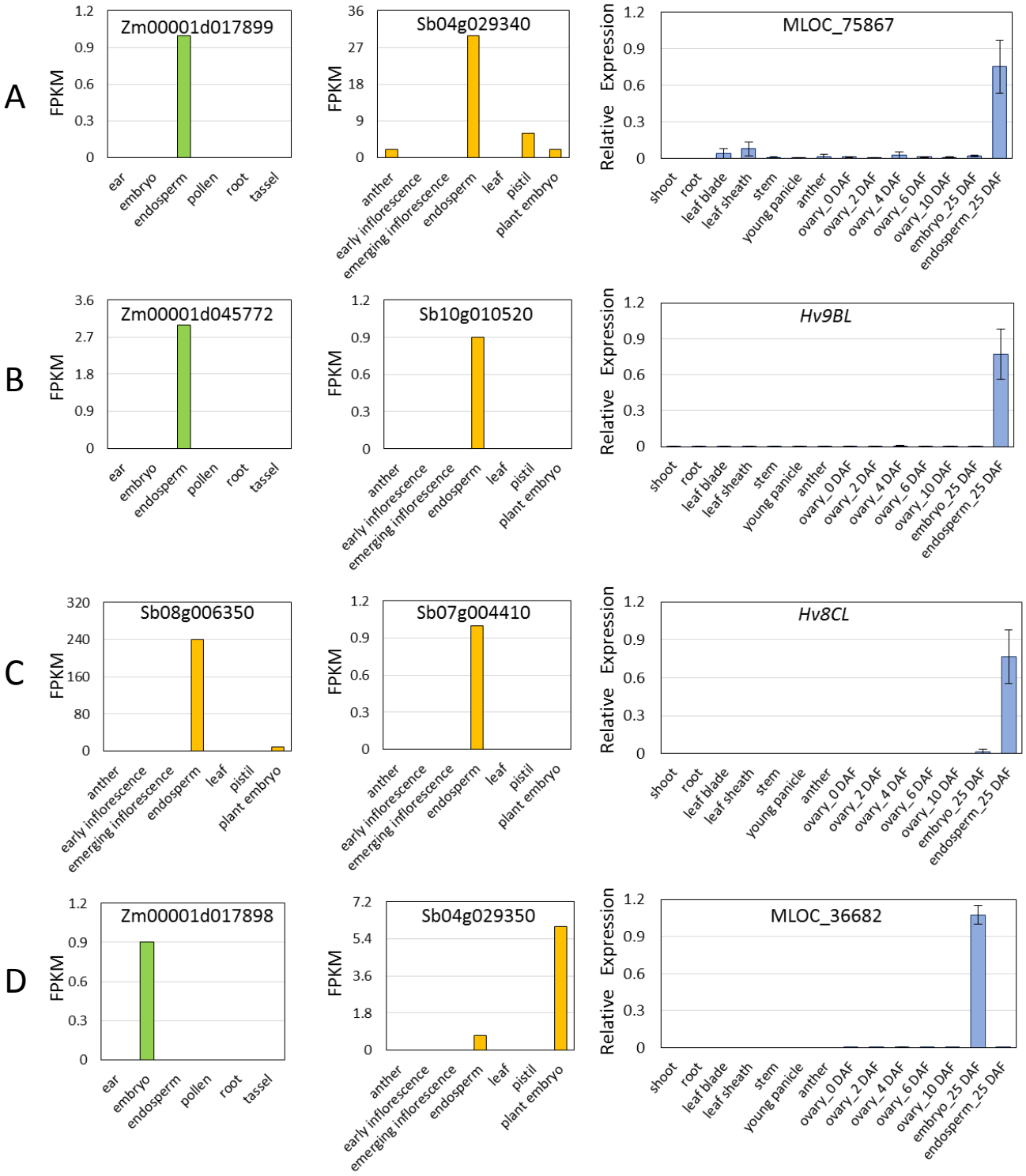


Figure 4. Gene expression of the *OsNF-YB1 Likes* (A), *OsNF-YB7 Likes* (B), *OsNF-YB9 Likes* (C) and *OsNF-YC8/12* Likes (D) in maize, sorghum and barley.

The green, yellow and blue bars indicate gene expression of the maize, sorghum and barley homologs, respectively. The expression abundance in maize and sorghum are indicated by FPKM (fragments per kilobase of exon per million fragments mapped). Data is obtained from the Expression Atlas. The relative expression in barley is detected by Real-time PCR assay. Y-axes indicate the tissues used for expression analysis. DAF, days after fertilization. Three biological replicates were used for analysis; error bars indicate standard deviations.

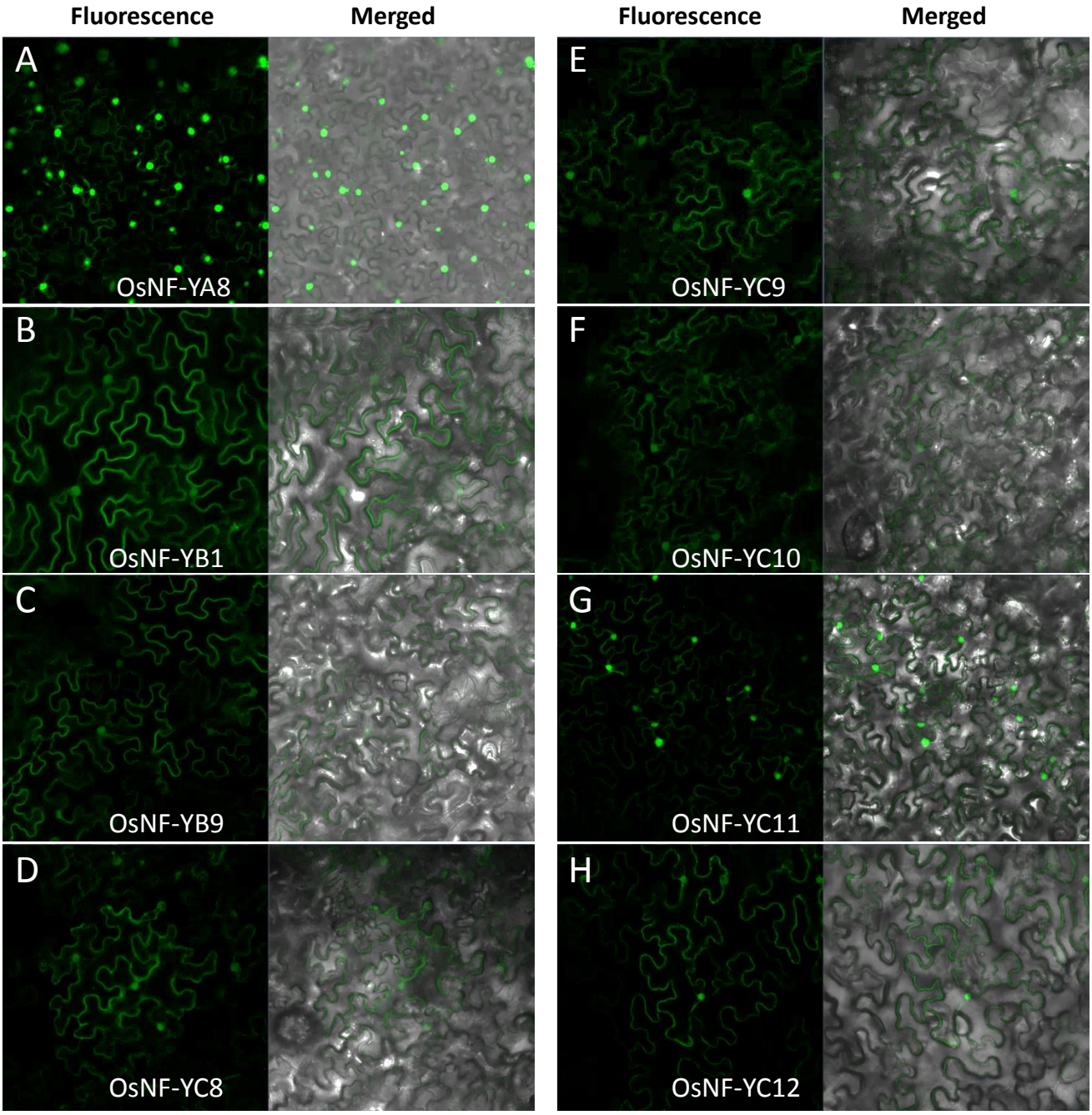


Figure 5. Subcellular localization of the endosperm-preferential NF-Ys of rice in tobacco epidermal cells.

The florescence signal of the OsNF-YA8:Venus fusion is predominantly expressed in nucleus **(A)** while these of the rest OsNF-Y:Venus fusions are expressed in cytoplasm, as well as in nucleus **(B-H)**. The NF-Ys are N-terminally fused to a venus tag.

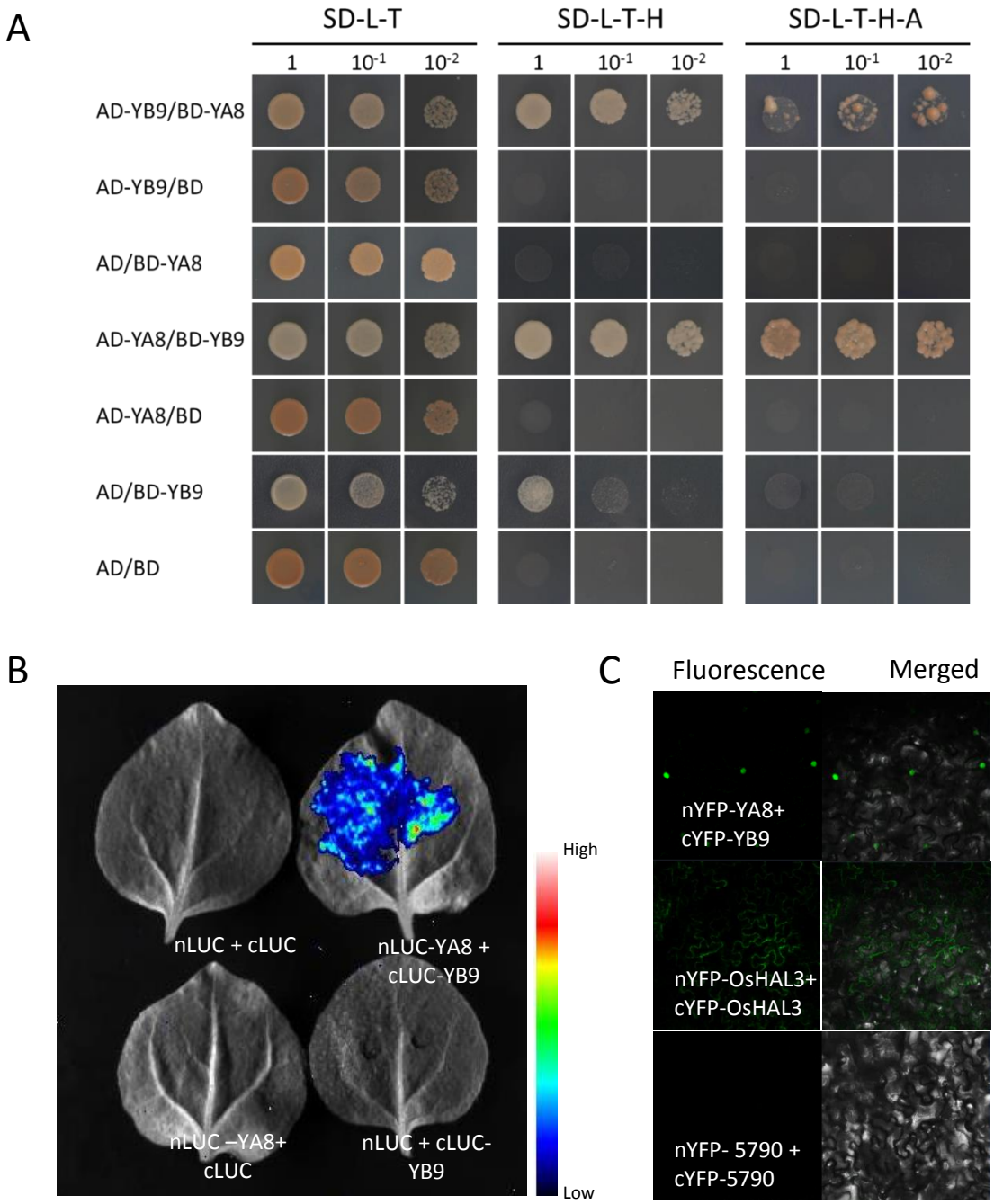


Figure 6. Interactions between the OsNF-YA8 and OsNF-YB9.

(A) Yeast-two-hybrid assay shows interaction between OsNF-YA8 and OsNF-YB9. AD and BD indicate the activation domain and the DNA binding domain of GAL4, respectively; AD-YA8, AD-YB9, BD-YA8 and BD-YB9 indicate AD:OsNF-YA8, AD:OsNF-YB9, BD:OsNF-YA8 and BD:OsNF-YB9 fusions, respectively. Serial dilutions of the yeast cells expressing the indicated proteins were plated on the non-selective medium (SD-L-T, synthetic dropout medium lacks Leu and Trp) or selective plates (SD-L-T-H and SD-L-T-H-A, synthetic dropout medium lacks Leu, Trp and His, and synthetic dropout medium lacks Leu, Trp, His and Ade). **(B)** Split luciferase complementation assay confirms the interaction between OsNF-YA8 and OsNF-YB9. nLUC, cLUC, nLUC-YA8 and cLUC-YB9 indicate the N-terminal of luciferase, the C-terminal of luciferase, the nLUC:OsNF-YA8 fusion and the cLUC:OsNF-YB9 fusion, respectively. Different constructs combinations are transiently co-expressed in the tobacco epidermal cells. **(C)** BiFC assay shows interactions between OsNF-YA8 and OsNF-YB9. OsNF-YA8 and OsNF-YB9 are fused with the N-terminal of YFP (nYFP) and the C-terminal of YFP (cYFP), respectively. OsHAL3, which can dimerize in the cytoplasm, is used as the positive control, while the gene LOC_Os01g05790 (5790), which cannot form a homo-dimer, is used as the negative control.

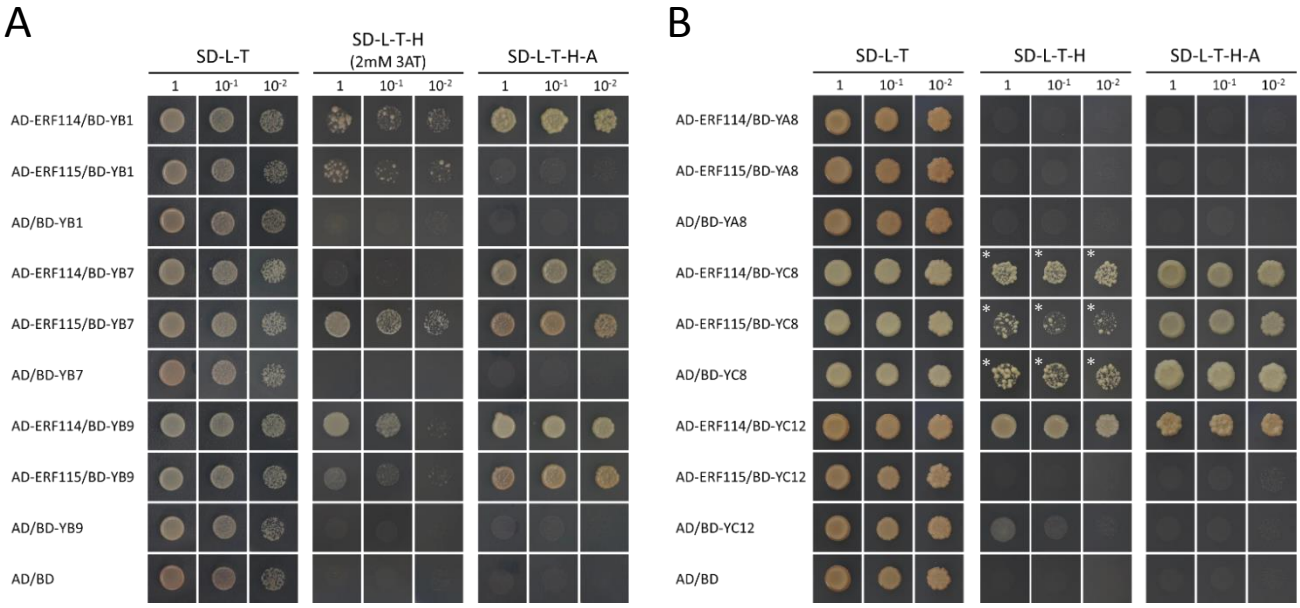


Figure 7. Interactions between the seed-preferential OsNF-Ys and ERFs.
(A) OsNF-YB1, OsNF-YB7 and OsNF-YB9 interact with OsERF114 or OsERF115. BD-YB1, BD-YB9 and BD-YB7 indicate that the OsNF-YB1, OsNF-YB9 and OsNF-YB7 are C-terminally in fusion with the DNA binding domain of GAL4 (BD), respectively. AD-ERF114 and AD-ERF115 indicate that the OsERF114 and OsERF115 are C-terminally in fused with the activation domain of GAL4 (AD), respectively. Serial dilutions of the yeast cells expressing the indicated proteins were plated on the non-selective medium (SD-L-T) or selective plates (SD-L-T-H with 2mM 3AT or SD-L-T-H-A). (B) OsNF-YC12 interacts with OsERF114 in yeast. BD-YA8, BD-YC8 and BD-YC12 indicate that the OsNF-YA8, OsNF-YC8 and OsNF-YC12 are C-terminally in fusion with the BD, respectively. The yeast grew on the selective triple dropout medium with 5mM 3AT are indicated by stars. High self-activation activity of OsNF-YC8 was observed; the yeast cells expressing the AD and the BD:OsNF-YC12 fusion was survivable on the selective medium.

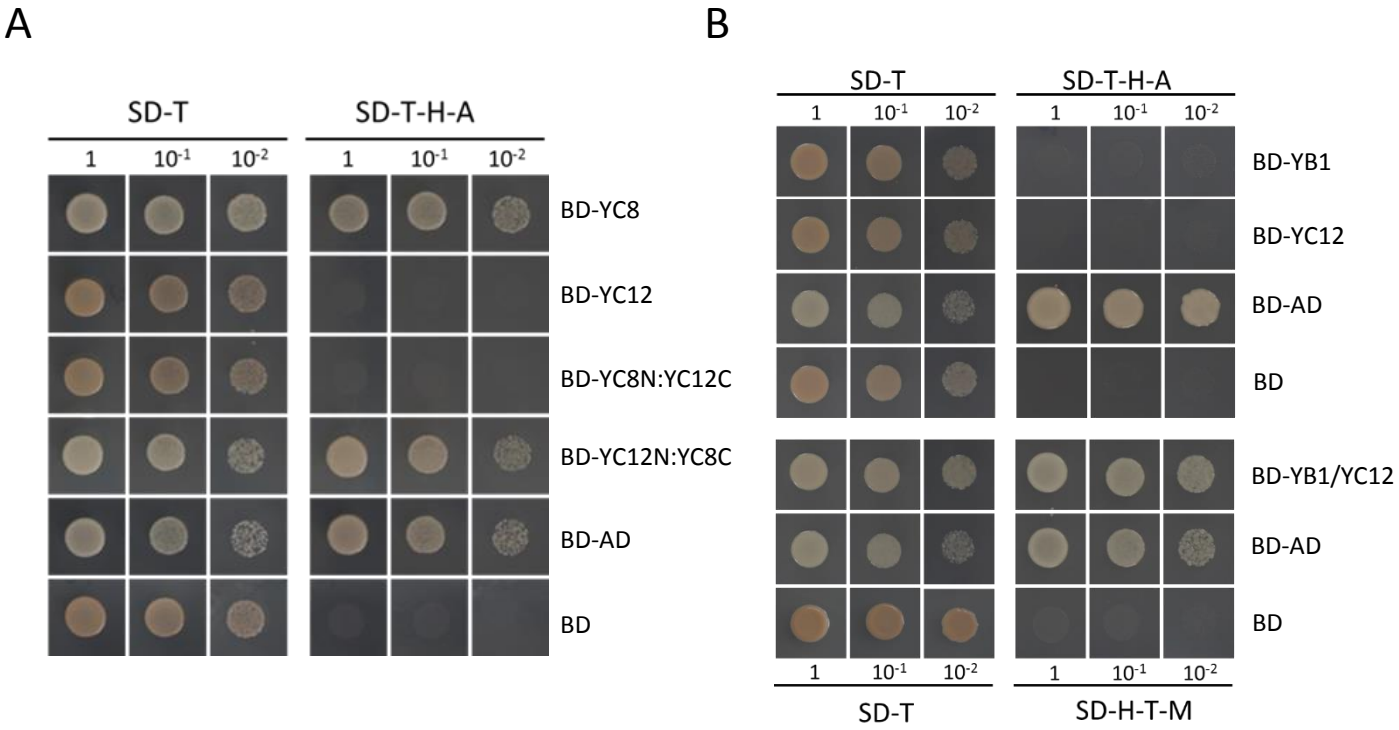


Figure 8. Transcriptional activation activities of the endosperm-preferential OsNF-Ys.
(A) C-terminal of OsNF-YC8 is important for the transcriptional activation activity. BD and AD indicate the DNA binding domain and activation domain of GAL4, respectively. The serially diluted yeast cells expressing BD:OsNF-YC8 fusions (BD-YC8) and BD:OsNF-YC12 fusions (BD-YC12) could grow on the nonselective medium (SD-T), but only the transformants expressing BD-YC8 survived on the selective medium (SD-T-H-A), like what the BD:AD (a positive control) expressing yeast cells performed. When using the C-terminal of the OsNF-YC12 (amino acid residuals 361-984) to replace the C-terminal of OsNF-YC8 (385-1392), the fusions (BD-YC8N:YC12C) lose transcriptional activation activity. In contrast, the OsNF-YC12 N-terminal (1-360) and OsNF-YC8 C-terminal (385-1392) fusion (BD-YC12N:YC8C) showed transcriptional activation activity. **(B)** The OsNF-YB1 and OsNF-YC12 dimer shows transcriptional activation activity in yeast. The BD:OsNF-YB1 and BD:OsNF-YC12 do not show transcriptional activation activity; but the yeast cells simultaneously expressing of BD:OsNF-YB1 and OsNF-YC12 (BD-YB1/YC12) could survive on the selective medium (SD-H-T-M).

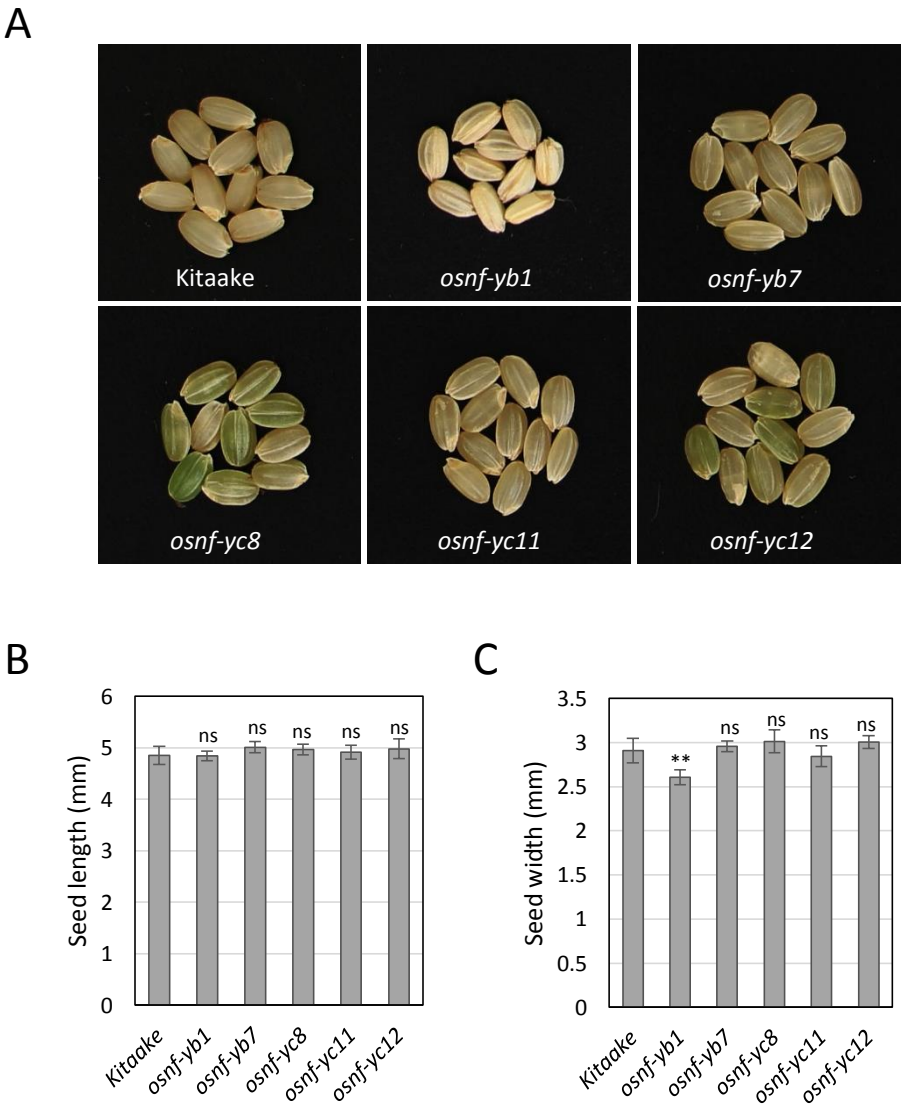
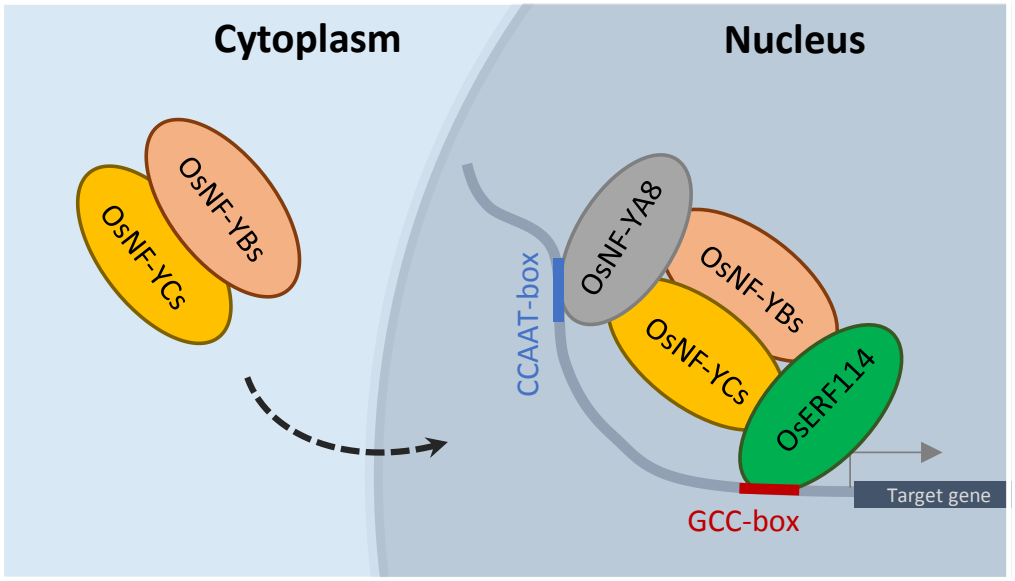


Figure 9. Seed morphologies of the seed-preferential *OsNF-Ys* mutants generated by CRISPR/Cas9 approach.

(A) Seed morphology of the *osnf-yb1*, *osnf-yb7*, *osnf-yc8*, *osnf-yc11*, *osnf-yc12* and the wild type (Kitaake). **(B-C)** Seed length **(B)** and seed width **(C)** of the *osnf-yb1*, *osnf-yb7*, *osnf-yc8*, *osnf-yc11*, *osnf-yc12* and the wild type (Kitaake). More than 30 well-filled seeds from the main stem were chose for the measurements; error bars indicate standard deviations. **, $p<0.01$; ns, no significance; t-test was used for the statistical analysis.



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