NF-YB2 and NF-YB3 Have Functionally Diverged and Differentially Induce Drought and Heat Stress-Specific Genes[1][OPEN]

Hikaru Sato,[a] Takamasa Suzuki,[b] Fuminori Takahashi,[a] Kazuo Shinozaki,[a] and Kazuko Yamaguchi-Shinozaki[c,2,3]

[a]Gene Discovery Research Group, RIKEN Center for Sustainable Resource Science (CSRS), Tsukuba, Ibaraki 305-0074, Japan

[b]College of Bioscience and Biotechnology, Chubu University, Kasugai, Aichi 487-8501, Japan

[c]Laboratory of Plant Molecular Physiology, Graduate School of Agricultural and Life Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

ORCID IDs: 0000-0001-7628-0414 (H.S.); 0000-0001-8817-8652 (F.T.); 0000-0002-6317-9867 (K.S.); 0000-0002-0249-8258 (K.Y.-S.).

Functional diversification of transcription factors allows the precise regulation of transcriptomic changes under different environmental conditions. The NUCLEAR FACTOR Y (NF-Y) transcription factor comprises three subunits, NF-YA, NF-YB, and NF-YC, and is broadly diversified in plant species, whereas Humans (*Homo sapiens*) have one protein for each subunit. However, there remains much to be learned about the diversified functions of each subunit in plants. Here, we found that NF-YB2 and NF-YB3, which have the greatest sequence similarity to each other among NF-YB family proteins in Arabidopsis (*Arabidopsis thaliana*), are functionally diversified and specifically activate dehydration-inducible and heat-inducible genes, according to environmental conditions. Overexpression of *NF-YB2* and *NF-YB3* specifically enhanced drought and heat stress tolerance, respectively, and each single knockout mutant showed adverse stress-sensitive phenotypes. Transcriptomic analyses confirmed that overexpression of *NF-YB2* and *NF-YB3* largely affected the transcriptomic changes under dehydration and heat stress conditions, respectively. The DNA-binding profiles of each protein in planta also suggested that dehydration and heat stress increased the DNA-binding activity of NF-YB2 and NF-YB3 to dehydration-inducible and heat stress-inducible target genes, respectively. Moreover, phylogenetic analysis suggested that the NF-YB proteins of angiosperm plants belong to divergent NF-YB2 and NF-YB3 subgroups. These results demonstrate the functional diversification of NF-Y through evolutionary processes and how plants adapt to various abiotic stresses under fluctuating environments.

Abiotic stresses such as drought, high and low temperature, and high salinity are important factors that affect plant growth and reproduction (Mickelbart et al., 2015). Recent extreme weather events have damaged global food production and security (Lesk et al., 2016; Schauberger et al., 2017). Plants have developed various types of molecular strategies through evolution

that are specifically induced according to environmental conditions. Transcriptomic analyses of plants treated with different abiotic stresses have revealed various stress-specific and common gene regulatory mechanisms (Rasmussen et al., 2013; Maruyama et al., 2017). For example, LATE EMBRYOGENESIS ABUNDANT (LEA) proteins and several sugar-biosynthetic enzymes are typical dehydration stress-inducible proteins that function as osmoprotectants and inducers of osmolytes, respectively (Hincha and Thalhammer, 2012; Keunen et al., 2013). Heat stress activates the expression of genes encoding the molecular chaperones HEAT SHOCK PROTEINs (HSPs; Jacob et al., 2017). These stress-specific transcriptomic changes are regulated by various transcription factors that are activated in response to specific abiotic stress (Song et al., 2016; Ohama et al., 2017).

Nuclear factor Y (NF-Y) is a transcription factor that is widely conserved among eukaryotes (Kumar et al., 2012; Li et al., 2016). A NF-Y trimer is composed of the NF-YA, NF-YB, and NF-YC subunits, and the trimer is known to bind to a CCAAT element on its target gene promoters to regulate their transcription (Myers and Holt, 2018). Humans (*Homo sapiens*) only have one

family protein for each NF-Y subunit. However, subunits of NF-Y are broadly diversified in plant species; Arabidopsis (*Arabidopsis thaliana*) has 10 NF-YAs, 10 NF-YBs, and 10 NF-YCs (Petroni et al., 2012). The NF-YB and NF-YC proteins contain conserved H2B and H2A domains (which are related to histone H2B and H2A proteins), respectively, and these domains are necessary to form a dimer of NF-YB and NF-YC before trimerization. DNA POLYMERASE II SUBUNIT B3 (DPB3) and NEGATIVE COFACTOR 2α family proteins also have the H2A domain, and DPB4 and NEGATIVE COFACTOR 2β family proteins have the H2B domain (Petroni et al., 2012). These proteins are also suggested to be involved in transcriptional regulation (Kim et al., 2011; Yoshida et al., 2011), and previous studies suggested that these H2A-containing and H2B-containing proteins can interact with each other across protein families (Kim et al., 2011). Moreover, recent studies have suggested that dimers of NF-YB and NF-YC can interact with not only NF-YAs but also with other proteins containing a CO, CO-LIKE, and TOC1 domain and that DNA binding preference of the trimer is changed by the CO, CO-LIKE, and TOC1-containing subunit (Yoshida et al., 2011). Diversification of plant NF-Ys and their interacting proteins raises the possibility that different combinations of trimers have different molecular functions, and many studies have actually revealed the wide variations in NF-Y functions in plants, including flowering induction (Fujita et al., 2009; Yoshida et al., 2015), seed maturation (Nambara et al., 1998; Iuchi et al., 2001), root nodule formation (Zanetti et al., 2010), and responses to abiotic stresses such as drought, heat, and cold (Yoshida et al., 2011). However, detailed insight into the functional diversification of each NF-Y subunit is still limited.

We previously identified that Arabidopsis DPB3-1, also annotated as NF-YC10, interacted with the transcription factor DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN2A (DREB2A), and we suggested that DPB3-1 forms a trimer with NF-YA2 and NF-YB3 to regulate heat-stress–responsive genes together with DREB2A (Sato et al., 2014). DREB2A is a transcription factor that regulates both dehydration-inducible and heat-stress–inducible genes according to environmental conditions (Qin et al., 2008; Morimoto et al., 2017). The DREB2A protein is stabilized under these abiotic stress conditions (Morimoto et al., 2013; Mizoi et al., 2019), whereas it is easily degraded under nonstress conditions through a 26S proteasome pathway (Sakuma et al., 2006a). A DREB2A protein lacking its negative regulatory domain was constitutively stable even under nonstress conditions (DREB2A CA; DREB2A constitutively active form; Sakuma et al., 2006a, 2006b). *DREB2A CA*-overexpressing plants show enhanced expression of both dehydration-inducible and heat-inducible DREB2A target genes under nonstress conditions and drought-tolerant and heat stress-tolerant phenotypes (Sakuma et al., 2006a, 2006b). Our previous findings provided insights into the molecular functions of NF-YA2, NF-YB3, and DPB3-1 to control target selectivity

by forming specific transcription complexes under specific environmental conditions (Sato et al., 2014). Meanwhile, we found that a trimer composed of NF-YA2, NF-YB2, and DPB3-1 also activated target promoters together with DREB2A in mesophyll protoplasts, but interestingly, expression patterns of *NF-YB2* and *NF-YB3* in plants the opposite of each other; *NF-YB3* was induced by heat stress and suppressed by dehydration stress, and *NF-YB2* was induced by dehydration stress and suppressed by heat stress (Sato et al., 2014). We therefore concluded that NF-YB3 is a candidate to form a transcriptional complex with NF-YA2, DPB3-1, and DREB2A during heat stress, although direct interaction between NF-YB3 and DREB2A was not detected in yeast cells (Sato et al., 2014). However, these results also suggested the functional diversification of NF-YB2 and NF-YB3, which have the greatest sequence similarity to each other among NF-YB family proteins (70% sequence identity). NF-YB2 and NF-YB3 are reported to have a redundant function: to induce flowering through activation of *FLOWERING LOCUS T* together with CONSTANS (CO; Siefers et al., 2009; Calvenzani et al., 2012; Sato et al., 2014). However, the functional diversification of NF-YB2 and NF-YB3 during abiotic stress is not well understood.

In this study, we revealed the functional diversification of NF-YB2 and NF-YB3 during dehydration and heat stress. Overexpressing plants and knockout mutants of *NF-YB2* and *NF-YB3* showed dehydration-specific and heat stress-specific phenotypes and gene expression patterns, respectively. Moreover, phylogenetic analysis revealed that proteins in divergent NF-YB2 and NF-YB3 subgroups are conserved among angiosperm plants but not bryophytes and lycophytes. These results provide important insight into the functional diversification of NF-Y proteins through evolution and provide a mechanistic understanding of the target selectivity of DREB2A under dehydration and heat stress conditions.

RESULTS

NF-YB2 and NF-YB3 Gene Expression Patterns Correlate with DREB2A under Dehydration and Heat Stress Conditions

We previously reported that NF-YA2, NF-YB3, and DPB3-1 (NF-YC10) form a trimer and cooperatively function with DREB2A to activate their target genes specifically under heat stress conditions, and we suggested that NF-YB2, which has a highly similar sequence to NF-YB3 (70% sequence identity), has different functions than NF-YB3 under dehydration and heat stress conditions (Sato et al., 2014). First, gene expression patterns of *NF-YB2* and *NF-YB3* were examined. Induction of the *NF-YB2* gene during dehydration stress was suppressed in the abscisic acid (ABA)-signaling mutants; triple mutants of the *SNF1-RELATED PROTEIN KINASE2D* (*SRK2D*), *SRKD2E*, and *SRK2I* (*srk2d/e/i*; Fujita

et al., 2009); and quadruple mutants of the *ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING PROTEIN1* (*AREB1*), *AREB2*, *ABSCISIC ACID RESPONSIVE ELEMENT-BINDING FACTOR1* (*ABF1*), and *ABF3* (*arebq*; Yoshida et al., 2010; Fig. 1A). The ABA-deficient mutants; knockout mutants of *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE3* (*nc3-2*) and *ABA DEFICIENT2* (*ABA2*; *aba2-2*; Nambara et al., 1998; Iuchi et al., 2001) also showed decreased expression of *NF-YB2* during dehydration stress (Fig. 1B). *NF-YB2* induction was not completely abolished in these mutants (Fig. 1, A and B), suggesting that the *NF-YB2* gene was induced through both ABA-dependent and ABA-independent mechanisms during dehydration stress as well as in a *DREB2A*-dependent manner (Kim et al., 2011). We also analyzed the expression levels of *NF-YB2* in these mutants treated with ABA. The expression level of *NF-YB2* was up-regulated by ABA treatment and was suppressed in the *srk2d/e/i* and *arebq* mutants (Supplemental Fig. S1A), confirming that the ABA-dependent pathway contributes to a part of

NF-YB2 gene induction. In the ABA-deficient mutants, the suppression of *NF-YB2* gene induction during ABA treatment was not observed (Supplemental Fig. S1B). These mutations had almost no effect on *NF-YB3* expression during dehydration and ABA treatment (Fig. 1, A and B; Supplemental Fig. S1, A and B). *DREB2A* is also induced by HEAT SHOCK FACTOR A1s (HSFA1s) in response to heat stress (Yoshida et al., 2011). The *hsfa1a/b/d* mutants (*hsfa1a hsfa1b hsfa1d* triple mutant) showed decreased expression levels of *NF-YB3* under heat stress conditions (Fig. 1C). This result showed that the expression of *NF-YB3* in response to heat stress was HSFA1 dependent. *NF-YB2* expression was not affected by the mutation of *HSFA1*s (Fig. 1C). AREB/ABFs and HSFA1s are known to bind to the ABA responsive element (ABRE) and heat shock element (HSE) on their target promoters, respectively (Kim et al., 2011; Yoshida et al., 2011). *NF-YB2* and *NF-YB3* also have ABRE and HSE sequences on their promoters, respectively (Supplemental Table S1), and expression of the *NF-YB2* and *NF-YB3* genes seems to
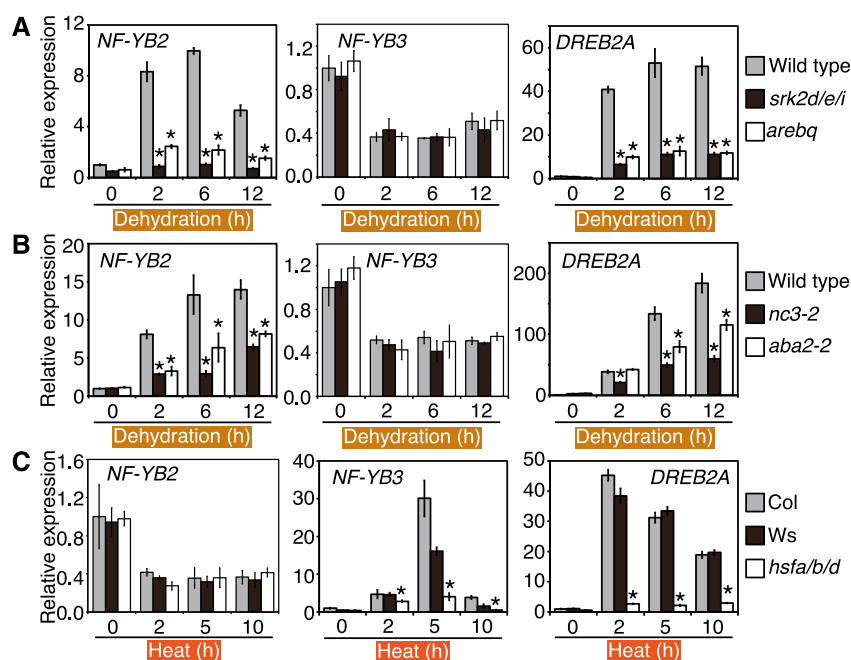

Figure 1. Expression patterns of *NF-YB2* and *NF-YB3* in ABA signaling, ABA-deficient, and *HSFA1* knockout mutants (*hsfa1a/b/d*) during dehydration and heat stress. A and B, Expression levels of *NF-YB2*, *NF-YB3*, and *DREB2A* during dehydration stress in the ABA-signaling mutants (A) and ABA-deficit mutants (B). The expression of *NF-YB2*, *NF-YB3*, and *DREB2A* genes was analyzed in the *SRK2D* triple mutants (*srk2d/e/i*) and *ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING PROTEIN/FACTOR*s quadruple mutants (*arebq*; A) and *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE3* (*NCED3*; *nc3-2*) and *ABA DEFICIENT2* (*ABA2*; *aba2-2*) knockout mutants (B) by reverse transcription quantitative PCR (RT-qPCR). The 14-d-old seedlings grown on agar plates were treated with dehydration stress. The expression levels of *NF-YB2*, *NF-YB3*, and *DREB2A* in wild-type plants before stress treatment (0 h) were defined as 1.0. The error bars indicate SD (*n* = three experiments, involving 7 individual plants for each line). The asterisk indicates the time point at which the expression levels of each gene were significantly decreased compared with those in the wild-type plants (*P* < 0.05, Bonferroni-corrected Student's *t* test). C, The expression levels of *NF-YB2*, *NF-YB3*, and *DREB2A* during heat stress in the *hsfa1a/b/d* mutants. The 14-d-old seedlings grown on agar plates were treated with heat stress (37°C). The expression levels of *NF-YB2*, *NF-YB3*, and *DREB2A* in the wild-type plants before stress treatment (0 h) were defined as 1.0. The error bars indicate SD (*n* = three experiments, involving 7 individual plants for each line). The asterisk indicates the time point at which the expression levels of each gene were significantly decreased compared with that of both wild-type plants (Col and Ws; *P* < 0.05, Bonferroni-corrected Student's *t* test).

be directly activated by AREB/ABFs and HSFA1s through these cis-elements, respectively. We also analyzed the conservation of these elements in the promoters of Arabidopsis *NF-YB2* and *NF-YB3* putatively orthologous genes across species and found that a DRE (GCCGAC) and an ABRE (ACGTGG) in the Arabidopsis *NF-YB2* promoter and an HSE-like (GAAnTTC) and an ABRE (CCACGT) in the Arabidopsis *NF-YB3* promoter are conserved among putatively orthologous promoters of *Brassica* sp. (Supplemental Fig. S2, A and B).

Overexpression of *NF-YB2* and *NF-YB3* Enhanced Drought and Heat Stress Tolerance, Respectively

Next, *NF-YB2*-overexpressing and *NF-YB3*-overexpressing plants were generated to analyze their functions (*35S:NF-YB2*-a, b and c, and *35S:NF-YB3*-a, b and c; Fig. 2A). These overexpressing plants showed early-flowering phenotypes (Supplemental Fig. S3, A and B) under long-day conditions (16-h photoperiod), as previously reported (Kumimoto et al., 2008). We therefore performed a drought stress tolerance test under short-day conditions (8-h photoperiod) to keep all the plant lines at the same developmental stages. After the drought stress treatment, the *NF-YB2*-overexpressing plants showed significantly higher survival rates, whereas the *NF-YB3*-overexpressing plants did not (Fig. 2, B and C). The overexpression of *NF-YB2* did not affect the heat stress tolerance (Fig. 2, D and E), whereas overexpression of *NF-YB3* significantly enhanced the heat stress tolerance (Fig. 2, F and G). Additionally, we analyzed water loss of detached leaves using overexpressors of *NF-YB2*, *NF-YB3*, and *DREB2A CA*. The results showed that overexpression of *NF-YB2*

for each line). B, Drought stress tolerance of the *NF-YB2*-overexpressing and *NF-YB3*-overexpressing plants. Plants grown on agar plates for 12 d were transferred to soil and grown for 2 d. Water was withheld for 24 d. Scale bars = 1 cm. Photographs were taken before drought (top row), after drought (middle row), and 7 d after rewatering (bottom row). C, Percentages of surviving plants after rewatering. The error bars indicate the SD from five experiments, each involving 7 individual plants for each line. The letters above the bars indicate significant differences among plant lines before and after drought stress treatment ($P < 0.05$, according to Tukey's multiple range test). D and F, Heat stress tolerance of the *NF-YB2*-overexpressing (D) and *NF-YB3*-overexpressing (F) plants. The 7-d-old seedlings were treated with or without 45°C for 54 min. Scale bars = 1 cm. The photographs were taken after a 7-d recovery period at 22°C with (right) or without (left) heat stress. E and G, Percentages of surviving plants after heat stress in the *NF-YB2*-overexpressing (E) and *NF-YB3*-overexpressing (G) plants. The error bars indicate the SD from three experiments, each involving 28 individual plants for each line. The survival rates of the *NF-YB2*-overexpressing plants after heat stress were evaluated using one-way ANOVA, but no significant differences were detected ($P > 0.05$). The letters above the bars in G indicate significant differences among plant lines with or without heat stress treatment ($P < 0.05$, according to Tukey's multiple range test).
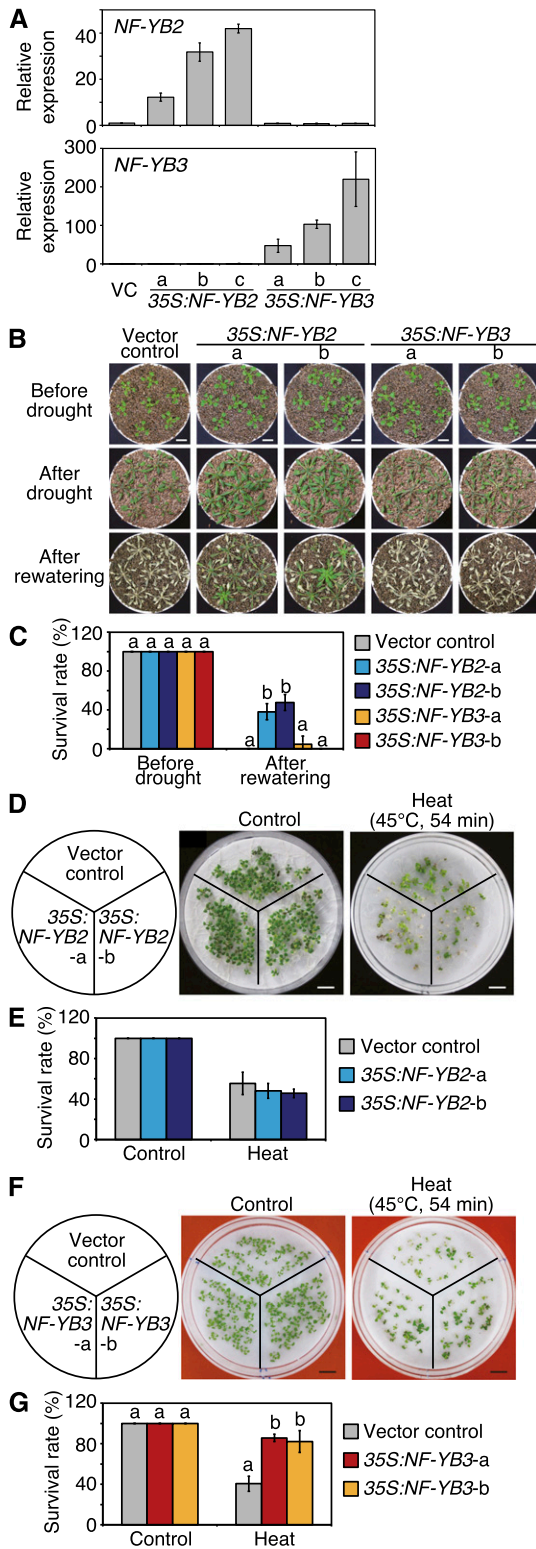
Figure 2. Drought and heat stress tolerance test in the *NF-YB2*-overexpressing and *NF-YB3*-overexpressing plants. A, Expression analysis of *NF-YB2* and *NF-YB3* in the *NF-YB2*-overexpressing and *NF-YB3*-overexpressing plants, respectively, by RT-qPCR. The expression levels of *NF-YB2* or *NF-YB3* in the VC plants were defined as 1.0. The error bars indicate SD (*n* = three experiments, involving 7 individual plants

and *DREB2A CA* suppressed water loss of leaves (Supplemental Fig. S3C). These results suggest that NF-YB2 and NF-YB3 are functionally differentiated and are positive regulators of drought and heat stress responses, respectively, despite the high protein sequence similarity.

Expression of DREB2A Target Genes was Enhanced in *NF-YB2*-Overexpressing and *NF-YB3*-Overexpressing Plants during Dehydration and Heat Stress, Respectively

The expression levels of DREB2A target genes during dehydration and heat stress were analyzed in the *NF-YB2*-overexpressing and *NF-YB3*-overexpressing plants. Under dehydration stress conditions, dehydration stress-inducible DREB2A target genes *LEA7* and *RESPONSIVE TO DESICCATION29B* (*RD29B*) were significantly up-regulated in *NF-YB2*-overexpressing plants, whereas the expression levels of *DREB2A* and *NF-YB3* were not altered in these plants (Fig. 3A). We also found that the expression of these dehydration-inducible DREB2A target genes was suppressed in *NF-YB3*-overexpressing plants, while *DREB2A* and *NF-YB2* expression levels were not altered (Fig. 3B). Under heat stress conditions, heat stress-inducible

DREB2A target genes *HSFA3* and *At1g75860* were significantly up-regulated in *NF-YB3*-overexpressing plants, whereas no differences were observed in the expression of *DREB2A* and *NF-YB2* (Fig. 3B). Overexpression of *NF-YB2* did not affect the expression of these heat stress-inducible genes under heat stress conditions (Fig. 3A). Accumulation of the DREB2A protein was also not changed by the overexpression of *NF-YB2* and *NF-YB3* under dehydration and heat stress conditions (Supplemental Fig. S4, A and B). These results suggest that NF-YB2 and NF-YB3 are functionally diversified in response to dehydration and heat stress; that they cooperatively activate their target genes with DREB2A specifically under dehydration and heat stress conditions, respectively; and that NF-YB3 has negative effects on the expression of dehydration stress-inducible genes under dehydration stress conditions. To analyze the genetic interactions between the NF-YBs and DREB2A, we overexpressed *NF-YB2* or *NF-YB3* in the *dreb2a-1* knockout mutants (Supplemental Fig. S4, C–F). As previously reported, gene induction of DREB2A-taget genes was suppressed during dehydration (*LEA7* and *RD29B*) or heat stress (*HSFA3* and *At1g75860*; Supplemental Fig. S4, G and H). The expression levels of the target genes were not affected by overexpression of *NF-YB2* and *NF-YB3* during
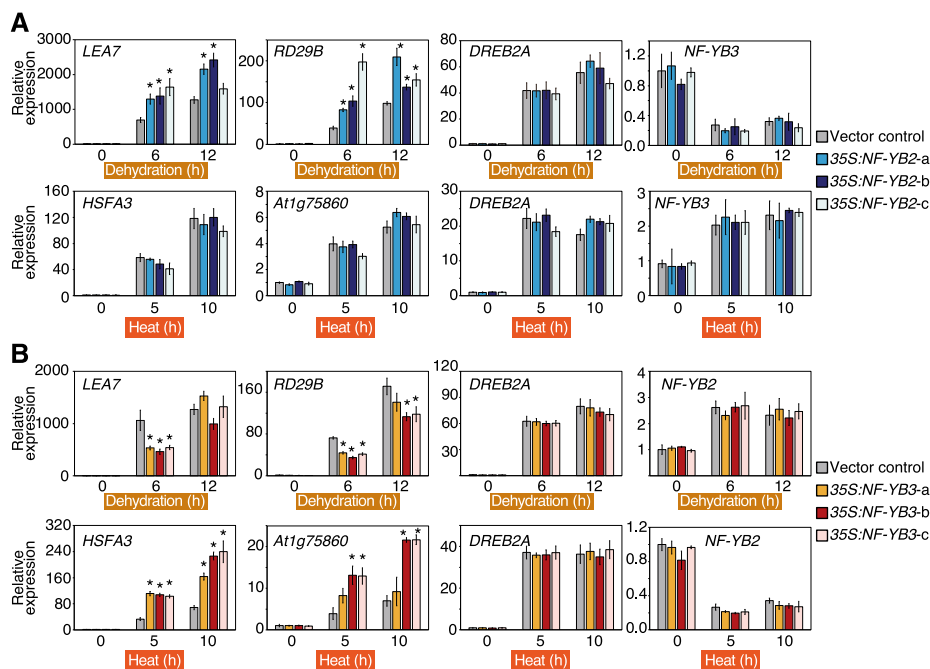

Figure 3. Expression patterns of dehydration-inducible and heat-inducible DREB2A target genes during dehydration and heat stress. A and B, The expression levels of dehydration-inducible genes (*LEA7*, *RD29B*), heat-inducible genes (*HSFA3* and *At1g75860*), *DREB2A*, *NF-YB2*, and *NF-YB3* during dehydration and heat stress in the *NF-YB2*–overexpressing (A) and *NF-YB3*–overexpressing (B) plants. The expression levels were analyzed by RT-qPCR. The 12-d-old plants grown on agar plates were treated with dehydration or heat stress. The expression levels of each gene in the VC plants under the control condition (0 h) were defined as 1.0. The error bars indicate SD (*n* = three experiments, involving 7 individual plants for each line). Asterisks indicate significant differences from the VC plants at each time point (*P* < 0.05, Bonferroni-corrected Student's *t* test), and the expression levels of each gene at other time points during dehydration and heat stress were evaluated using one-way ANOVA, but no significant differences were detected (*P* > 0.05).

dehydration and heat stress, respectively, in the *dreb2a-1* mutants (Supplemental Fig. S4, G and H). These results suggested that NF-YB2 and NF-YB3 function to enhance the expression levels of these target genes in a DREB2A-dependent manner.

Transcriptomic Analysis Confirmed Stress-Specific Functions of NF-YB2 and NF-YB3

Transcriptomic analysis was performed in the *NF-YB2*-overexpressing and *NF-YB3*-overexpressing plants (*35S:NF-YB2*-b and *35S:NF-YB3*-b) relative to the vector control (VC) plants under 6-h dehydration stress, 5-h heat stress, and nonstress conditions to reveal the stress-specific functions of NF-YB2 and NF-YB3 by RNA sequencing (Supplemental Data Sets S1–S6). We detected 2427 and 1582 genes up-regulated by dehydration and heat stress, respectively, reflecting a more than 3-fold increase in response to dehydration and heat stress in the VC plants (VC [control] vs VC [dry], and VC [control] vs VC [heat]; $P < 0.05$; Fig. 4, A and B). Previous studies revealed up-regulated genes by overexpression of *DREB2A CA* (Sakuma et al., 2006b; Mizoi et al., 2013). Under dehydration stress, overexpression of *NF-YB2* and *NF-YB3* resulted in up-regulation of 294 and 131 genes, respectively, more than 1.8-fold of the levels in the VC plants (VC [dry] vs NF-YB2ox [dry], and VC [dry] vs NF-YB3ox [dry]; $P < 0.05$; Fig. 4A). The proportion of dehydration-stress–inducible genes specifically up-regulated in the *NF-YB2*–overexpressing plants (88 genes; 30%) among the 294 genes up-regulated by NF-YB2 was significantly higher than that specifically up-regulated in the *NF-YB3*-overexpressing plants (14 gene; 11%) among the 131 genes up-regulated by NF-YB3 ($P < 0.0001$, Fisher's exact test). Meanwhile, under heat stress, 195 and 262 up-regulated genes were detected in the *NF-YB2*-overexpressing and *NF-YB3*-overexpressing plants, respectively, reflecting more than 1.8-fold greater levels than those in the VC plants (VC [heat] vs NF-YB2ox [heat], and VC [heat] vs NF-YB3ox [heat]; $P < 0.05$;

during heat stress. The sums of numbers in black squares represent the NF-YB2 (14+1) –specific or NF-YB3 (48+14) –specific up-regulated genes with heat inducibility. The total numbers of each set of genes are presented in parentheses. C and D, The expression analysis of several dehydration-inducible or heat-inducible genes during dehydration or heat stress in the *NF-YB2*–overexpressing (C) and *NF-YB3*–overexpressing (D) plants. The expression levels of candidate target genes of DREB2A with dehydration-inducibility or heat-inducibility that were specifically up-regulated in the *NF-YB2*–overexpressing {*LEA76* and *CYSTM1*) or *NF-YB3*–overexpressing (*HSP70B* and *At1g53540*) plants were analyzed by RT-qPCR. The expression levels of each gene in the VC plants were under the control condition (0 h) defined as 1.0. The error bars indicate SD (*n* = three experiments, involving 7 individual plants for each line). Asterisks indicate significant differences from the VC plants at each time point (*P* < 0.05, Bonferroni-corrected Student's *t* test).
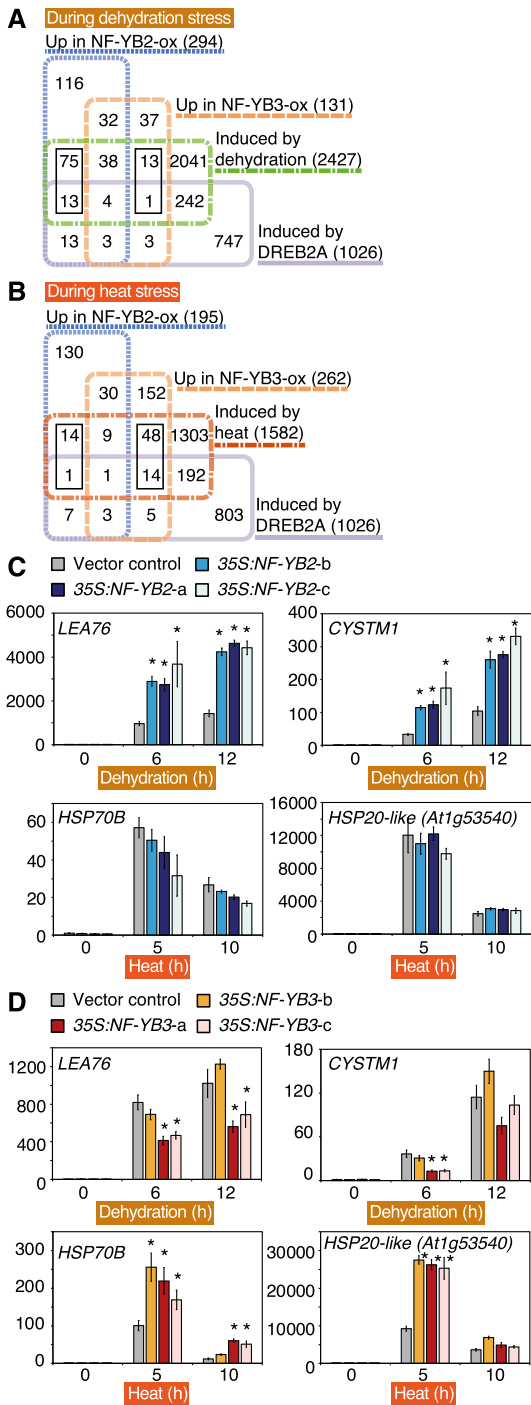
Figure 4. Transcriptomic analysis of *NF-YB2*-overexpressing and *NF-YB3*-overexpressing plants during dehydration and heat stress. A, Venn diagram comparing the dehydration-inducible genes, DREB2A CA (DREB2A constitutively active form)-inducing genes, and the up-regulated genes in the *NF-YB2*–overexpressing and *NF-YB3*–overexpressing plants during dehydration stress. The sums of numbers in black squares represent the NF-YB2 (75+13) –specific or NF-YB3 (13+1) –specific up-regulated genes with dehydration inducibility. The total numbers of each set of genes are presented in parentheses. B, Venn diagram comparing the heat-inducible genes, DREB2A CA-inducing genes, and the up-regulated genes in the *NF-YB2*–overexpressing and *NF-YB3*–overexpressing plants

Fig. 4B). The proportion of heat-stress–inducible genes specifically up-regulated in the *NF-YB3*-overexpressing plants (62 genes; 24%) among the 262 genes up-regulated by NF-YB3 was significantly higher than that specifically up-regulated in the *NF-YB2*-overexpressing plants (15 gene; 8%) among the 195 genes up-regulated by NF-YB2 ($P < 0.0001$, Fisher's exact test). These results suggest that NF-YB2 and NF-YB3 have specific functions under dehydration and heat stress, respectively, and that overexpression of these genes has greater effects on the transcriptomic changes under each stress. Moreover, the proportion of DREB2A CA-inducible genes among the 294 genes up-regulated in the *NF-YB2*-overexpressing plants during dehydration stress (33 genes; 11%) was significantly higher than that among Arabidopsis whole genes (1026 genes; 4%; $P < 0.0001$; Fisher's exact test), whereas that among the 131 genes up-regulated in the *NF-YB3*-overexpressing plants during dehydration stress (11 genes; 8%) was not significantly higher ($P > 0.01$, Fisher's exact test). During heat stress, the proportion of DREB2A CA-inducible genes among the 262 genes up-regulated in the *NF-YB3*-overexpressing plants (23 genes; 9%) was significantly higher than that among Arabidopsis whole genes (1026 genes; 4%; $P < 0.0001$; Fisher's exact test), while that among the 195 genes up-regulated in the *NF-YB2*-overexpressing plants during heat stress (12 genes; 6%) was not significantly higher ($P > 0.01$, Fisher's exact test). These results suggest that NF-YB2 and NF-YB3 share their downstream genes with DREB2A during dehydration and heat stress, respectively. Eighty-eight NF-YB2–specific up-regulated genes during dehydration stress were also dehydration-inducible genes, and among them, 13 genes were up-regulated in the *DREB2A CA*-overexpressing plants (Fig. 4A). Sixty-two NF-YB3–specific genes up-regulated during heat stress were also heat-inducible genes, and among them, 14 genes were up-regulated in the *DREB2A CA*-overexpressing plants (Fig. 4B). These genes are candidates for regulation by DREB2A together with NF-YB2 and NF-YB3 under dehydration and heat stress conditions, respectively (Supplemental Tables S2 and S3). Among them, several genes have DRE and CCAAT elements on their 1 kb-promoters, and expression of these genes was analyzed by qPCR because eukaryotic NF-Y trimers have been suggested to bind to CCAAT elements (Dolfini et al., 2012; Myers and Holt, 2018). The *LEA76* and *CYS-RICH TRANS-MEMBRANE MODULE 1* (*CYSTM1*) genes were candidates regulated by NF-YB2 and DREB2A in the transcriptomic analysis (Supplemental Table S2) and were significantly up-regulated in *NF-YB2*–overexpressing plants during dehydration stress. The *HSP70B* and *At1g53540* (*HSP20-like*) genes were candidates regulated by NF-YB3 and DREB2A (Supplemental Table S2) and were significantly up-regulated in the *NF-YB3*–overexpressing plants during heat stress (Fig. 4, C and D). Moreover, the overexpression of *NF-YB3* suppressed the expression of *LEA76* and *CYSTM1* during dehydration stress (Fig. 4D).

Knockout Mutants of *NF-YB2* and *NF-YB3* Showed Dehydration and Heat Stress-Sensitive Phenotypes, Respectively

To analyze the contribution of NF-YB2 and NF-YB3 to stress responses under dehydration and heat stress conditions, single-knockout mutants of *NF-YB2* (*nf-yb2*; salk_025666) and *NF-YB3* (*nf-yb3*; salk_062245) and a double-knockout *nf-yb2 nf-yb3* mutant were isolated (Supplemental Fig. S5, A and B). As previously reported (Kumimoto et al., 2008), these knockout mutants showed late-flowering phenotypes under long-day conditions (Supplemental Fig. S5C). Drought stress tolerance tests were therefore performed under short-day conditions to keep all lines at the same developmental stages. The results revealed that the *nf-yb2* plants showed drought stress sensitivity. Meanwhile, the *nf-yb3* and *nf-yb2 nf-yb3* plants showed no significant changes in drought stress tolerance compared with that of the wild-type plants (Fig. 5, A and B). A previous paper showed that *nf-yb3* knockout mutants were sensitive to drought stress (Zhang et al., 2015). These differences probably resulted from the experimental conditions; we focused on phenotypic differences between the *nf-yb2* and *nf-yb3* mutants. A heat stress tolerance test revealed that the *nf-yb3* and *nf-yb2 nf-yb3* plants were sensitive to heat stress and that the *nf-yb2* plants did not show significant changes relative to the wild-type plants (Fig. 5, C and D). These results indicated that NF-YB2 and NF-YB3 play different roles and are important factors for survival under drought and heat stress conditions, respectively. In water loss tests of detached leaves, the *nf-yb3* and *nf-yb2 nf-yb3* plants showed significant suppression of water loss, while the *nf-yb2* plants did not show phenotypic differences (Supplemental Fig. S5D). The phenotype of *nf-yb3* coincides with the suppressive effect of NF-YB3 on dehydration stress-inducible genes (Fig. 3B).

NF-YB2 and NF-YB3 Play Important Roles in the Full Expression of Dehydration-Inducible and Heat Stress-Inducible DREB2A Target Genes, Respectively

The expression levels of dehydration-inducible and heat stress-inducible genes were analyzed in the *nf-yb2*, *nf-yb3*, and *nf-yb2 nf-yb3* knockout mutants. During dehydration stress, induction of the dehydration stress-inducible DREB2A target genes was significantly suppressed in the *nf-yb2* mutants (Fig. 6A). Meanwhile, induction of the heat-stress-inducible DREB2A target genes was significantly suppressed in the *nf-yb3* mutants (Fig. 6B). The expression levels (Fig. 6) and protein accumulation (Supplemental Fig. S6, C and D) of DREB2A were not different among the wild-type and knockout mutants. These results suggest that NF-YB2 and NF-YB3 are necessary for the full expression of these target genes specifically under dehydration and heat stress conditions, respectively. The *nf-yb3* mutants showed enhanced expression of dehydration
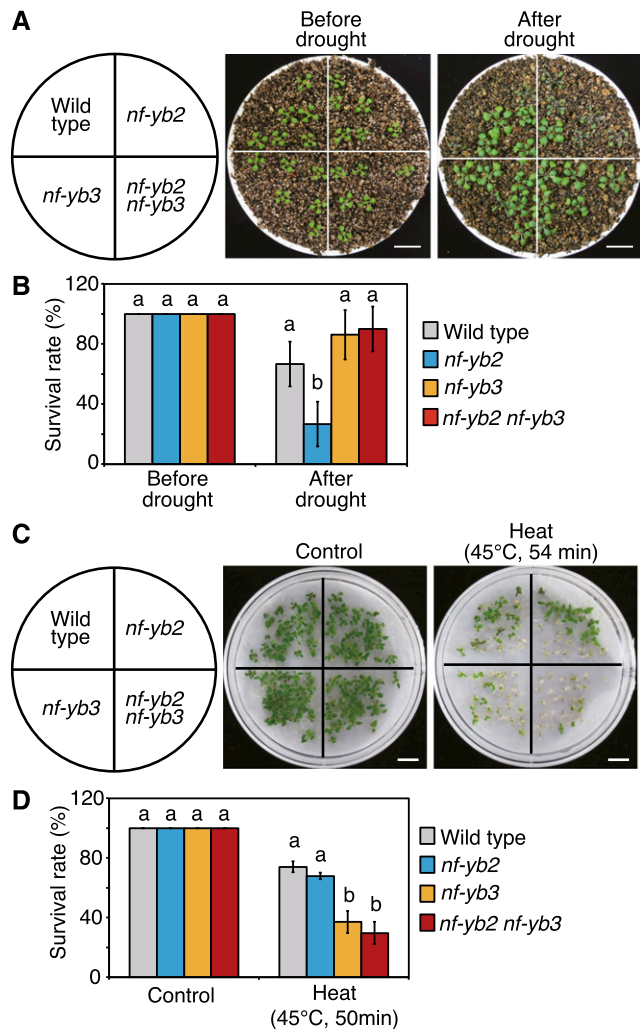
Figure 5. Drought and heat stress tolerance in the *nf-yb2*, *nf-yb3*, and *nf-yb2 nf-yb3* knockout mutants. A, Drought stress tolerance of the *nf-yb2*, *nf-yb3*, and *nf-yb2 nf-yb3* knockout mutants. Plants grown on agar plates for 10 d were transferred to soil and grown for 2 d. Water was withheld for 22 d. Scale bars = 1 cm. Photographs were taken before drought (left) and 7 d after rewatering (right). B, Percentages of surviving plants after rewatering. The error bars indicate the SD from six experiments, each involving 6 individual plants for each line. The letters above the bars indicate significant differences among plant lines before or after drought stress treatment ($P < 0.05$, according to Tukey's multiple range test). C, Heat stress tolerance of the *nf-yb2*, *nf-yb3*, and *nf-yb2 nf-yb3* mutants. The 7-d-old seedlings were treated with or without 45°C for 54 min. Scale bars = 1 cm. The photographs were taken after a 7-d recovery period at 22°C with (right) or without (left) heat stress. D, Percentages of surviving plants after heat stress. The error bars indicate the SD from three experiments, each involving 28 individual plants for each line. The letters above the bars indicate significant differences among plant lines with or without heat stress treatment ($P < 0.05$, according to Tukey's multiple range test).

stress-inducible genes during dehydration stress (Fig. 6A), suggesting that NF-YB3 has negative effects on the induction of these dehydration stress-inducible genes. However, knockout of *NF-YB2* did not affect the expression of heat stress-inducible genes (Fig. 6B).

These data also indicated the functional diversity of NF-YB2 and NF-YB3 in terms of the repressive effects on their target genes under stress conditions. In the *nf-yb2 nf-yb3* mutants, some heat-inducible genes such as *At1g75860* and *HSP70B* were down-regulated, and some dehydration-inducible genes such as *LEA7* and *RD29B* were up-regulated (Fig. 6). These data suggested that knockout of *NF-YB3* had greater effects than did knockout of *NF-YB2* on the regulation of dehydration-inducible genes during dehydration stress.

NF-YB2 and NF-YB3 Show Stress-Specific DNA-Binding Profiles to the Promoter of DREB2A Target Genes

To analyze the DNA-binding profiles of NF-YB2 and NF-YB3 to target gene promoters during stress, transgenic *nf-yb2* or *nf-yb3* plants expressing NF-YB2 or NF-YB3 fused to GFP driven by its native promoter were generated (*NF-YB2p:GFP-NF-YB2/nf-yb2*-a, b and *NF-YB3p:GFP-NF-YB3:nf-yb3*-a, b; Supplemental Fig. S7, A and B). The complementation of GFP-NF-YB2 and GFP-NF-YB3 recovered the drought-sensitive phenotypes of *nf-yb2* (Supplemental Fig. S7, C and D) and heat-sensitive phenotypes of *nf-yb3* (Supplemental Fig. S7, E and F), respectively, indicating that NF-YB2 and NF-YB3 fused to GFP were functional. The suppressed expression levels of *LEA7* and *HSFA3* during dehydration and heat stress were also recovered by complementation of *NF-YB2* and *NF-YB3*, respectively (Supplemental Fig. S7, G and H). *LEA7* and *LEA76* are candidate target genes with dehydration stress inducibility, and *HSFA3* and *HSP70B* have heat stress inducibility (Fig. 6). These genes have DRE and CCAAT sequences on their promoters within 1 kb upstream of translational start sites (Fig. 7, A and B). Chromatin immunoprecipitation (ChIP) assays revealed that dehydration stress increased the DNA binding of NF-YB2 to the *LEA7* and *LEA76* promoters (Fig. 7A), while heat stress increased the DNA binding of NF-YB3 to the *HSFA3* and *HSP70B* promoters (Fig. 7B). These results suggest the possibility that these dehydration-inducible and heat-inducible genes are direct targets of NF-YB2 and NF-YB3 under dehydration and heat stress conditions, respectively. Moreover, heat stress decreased the DNA binding of NF-YB2 to the promoters of the dehydration-inducible genes (Fig. 7A), and dehydration stress decreased that of NF-YB3 to those of heat-inducible genes (Fig. 7B). It is suggested that the transcriptional complexes, including NF-YB2 and NF-YB3, were dissociated from the promoters of target genes that were not induced during specific stress conditions. We also found differences in the DNA-binding profiles of NF-YB2 and NF-YB3; heat stress did not affect the DNA-binding of NF-YB2 to the promoters of the heat-inducible genes (Fig. 7B), while dehydration stress clearly suppressed that of NF-YB3 to those of the dehydration-inducible genes (Fig. 7A). These data suggested differences in the molecular functions of NF-YB2 and NF-YB3 toward heat-inducible
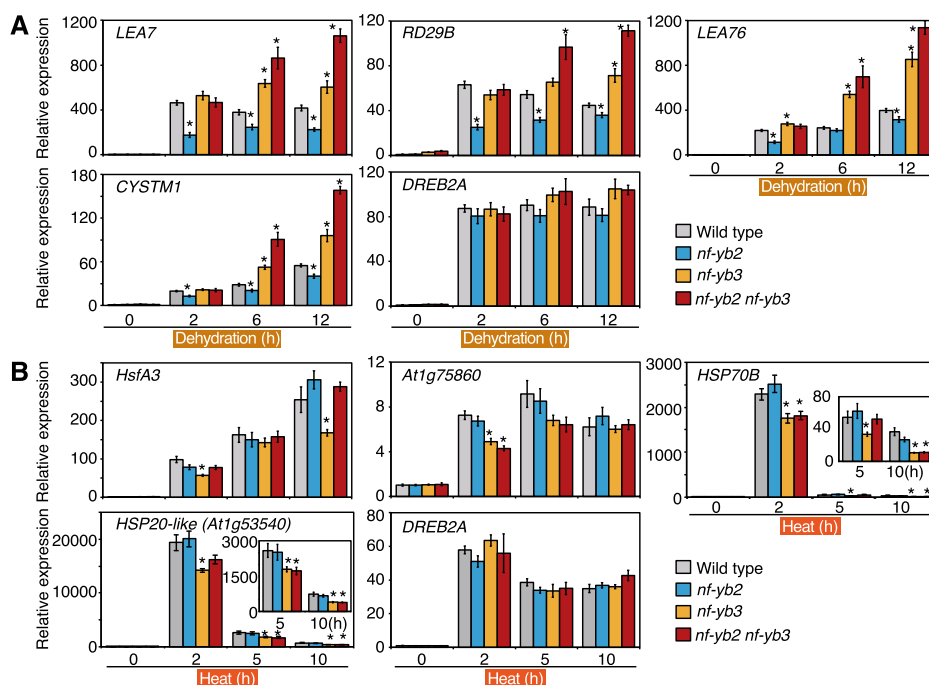
Figure 6. Expression patterns of dehydration-inducible and heat-inducible DREB2A target genes during dehydration and heat stress. A and B, Expression analysis of dehydration-inducible (A) or heat-inducible (B) DREB2A target genes during dehydration and heat stress in the *nf-yb2*, *nf-yb3*, and *nf-yb2 nf-yb3* knockout plants. The expression levels of *DREB2A* and dehydration-inducible (*LEA7*, *RD29B*, *LEA76*, and *CYSTM1*) and heat-inducible (*HSFA3*, *At1g75860*, *HSP70B*, and *At1g53540*) DREB2A target genes during dehydration and heat stress were analyzed by RT-qPCR. The 14-d-old plants grown on agar plates were treated with dehydration or heat stress. The expression levels of each gene in the VC plants under the control condition (0 h) were defined as 1.0. The error bars indicate SD (*n* = three experiments, involving 7 individual plants for each line). Asterisks indicate significant differences from the VC plants at each time point ($P < 0.05$, Bonferroni-corrected Student's *t* test). Small values of *HSP70B* and *At1g53540* (*HSP20-like*) gene expression under 5- and 10-h heat stress conditions were enlarged. The expression levels of *DREB2A* at each time point during dehydration and heat stress were evaluated using one-way ANOVA, but no significant differences were detected ($P > 0.05$).

and dehydration-inducible genes, respectively. In our previous study, direct interactions between NF-YBs (NF-YB2 and NF-YB3) and DREB2A were not detected in yeast cells (Sato et al., 2014). To analyze whether they form a transcriptional complex in plants, transgenic plants overexpressing one fusion protein consisting of DREB2A and the C-terminal half of SCFP3A with a he-magglutinin (HA) tag (DREB2A-HA-SCFP3AC) and one fusion protein consisting of NF-YBs (NF-YB2 or NF-YB3) and the N-terminal half of Venus with a myc tag (NF-YB2-myc-VenusN or NF-YB3-myc-VenusN) were generated. In bimolecular fluorescence complementation (BiFC) assays, fluorescent signals that indicated the interaction between DREB2A and NF-YB3 were detected in nuclei during heat stress (37°C, 5 h; Supplemental Fig. S7I). This suggested that DREB2A and NF-YB3 form a transcriptional complex during heat stress. Signals indicating the interaction between DREB2A and NF-YB2 could not be detected even under dehydration stress conditions (6 h), probably because the accumulated levels of the DREB2A protein were too low to be detected during drought conditions, or water loss from cells decreased signal intensity. We therefore performed coimmunoprecipitation (IP) assays using the same transgenic

lines after crosslinking to identify the proteins in transcriptional complexes. After IP of DREB2A-HA-SCFP3AC using an anti-DREBB2A antibody after dehydration stress (6 h), the NF-YB2-myc-VenusN protein as well as the NF-YB3-myc-VenusN protein were detected after heat stress (37°C, 5 h; Fig. 7C). The differences in the protein levels of NF-YB2 and NF-YB3 immunoprecipitated during dehydration and heat stress suggested the stress-specific formation of transcriptional complexes with DREB2A.

Phylogenetic Analysis Suggests the Evolutionary Diversification of NF-YB2 and NF-YB3 Subgroups

Our current studies indicated that Arabidopsis NF-YB2 and NF-YB3 have dehydration-specific and heat-stress–specific functions. To speculate on the evolutionary conservation of NF-YB2–specific and NF-YB3–specific functions in various plant species, phylogenetic analysis was performed. The phylogenetic tree of NF-YB indicated that all analyzed angiosperm plants have NF-YB family proteins that belong to divergent NF-YB2 and NF-YB3 subgroups (Supplemental Fig. S8). Another subgroup to which Arabidopsis NF-YB1, NF-YB8,
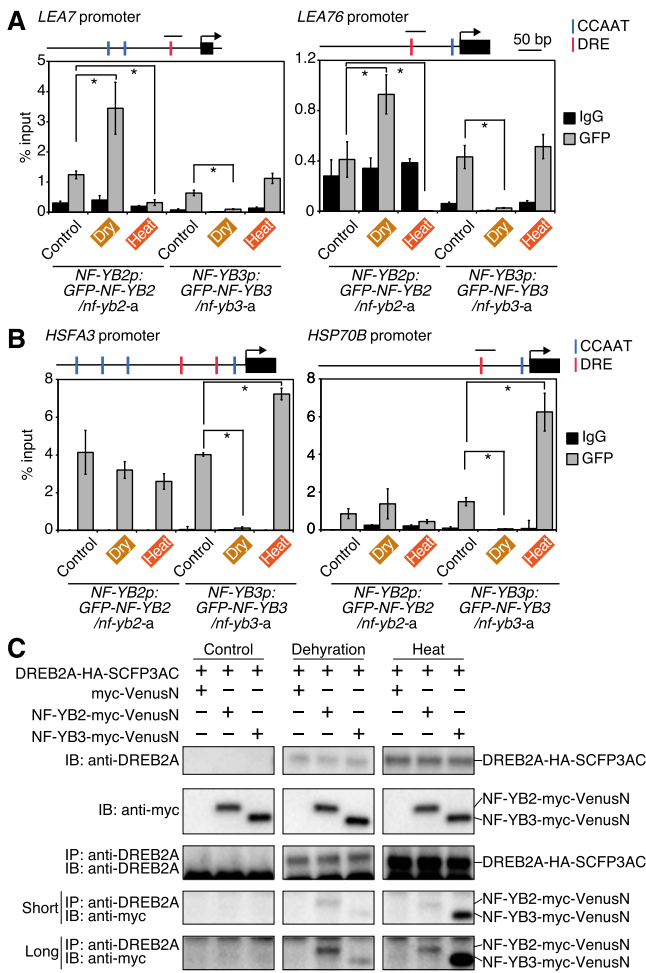
Figure 7. DNA-binding profiles of NF-YB2 and NF-YB3 to the target gene promoters, and protein interactions of NF-YB2 and NF-YB3 with DREB2A during dehydration and heat stress. A and B, ChIP-RT-qPCR assays of the NF-YB2 and NF-YB3 proteins under nonstress (control), dehydration (dry,) and heat stress conditions to the promoters of the dehydration-inducible (A) and heat-inducible (B) DREB2A target genes in the *NF-YB2p:GFP-NF-YB2/nf-yb2* and *NF-YB3p:GFP-NF-YB3/nf-yb3* plants. A schematic diagram displays the models of each target gene and amplified regions by RT-qPCR. The coding regions and CCAAT and DRE sequences are indicated as closed boxes and blue and red lines, respectively. The 14-d-old plants grown on agar plates were treated with or without dehydration or heat stress. IP was performed using an anti-GFP antibody or normal mouse igG (IgG; negative control). The error bars indicate the SD from six experiments. Asterisks indicate significant differences between two values ($P < 0.05$, Student's t test). C, Co-IP of NF-YB2-myc-VenusN and NF-YB3-myc-VenusN with DREB2A-HA-SCFP3AC in the transgenic plants under nonstress, dehydration, and heat stress conditions. The 14-d-old plants grown on agar plates were treated with or without dehydration or heat stress. Protein extracts before and after IP were analyzed by immunoblotting using anti-DREB2A or antimyc antibodies. Short and long exposures of the immunoblots using the antimyc antibody after IP are shown.

and NF-YB10 belong also contains NF-YB proteins of all angiosperms (Supplemental Fig. S8). Meanwhile, green algae (*Chlamydomonas reinhardtii*) has only one NF-YB protein among these three subgroups (Supplemental

Fig. S8). Bryophytes (*Physcomitrella patens*) and lycophytes (*Selaginella moellendorffii*) have NF-YB proteins that are closely related to the subgroup of Arabidopsis NF-YB1, NF-YB8, and NF-YB10; however, they do not have diversified NF-YB proteins that differentially belong to the NF-YB2 and NF-YB3 subgroups (Supplemental Fig. S8). These data suggest that functional diversification between NF-YB2 and NF-YB3 is conserved among angiosperms.

DISCUSSION

The NF-Y family proteins in plant species are more widely diversified compared with those in *Homo sapiens* (Petroni et al., 2012). The NF-Y transcription factors form a trimer composed of NF-YA, NF-YB, and NF-YC subunits when they work together, and wide diversification of plant NF-Y proteins raises the possibility that different combinations of these three subunits result in trimers with different molecular functions (Siefers et al., 2009; Myers and Holt, 2018). Previous studies have revealed the functions of NF-Y subunits involved in several biological phenomena such as flowering, embryo development, and abiotic stress responses (Nelson et al., 2007; Kumimoto et al., 2008; Yamamoto et al., 2009). Our current study revealed functional diversification between NF-YB2 and NF-YB3, which have the greatest sequence similarity (70% sequence identity) among all NF-YB proteins in Arabidopsis. Overexpression of *NF-YB2* and *NF-YB3* resulted in enhanced drought and heat stress tolerance, respectively (Fig. 2), and increased expression levels of several stress-specific inducible genes under each stress condition (Fig. 3). Moreover, the *nf-yb2* and *nf-yb3* knockout mutants were drought sensitive and heat stress sensitive, respectively (Fig. 5), and showed significant decreases in the expression of several stress-inducible genes during dehydration and heat stress (Fig. 6). RNA-sequencing also confirmed that NF-YB2 and NF-YB3 regulated their specific target genes during dehydration and heat stress, and overexpression of these genes had greater effects on transcriptomic changes in dehydration and heat stress responses, respectively (Fig. 4). These results clearly indicated that NF-YB2 and NF-YB3 were functionally diversified and activated target genes specifically in response to dehydration and heat stress. The DNA binding profiles also confirmed the stress-specific dynamics of NF-YB2 and NF-YB3 on the target gene promoters. Dehydration stress increased the DNA binding of NF-YB2 to the dehydration-inducible gene promoters (Fig. 7A) but did not affect that to the heat-inducible gene promoters (Fig. 7B). In contrast, heat stress increased NF-YB3 binding to the heat-inducible gene promoters (Fig. 7B) but did not affect that to the dehydration-inducible gene promoters (Fig. 7A). The gene expression patterns of *NF-YB2* and *NF-YB3* also coincide with the stress-specific functions of NF-YB2 and NF-YB3 (Fig. 1).

Moreover, unexpected functions of NF-YB3 during dehydration stress were indicated. The *nf-yb3* knockout

mutants showed enhanced expression of dehydration-inducible genes during dehydration stress (Fig. 5A), and overexpression of *NF-YB3* resulted in decreased expression of those genes (Fig. 3). Meanwhile, knockout and overexpression of *NF-YB2* did not affect the expression of heat-inducible genes (Figs. 3 and 5B). These data suggested that NF-YB3 had negative effects on the induction of dehydration-inducible genes during dehydration stress, but NF-YB2 did not have negative effects on heat-inducible genes during heat stress. In the ChIP assays, heat stress did not affect NF-YB2 binding to the heat-inducible gene promoters (Fig. 7B), whereas dehydration stress decreased the binding of NF-YB3 to the dehydration-inducible promoters (Fig. 7A). These data also suggested the negative functions of NF-YB3 in dehydration-inducible genes, but NF-YB2 did not show such negative functions in heat-inducible genes. It is still possible that these repressive effects on the expression of the dehydration-inducible genes may be caused indirectly by *NF-YB3* knockout. Further studies will be necessary to reveal the more detailed mechanisms by which NF-YB3 exerts negative effects on dehydration-inducible genes and what causes differences between NF-YB2 and NF-YB3 functions toward their target genes.

The possibility of functional conservation among plant species opens the door to the application of NF-YB2 and NF-YB3 to improve drought and heat stress tolerance in agriculturally important crops. We previously reported that Arabidopsis DPB3-1 (NF-YC10) and rice (*Oryza sativa*) OsDPB3-2 had conserved functions and that overexpression of Arabidopsis *DPB3-1* enhanced heat stress tolerance in both Arabidopsis and rice (Sato et al., 2014, 2016). Other studies have provided evidence that NF-Y could be a useful target for improving agricultural traits in response to abiotic stress: Arabidopsis NF-YB1 conferred drought stress tolerance in maize (*Zea mays*; Nelson et al., 2007), overexpression of rice *NF-YA7* increased drought tolerance in rice (Lee et al., 2015), and so on. Arabidopsis NF-YB2 and NF-YB3 or other NF-YB proteins with conserved functions in other plant species might be important factors for manipulating drought and heat stress tolerance in crops.

Our current study also suggested the mechanisms underlying the target selectivity of DREB2A, which regulated both dehydration-inducible and heat stress-inducible genes in response to environmental conditions. The genes that were differentially expressed in *NF-YB2*-overexpressing and *NF-YB3*-overexpressing or knockout plants during dehydration and heat stress, respectively, are also known to be target genes of DREB2A (Sakuma et al., 2006b; Figs. 3 and 6). Genetic interaction and physical protein interaction assays suggested that NF-YB2 and NF-YB3 cooperatively activated their target genes together with DREB2A specifically during dehydration and heat stress, respectively (Fig. 7C; Supplemental Fig. S4, G and H). Moreover, transcriptomic data showed that NF-YB2 and NF-YB3 shared a significant proportion of their downstream genes with DREB2A CA during dehydration and heat stress, respectively (Fig. 4, A and B). These results indicated that there is an overlap between stress-specific target genes of DREB2A and NF-YBs (NF-YB2 or NF-YB3). Moreover, our transcriptomic analysis revealed that overexpression of *NF-YB2* and *NF-YB3* enhanced the expression levels of dehydration-inducible and heat-inducible genes, respectively, that were not downstream of DREB2A (Fig. 4, A and B), suggesting that NF-YB2 and NF-YB3 are involved in DREB2A-independent pathways, for example, ABA-dependent pathways during dehydration stress (Yoshida et al., 2014) or HSFA2 pathways during heat stress (Ohama et al., 2017). Future studies are needed to reveal what factors other than DREB2A form transcriptional complexes with NF-YB2 and NF-YB3, and enhance drought and heat stress responses, respectively.

Our present study also raised several questions about the molecular functions of NF-YB2 and NF-YB3. First, it is important to identify dehydration-specific and heat stress-specific trimers including NF-YB2 or NF-YB3. Our previous work suggested that a heat stress–specific trimer composed of NF-YA2, NF-YB3, and DPB3-1 (NF-YC10) worked in cotyledons during heat stress (Sato et al., 2014); however, *NF-YB3* is also expressed in true leaves (Siefers et al., 2009; Sato et al., 2014), and the expression patterns of several DREB2A target genes were altered in 14-d-old *nf-yb3* mutants (Fig. 5B). These results imply that another NF-YC protein forms a transcriptional complex with NF-YB3 in true leaves during heat stress. In conclusion, we found that the NF-YB2 and NF-YB3 proteins, which had high sequence similarity to each other, had different functions in response to environmental conditions (Supplemental Fig. S9). These results provide insights into the evolution and diversification of NF-Y proteins in plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) Heynh. ecotype Columbia and Wassilewskija plants were grown on germination medium (GM) agar plates at 22°C under a 16-h or 8-h photoperiod at a photon flux density of 40 μm^{-2}s^{-1} as described previously in Sakuma et al. (2006a, 2006b). The 16-d-old plants were transferred to perlite-containing soil (Dio Chemicals) and grown under similar conditions. For analysis of flowering time, five plants were transferred to soil in a plastic pot with a diameter of 7.5 cm. The T-DNA insertion lines for *NF-YB2* and *NF-YB3* were obtained from the Arabidopsis Biological Resource Center (The Ohio State University, Columbus; *nf-yb2*; salk_025666, *nf-yb3*; salk_062245). The T-DNA insertions were detected by amplifying the insert-flanking region of the genomic DNA using the primers shown in Supplemental Table S4. The double mutant *nf-yb2 nf-yb3* was constructed by genetic crosses between *nf-yb2* and *nf-yb3*. The triple-knockout mutant *srk2d srk2e srk2i* (*srk2d/e/i*), quadruple mutant *areb1 areb2 abf1 abf3* (*arebq*), triple-knockout mutant *hsfa1a hsfa1b hsfa1d* (*hsfa1a/b/d*), *nc3-2*, *aba2-2* and *dreb2a-1* mutants, and *35S:DREB2A CA* transgenic plants were previously described (Nambara et al., 1998; Iuchi et al., 2001; Fujita et al., 2009; Yoshida et al., 2011, 2015). Transgenic plants were generated by the floral dip method (Clough and Bent, 1998) using *Agrobacterium tumefaciens* GV3101 (pMP90) cells.

Stress Treatment

The dehydration, heat stress, and ABA treatments were performed as described previously (Liu et al., 1998; Sato et al., 2014) with minor modifications.

For dehydration stress, the plants grown on GM agar plates were transferred to petri dishes covered with Parafilm M. For heat stress, the GM agar plates on which plants were grown were transferred to a 37°C incubator (LPH-350SP; NK system). The heat and drought stress tolerance tests were performed as previously described in Sakuma et al. (2006a, 2006b), with minor modifications. For the drought stress tolerance test, the plants grown on GM agar plates under an 8-h photoperiod were transferred to soil in plastic pots and grown for 2 d under the same conditions. Water supply was withheld until plants withered. The survival rates of plants were calculated 7 d after rewatering under control conditions. For the heat stress tolerance test, plants were grown on two layers of filter paper containing 4 ml of liquid GM medium in 90-mm plastic Petri dishes under control conditions with supplying 2 ml of sterilized water every 3 d. The 7-d-old plants were subjected to heat stress (45°C, 54 min) in a paper box (width 13 mm × depth 130 mm × height 50 mm) in an incubator (SLC-25A; Mitsubishi Electric). For ABA treatment, 14-d-old plants were transferred to a Petri dish filled with distilled water containing 100 μM ABA (Sigma-Aldrich). For measurement of water loss, rosette leaves of 14-d-old plants were detached and placed on a petri dish. The loss of fresh weight was monitored at the indicated time.

RNA Preparation and RT-qPCR

The total RNA from seedlings was isolated with RNAiso plus (TaKaRa) according to the supplier's instructions. The complementary DNA (cDNA) was synthesized using SuperScript IV (Thermo Fisher), and RT-qPCR was performed using an Applied Biosystems 7500 Fast Real-Time PCR system and THUNDERBIRD SYBR qPCR Mix (TOYOBO) according to the supplier's instructions. Triplicate measurements were made for each cDNA sample, and the values were normalized according to the amounts of *18S* rRNA. The primers used for RT-qPCR are shown in Supplemental Table S4.

Protein Immunoblot Analysis

Protein immunoblot analysis was performed as previously described (Morimoto et al., 2013) with minor modifications. The total proteins were extracted from seedlings using protein extraction buffer (8 M urea, 5 mM [v/v] 2-mercaptoethanol, 2 mM EDTA, 1% [w/v] SDS, 50 mM Tris-HCl [pH 6.8]), and mixtures were centrifuged at 20,000g for 10 min at room temperature. The supernatant was boiled at 95°C for 3 min. The resultant extracts, which corresponded to a fresh weight of 4 mg seedling, were separated by SDS-PAGE. The separated proteins were blotted to an immune-blot polyvinylidene difluoride membrane (BIO RAD) using a Trans-Blot Turbo Transfer System (BIO RAD). For immunoblotting, a polyclonal anti-DREB2A antibody (1: 2500 dilution; Morimoto et al., 2013) or antimyc-antibody (1: 2000 dilution; Millipore) was used as a primary antibody, and goat antirabbit or antimouse IgG peroxidase-conjugate (Thermo Fisher) was used as a secondary antibody. The signals were developed by SuperSignal West Dura Extended Duration Substrate (Thermo Fisher) according to the manufacturer's protocol and detected with an image analyzer (ChemiDoc MP, Bio-Rad). The Rubisco large subunit (rbcL) was detected by Ponceau S (APRO SCIENCE) according to the manufacturer's instructions.

RNA Sequencing and Data Analysis

cDNA libraries were constructed using TruSeq RNA Sample Preparation Kit v2 (Illumina), and the libraries were sequenced by NextSeq 500 (Illumina). The produced bcl files were converted to fastq files by bcl2fastq (Illumina). The reads were analyzed as previously described (Notaguchi et al., 2015). The data were deposited to the DNA Data Bank of Japan (DRA007270). The genes upregulated by DREB2A CA were reported in previous works (Sakuma et al., 2006a; Mizoi et al., 2013).

ChIP Assay

ChIP assays were performed using an EpiQuik Plant ChIP kit (Epigenetek) according to the user guide. Plant samples (one gram fresh weight) with or without stress treatment were collected. The polyclonal anti-GFP antibody (Tanaka et al., 2012) was used for IP of the DNA-protein complex.

BiFC and Co-IP Assays

GFP fluorescence was observed by using a confocal laser scanning microscope (Olympus FLUOVIEWV FV 1000) as described previously (Sato et al., 2018). For co-IP assays, 2 g of plant materials crosslinked by 1% (w/v) formaldehyde were ground in liquid nitrogen and dissolved in an extraction buffer containing 10 mM Tris-HCl (pH 8.0), 0.4 M Suc, 5 mM 2-mercaptoethanol, and a protease inhibitor mixture (cOmplete, EDTA-free; Roche Applied Science). The mixture was filtered through two layers of Miracloth (Merck) and centrifuged at 3,000g for 20 min at 4°C. The nuclear pellet was resuspended with 1 ml of a lysis buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.2 M NaCl, 1% (v/v) Triton X-100, and a protease inhibitor mixture (cOmplete, EDTA-free; Roche Applied Science) and placed on ice for 5 min. The solution was centrifuged at 3,000g for 10 min at 4°C. The pellet was resuspended with 500 μL of a sonication buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.15 M NaCl, 0.1% (w/v) SDS, 100 μM MG132 (Calbiochem), and a protease inhibitor mixture (cOmplete, EDTA-free; Roche Applied Science), sonicated, and centrifuged at 12,000g for 10 min at 4°C. The supernatant was incubated with 3 μg of an anti-DREB2A antibody (Morimoto et al., 2013) for 1 h at 4°C with rotation, and 50 μL of Protein G Sepharose 4 Fast Flow (GE Healthcare) was added and incubated for 1 h at 4°C with rotation. The resin was collected after centrifugation at 12,000g for 20 s at 4°C and washed with 3 mL of sonication buffer. Proteins were eluted with 50 μL of an elution buffer containing 50 mM Tris-HCl (pH 7.5), 1% (w/v) SDS, and 100 mM dithiothreitol at 95°C for 3 min and subjected to immunoblot analysis.

Sequence Alignment and Phylogenetic Analysis

The peptide and promoter sequences of the NF-YB family were obtained through Phytozome 12 (https://phytozome.jgi.doe.gov/pz/portal.html) using PANTHER ID (PTHR11064). The family proteins were aligned using the MUSCLE program by SnapGene software (https://www.snapgene.com/). The resultant alignments were manually adjusted and visualized through the BoxShade program (https://embnet.vital-it.ch/software/BOX_form.html). The neighbor-joining phylogenetic tree was constructed using MEGA6 software (http://www.megasoftware.net/) with the Poisson model, 10,000 bootstrap replicates, and pairwise deletion for gap/missing data treatment using conserved regions of the NF-YB proteins. The accession numbers of NF-YB family proteins used to construct the phylogenetic tree are shown in Supplemental Table S5.

Plasmid Constructions

To generate the *35S:NF-YB2* and *NF-YB3* constructs, the *NF-YB2* and *NF-YB3* coding sequences were inserted into the *Xba*I and *Xho*I sites of the pGKX or pGHX vector (Qin et al., 2008). To generate the *NF-YB2p:GFP-NF-YB2* and *NF-YB3p:GFP-NF-YB3* constructs, the *NF-YB2* and *NF-YB3* coding sequences were initially inserted into the *Xba*I and *Xho*I sites of the pGKX-NsGFP vector (Qin et al., 2008). The *GFP-NF-YB2-Nos* terminator (*Nos*T) or *GFP-NF-YB3-Nos*T sequences and 1-kb upstream promoter sequences from translational start sites of *NF-YB2* and *NF-YB3* were inserted into the *Sal*I and *Kpn*I sites and *Sac*I and *Pst*I sites of the pGreen0129 vectors (Hellens et al., 2000), respectively. To generate the constructs for BiFC and co-IP, *NF-YBs* (*NF-YB2* and *NF-YB3*) and *DREB2A* coding sequences were inserted into the *Xba*I and *Xho*I sites or *Spe*I and *Cla*I sites of the pVYNE and pSCYCE vector (Waadt et al., 2008), respectively.

Statistical Analysis

The significant differences between the control plants and transgenic plants, control conditions, and stress conditions were analyzed by Bonferroni-corrected Student's t test, Student's t test, one-way ANOVA, Tukey's multiple range test, or Fisher's exact test. P values < 0.01 were considered to indicate statistical significance in Fisher's exact test, and P values < 0.05 were considered to indicate statistical significance in other tests.

Accession Numbers

The sequence data of genes in this article can be obtained in Phytozome 12 (https://phytozome.jgi.doe.gov/pz/portal.html) under the following accession numbers: *NF-YB2* (*At5g47640*), *NF-YB3* (*At4g14540*), *DREB2A* (*At5g05410*), *LEA7* (*At1g52690*), *RD29B* (*At5g52300*), *RD17* (*At1g20440*), *HSFA3* (*At5g03720*),

At1g75860, *LEA76* (*At3g15670*), *CYSTM1* (*At1g05340*), *HSP79B* (*At1g16030*), and *At1g53540*.

SUPPLEMENTAL DATA

The following supplemental materials are available.

Supplemental Figure S1. Expression levels of *NF-YB2*, *NF-YB3* and *DREB2A* in the ABA-signaling and ABA-deficient mutants during ABA treatment.

Supplemental Figure S2. Alignments of promoters of *NF-YB* family genes among *Brassica* species.

Supplemental Figure S3. Early-flowering phenotypes under long-day conditions and water loss of the *NF-YB2*-overexpressing and *NF-YB3*-overexpressing plants.

Supplemental Figure S4. Protein accumulation of DREB2A in the *NF-YB2*-overexpressing and *NF-YB3*-overexpressing plants during dehydration and heat stress, and genetic interactions between *NF-YBs* (*NF-YB2* and *NF-YB3*) and *DREB2A*.

Supplemental Figure S5. Isolation of *nf-yb2* and *nf-yb3* knockout mutants and late-flowering phenotypes under long-day conditions and water loss of the *nf-yb2*, *nf-yb3* and *nf-yb2 nf-yb3* mutants.

Supplemental Figure S6. Protein accumulation of DREB2A in the *nf-yb2*, *nf-yb3* and *nf-yb2 nf-yb3* mutants during dehydration and heat stress.

Supplemental Figure S7. Recovery of drought and heat stress tolerance by the complementation of GFP-NF-YB2 in *nf-yb2* and GFP-NF-YB3 in *nf-yb3*, respectively, and protein interaction between DREB2A and NF-YB2 or NF-YB2 during dehydration and heat stress.

Supplemental Figure S8. A phylogenetic tree of NF-YB family proteins across various plant species.

Supplemental Figure S9. A current model of how NF-YB2 and NF-YB3 regulate their target genes together with of DREB2A under drought and heat stress conditions.

Supplemental Table S1. Numbers and positions of cis-acting elements related to abiotic stress on promoters of *NF-YB2* and *NF-YB3*.

Supplemental Table S2. Candidate target genes by DREB2A and NF-YB2 during dehydration stress.

Supplemental Table S3. Candidate target genes by DREB2A and NF-YB3 during heat stress.

Supplemental Table S4. Primers used in this study.

Supplemental Table S5. NF-YB family proteins across various plant species belonging to the same subgroups of Arabidopsis thaliana NF-YB2, NF-YB3, NF-YB1, NF-YB8 and NF-YB10.

Supplemental Dataset S1. Dehydration-stress-inducible genes in the control plants.

Supplemental Dataset S2. Heat-stress-inducible genes in the control plants.

Supplemental Dataset S3. Upregulated genes in the *NF-YB2*-overexpressing plants during dehydration stress.

Supplemental Dataset S4. Upregulated genes in the *NF-YB3*-overexpressing plants during dehydration stress.

Supplemental Dataset S5. Upregulated genes in the *NF-YB2*-overexpressing plants during heat stress.

Supplemental Dataset S6. Upregulated genes in the *NF-YB3*-overexpressing plants during heat stress.

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