Multiple NUCLEAR FACTOR Y Transcription Factors Respond to Abiotic Stress in *Brassica napus* L.

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**Abstract**

Members of the plant NUCLEAR FACTOR Y (NF-Y) family are composed of the NF-YA, NF-YB, and NF-YC subunits. In *Brassica napus* (canola), each of these subunits forms a multimember subfamily. Plant NF-Ys were reported to be involved in several abiotic stresses. In this study, we demonstrated that multiple members of thirty three BnNF-Ys responded rapidly to salinity, drought, or ABA treatments. Transcripts of five BnNF-YAs, seven BnNF-YBs, and two BnNF-YCs were up-regulated by salinity stress, whereas the expression of thirteen BnNF-YAs, ten BnNF-YBs, and four BnNF-YCs were induced by drought stress. Under NaCl treatments, the expression of one BnNF-YA10 and four NF-YBs (BnNF-YB3, BnNF-YB7, BnNF-YB10, and BnNF-YB14) were greatly increased. Under PEG treatments, the expression levels of four NF-YAs (BnNF-YA9, BnNF-YA10, BnNF-YA11, and BnNF-YA12) and five NF-YBs (BnNF-YB1, BnNF-YB8, BnNF-YB10, BnNF-YB13, and BnNF-YB14) were greatly induced. The expression profiles of 20 of the 27 salinity- or drought-induced BnNF-Ys were also affected by ABA treatment. The expression levels of six NF-YAs (BnNF-YA1, BnNF-YA7, BnNF-YA9, BnNF-YA10, and BnNF-YA12) and seven BnNF-YB members (BnNF-YB2, BnNF-YB3, BnNF-YB7, BnNF-YB10, BnNF-YB11, BnNF-YB13, and BnNF-YB14) and two NF-YC members (BnNF-YC2 and BnNF-YC3) were greatly up-regulated by ABA treatments. Only a few BnNF-Ys were inhibited by the above three treatments. Several NF-Y subfamily members exhibited collinear expression patterns. The promoters of all stress-responsive BnNF-Ys harbored at least two types of stress-related cis-elements, such as ABRE, DRE, MYB, or MYC. The cis-element organization of BnNF-Ys was similar to that of *Arabidopsis thaliana*, and the promoter regions exhibited higher levels of nucleotide sequence identity with *Brassica rapa* than with *Brassica oleracea*. This work represents an entry point for investigating the roles of canola NF-Y proteins during abiotic stress responses and provides insight into the genetic evolution of *Brassica* NF-Ys.

**Introduction**

Abiotic stress, such as salinity, drought or dramatic temperature change, adversely affects plant growth and productivity. Plants, being sessile, have evolved a range of strategies to adapt to detrimental environmental conditions. Plants respond to unfavorable conditions through two pathways, i.e., the abscisic acid (ABA)-dependent and ABA-independent pathway [1–3]. Transcription factors (TFs) play vital roles in mediating stress tolerance in both of these pathways. NUCLEAR FACTOR Y (NF-Y) TFs are ubiquitous in higher eukaryotes and belong to the CCAAT-binding factor (CBF) family, also known as the Heme Activator Protein (HAP) family in yeast. The NF-Y heterocomplex consists of at least three subunits, namely NF-YA (also named CBF-B or HAP2), NF-YB (CBF-A or HAP5), and NF-YC (CBF-C or HAP3) [4]. In plants, each NF-Y subunit is encoded by a multi-member family, and there are at least 10 annotated members in each NF-Y subfamily in *Arabidopsis* [5–7]. Rice harbors at least 10 NF-YA genes, 12 NF-YB genes, and 8 NF-YC genes [8]. In a previous study, we identified 14 NF-YA genes, 14 NF-YB genes, and 5 NF-YC genes in *Brassica napus* L. [9]. NF-Y family members are extensively involved in plant development and stress responses, as summarized in a recent review [10].

Increasing evidence suggests that NF-Y subunits are important regulators of abiotic stress responses [11–14]. Overexpression of NF-YA1 in *Arabidopsis* was shown to result in hypersensitivity to salt stress during early postgerminative growth [15]. Real-time PCR analysis revealed that *Glycine max* (soybean) *GmNF-YA3* was induced by ABA and other abiotic stresses, and that overexpression of *GmNF-YA3* in *Arabidopsis* enhanced drought resistance [16]. A microarray analysis identified *Arabidopsis* NF-YB2 as one of the most strongly induced genes under multiple treatments, including cold, mannitol (dehydration stress), and NaCl (salinity) [17]. Further analysis confirmed that AtNF-YB2 functions in early flowering under osmotic stress [12]. AtNF-YB1 was found to

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improve plant performance under drought conditions and its ortholog in maize, ZmNF-YB2, was shown to resist drought stress in the field [11]. Several drought-related NF-YB genes were identified in *Tritium aestivum* (wheat), *Populus euphratica* (poplar), and *Hordeum vulgare* (barley) [18–20]. In the NF-YYC subfamily, the transcript level of *AtNF-YYC2* was highly induced by light, oxidative, heat, cold, and drought stress [21]. Overexpression of *PpHAP5* (homolog of *Arabidopsis* NF-YYC2) from the conifer *Picea wilsonii* partially rescued the increased sensitivity of *nf-y*c2 to salt, drought, and ABA treatments [22].

In addition, several members of the NF-Y family were shown to be regulated by the microRNA169 (miR169) family, suggesting that a complex regulatory cascade is activated under stress conditions [13, 14, 23]. Li et al. (2008) demonstrated that *AtNF-YA5* is a target of miRNA169a or miRNA169c [13]. At least six other miR169 targets were predicted to exist in the AtNF-YA family [24]. Of those six NF-YAs, NF-YA2, 3, 7, and 10 were found to be involved in multiple abiotic stresses, especially those involving low nutrient availability [25]. In rice, an NF-YA gene (*Os03g29760*) known as a target of the miR169 family was found to be induced by high salinity [14]. The above results suggest that plant NF-Ys play a crucial role in the plant’s response to abiotic stress. However, the roles of BnNF-Ys under stress have not been examined in detail.

Canola (*B. napus*) constitutes the third largest vegetable oil source in the world. However, salinity and drought stress have a major limiting impact on its production and yield stability. Drought accounts for at least a 30% yield loss in canola every year [26]. In this study, we examined the expression patterns of *BnNF-Ys* under drought, high salinity, and ABA conditions. Our results suggest that 27 of 33 putative *BnNF-Ys* were induced by salt or drought stresses. Furthermore, 20 *BnNF-Ys* may be involved in the ABA-related pathway. We cloned the promoters of 20 *BnNF-Ys* from canola, which mostly included regions ~1000 bp upstream of the coding sequences, and identified their stress-related cis-elements. Taken together, our results suggest that most *BnNF-Y* members are regulated by abiotic stresses in an abscisic acid (ABA)-dependent or ABA-independent manner and serve as an entry point to investigate the roles of these genes in the plant’s response to environmental stresses.

**Materials and Methods**

**Plant materials and growth conditions**

Seeds of oilseed rape (*Brassica napus* ‘Nanyanyou 1’, kindly provided by Professor Zhaopu Liu at Nanjing Agricultural University) were kept in darkness for 2 days and germinated in Hoagland nutrient solution. The plants were grown for 3 weeks in the greenhouse with a light intensity of 350–415 μmol m⁻² s⁻¹ during a daily cycle consisting of 16 h of light at 25°C and 8 h of darkness at 18°C. For dehydration and salt stress treatments, plants were grown hydroponically in solution containing 15% (w/v) PEG6000 and 150 mM NaCl plus ½ Hoagland nutrient solutions for 1 or 3 h, respectively. For ABA treatments, 100 μM ABA was added to the ½ Hoagland nutrient solution and the plants were treated for 1 or 3 h. Seedlings growing on ½ Hoagland nutrient solution for various periods of time were used as the control. The harvested samples were immediately frozen in liquid nitrogen and stored at −80°C until used for gene expression analysis. All experiments were performed in biological triplicate.

**Genomic DNA extraction, total RNA isolation, and primary cDNA synthesis**

Genomic DNA was extracted from oilseed rape using a method from Rogers and Bendich's CTAB-based protocol [27]. Total RNA of each sample was extracted using an E.Z.N.A. Plant RNA Kit (Omega Biotek, Cat# R8627, USA). The quality and quantity of extracted DNA and RNA from all samples were confirmed by both agarose gel electrophoresis and spectrophotometry (Thermo Scientific, NanoDropTM 1000, USA). Total RNA samples were pretreated with an RNase-Free DNase Set (Omega Biotek, Cat# E1091, USA) to eliminate residual genomic DNA. Primary cDNA was synthesized using a PrimeScript RT-PCR Kit (TaKaRa Code: D6110A, Japan), according to the manufacturer’s instructions.

**Analysis of Gene Expression by Quantitative real-time PCR (qRT-PCR)**

Quantitative real-time PCR was performed on an Applied Biosystems 7500 real-time PCR system using the SYBR Premix Ex TaqTM Kit (TaKaRa Code: DRR041A, Japan). Sequence data, primers, and the PCR procedure used in this study were provided in our previous study [9]. All sequences of qRT-PCR amplification products were verified by sequencing. 18S rRNA was used as an internal control for expression analysis. Data were processed using the 2^(-ΔΔCt) method [28, 29].

The transcript levels of each *BnNF-Y* gene in each tissue were first normalized to those of the housekeeping gene 18S and then compared to the transcript levels of each time-point control to obtain the relative gene expression level (i.e., the levels of expression in the same untreated tissue at the corresponding time point). Each data point represents the mean ±SE of three independent experiments. For the figures S1-S5, the transcript levels of each *BnNF-Y* gene were first normalized to those of the housekeeping gene 18S and then compared to the levels in the 0-h leaf control.

**Statistical Analysis and Correlation Analysis**

Significant differences between three treated samples and three untreated controls (same tissue and time-point only) are indicated by a single (P<0.05) or double (P<0.01) asterisk, according to Dunnett’s method of one-way ANOVA in SPSS 18.0 (SPSS Corp., Chicago, IL, USA). Figures were created using SigmaPlot 10.0 (Systat Software, Inc. Germany). Correlation analysis of expression patterns was determined using relative gene expression levels from Fig. S1, Fig. S2, and Fig. S3. The regression coefficient was calculated in Microsoft Excel.

**Measurements of Relative Water Content (RWC) from Drought-Stressed Plants**

RWC measurements under PEG treatments were conducted as previously described [20].

**Isolation of NF-Y promoters and Identification of their regulatory elements**

To identify the promoter regions of *NF-Y* from canola, each *BnNF-Y* coding region was used as a query in a BLASTN search against the *Brassica* database (http://brassicadb.org/brad/index. php), which includes *B. rapa* and *B. oleracea* sequences, and the highest hits were identified. Based on *BnNF-Y* coding sequences and the promoter sequences of the retrieved orthologous *Brassica* *NF-Y* s, most promoter regions of *BnNF-Y* sequences could be amplified with the primers listed in Table S1. Others promoter regions were amplified using a Genome Walking Kit (TaKaRa
Code: D316, Table S1). In addition, the promoters of Arabidopsis NF-Y sequences were downloaded from the Plant Promoter Database [http://133.66.216.33/ppdb/cgi-bin/index.cgi]. Sequences ~1000 bp upstream of the ATG start codon of each gene were retrieved from the genome, and known elements were identified using Plant cis-element Regulatory DNA Elements (PLACE) [http://www.dna.afrc.go.jp/PLACE/] and PlantCARE [http://bioinformatics.psb.ugent.be/webtools/plantcare/html/].

Phylogenetic tree analysis

The phylogenetic tree based on the promoter regions of plant NF-Ys was constructed using the neighbor-joining method implemented in MEGA software (version 4.1) with the following parameters: Jones–Taylor–Thornton (JTT) model and 1,000 bootstrap replicates.

Results

Expression Patterns of BnNF-Y Genes in the Leaves and Roots of Canola in Response to Salinity Stress

We previously identified BnNF-Y TF families in canola (B. napus L.) [9]. Since numerous reports have demonstrated that plant NF-Ys function in abiotic stress responses [13,15], we examined whether BnNF-Ys had similar roles. To investigate whether BnNF-Y genes were responsive to salt stress, we monitored the transcript levels of all 33 BnNF-Ys in both the leaves and roots up to 3 h of exposure to high salinity. First, we quantified the transcript levels of each BnNF-Y gene in different tissues and expressed these levels relative to those in 0-h untreated leaves. In this study, a gene was considered to be induced or down-regulated if its expression levels were both significantly different from those of an untreated control and >2-fold higher or lower than the control values, respectively. As shown in Fig. S1A, S2A and S3A, in the absence of any stress, the expression levels of most BnNF-YA subfamily members were not significantly altered during a 3-h observation period, except for BnNF-YA4/5, BnNF-YA11, BnNF-YA12, and BnNF-YA13. The transcription levels of BnNF-YA4/5 (BnNF-YA4 and BnNF-YA5 are alternative splice variants, with BnNF-YA4 being 11 amino acids shorter than BnNF-YA5) [9], and BnNF-YA11 were up-regulated in the 3-h root samples, while levels of BnNF-YA12 and BnNF-YA13 were down-regulated in 1-h leaf samples. This implies that these BnNF-YAs are subject to circadian clock regulation. To minimize the effect of circadian-mediated changes, we recalculated the expression levels by dividing the expression level of each gene to the corresponding gene expression level in the untreated control at each time point and comparing values in the same tissues. As shown in Fig. 1A, the transcript levels of BnNF-YA11, BnNF-YA2, BnNF-YA8 and BnNF-YA10 in leaves were higher than those of the control after 1 h of NaCl treatment and remained elevated up to 3 h of treatment, whereas those of, BnNF-YA7 and BnNF-YA9 in leaves were higher than those of the control up to 3 h of treatment. In roots, two BnNF-YA subfamily members (BnNF-YA9 and BnNF-YA10) were induced at 1 h or 3 h. Most salt-responsive BnNF-YAs were only moderately up-regulated (2~4 fold) upon exposure to salt stress, whereas BnNF-YA3 and BnNF-YA4/5 were slightly down-regulated in roots upon NaCl treatment.

We found that the expression of only two members of the BnNF-YB subfamily (i.e., BnNF-YB10, and BnNF-YB13) increased during a 3-h period in the absence of any treatments (Fig. S1B, S2B, and S3B). In leaves, the transcript levels of six BnNF-YBs (i.e., BnNF-YB3, BnNF-YB7, BnNF-YB8, BnNF-YB9, BnNF-YB10, and BnNF-YB14) were elevated after 1 h or 3 h of NaCl stress (Fig. 1B). In roots, NaCl treatments increased the transcript levels of BnNF-YB3, BnNF-YB13, and BnNF-YB14. Our results show that the levels of BnNF-YB1, BnNF-YB5, BnNF-YB6, BnNF-YB9, BnNF-YB11, and BnNF-YB12 transcripts were mostly not affected by salinity. Only the expression level of BnNF-YB4 in the roots was decreased at 3 h.

None of the five BnNF-YC subunits was regulated by the biological clock, since the expression levels of these genes remained constant in the absence of any treatments (Fig. S1C, S2C, and S3C). Only BnNF-YC5 was induced in the leaf samples both at the 1- and 3-h time-points under salinity (Fig. 1C). BnNF-YC2 transcripts were more abundant in roots subjected to NaCl treatments than in untreated roots.

Expression Patterns of BnNF-Y Genes in Leaves and Roots of Canola in Response to drought Stress

As shown in Fig. 2, all BnNF-YA members were responsive to PEG6000 treatments, which mimic drought stress, in leaf tissues. A 15% PEG treatment generally caused a 72% relative water content loss within 24 h (Table S2). The expression levels of BnNF-YA10, BnNF-YA11 and BnNF-Y12 in leaves were dramatically up-regulated to more than four-fold the levels observed in the untreated control (Fig. 2A). BnNF-YA8, BnNF-YA9, BnNF-YA10 and BnNF-YA13 were induced in roots under drought stress. Four BnNF-YAs (BnNF-YA8, -Y9, -Y10, and -YA13) responded to drought stress both in leaves and roots, particularly up to 3 h of treatment. The expression levels of five BnNF-YAs (BnNF-YA1, BnNF-YA2, -Y3, -Y4/5, and -YA6) decreased in leaves at 1 h and increased at 3 h.

Most members of the BnNF-YB subfamily also responded to PEG treatments, except for BnNF-YB12, while the expression level of BnNF-YB14 increased 80-fold in leaves after 1 h of treatment (Fig. 2B). As in the BnNF-YA subfamily, the transcripts of BnNF-YB were induced more strongly in leaves than in roots, since only 3 of 14 BnNF-YB members (BnNF-YB7, BnNF-Y10, and BnNF-Y14) were induced in roots subjected to drought stress. Four BnNF-YBs (BnNF-YB4, -B9, -B10 and -B11) exhibited decreased expression in the roots after 1 h of treatment. The transcript levels of four BnNF-YC members (BnNF-YC1, BnNF-YC2, BnNF-YC4, and BnNF-YC5) were elevated in both the leaf and root samples up to 3 h of drought treatment, while those of three BnNF-YCs (BnNF-YC1, BnNF-YC2, and -YC3) were initially inhibited in leaves at the 1-h time point (Fig. 2C).

Expression Patterns of BnNF-Y Genes of Canola in Leaves and Roots in Response to ABA Stress

Plants respond to abiotic stresses via both the ABA-dependent and ABA-independent pathways. Some NF-Ys were reported to be involved in ABA-dependent pathways, whereas others were not [11,13,15]. To determine whether canola NF-Ys function via the ABA pathway, we further examined the expression profiles of BnNF-Ys that were found to be responsive to salinity or drought stress (i.e., all BnNF-YA members and most BnNF-YBs and BnNF-YCs; Figs 1 and 2) under ABA treatments. All BnNF-YAs, except for BnNF-YA4/5 and BnNF-YA13, were responsive to 3-h of ABA treatment (Fig. 3A). The transcript levels of BnNF-YA1, -Y2, -Y3, -Y6, -Y7, -Y8, -Y9, -Y10 and BnNF-YA11 were elevated at 1 h. Also, most NaCl- or PEG- responsive BnNF-YB members were induced by ABA treatments, with the exception of BnNF-YB1 and BnNF-YB9 (Fig. 3B). The transcript level of BnNF-YB2 was inhibited in leaves after 1 h of ABA treatment. Interestingly, several BnNF-YBs (BnNF-YB2, -Y3, -Y7, -Y10, -Y11, -Y13, and -Y14) were greatly up-regulated by ABA treatment. Amongst the BnNF-YC members, only the transcripts
of BnNF-YC2 and BnNF-YC5 were increased in response to ABA treatment, and the upregulation was greater in the roots than in the leaves (Fig. 3C). The transcript levels of BnNF-YC1 remained almost constant from 1 h to 3 h of ABA treatment in all tissues examined.

To compare the gene expression levels of different BnNF-Ys in canola, the expression level of each NF-Y gene in the 0-h leaf or root samples (arbitrarily chosen as baseline in the real-time PCR analysis) was also determined by semi-quantitative RT-PCR analysis (35 cycles; Fig. S4). The BnNF-Ys showed diverse expression profiles. Interestingly, most BnNF-Ys were expressed at similar levels in 0-h leaves and 0-h roots.

Correlation between the gene expression patterns of BnNF-Y genes

Since NF-Y TFs are known to form functional complexes in plants [30,31], one would expect that NF-Ys with correlated expression profiles would be involved in the same regulatory processes under abiotic stress conditions. Extensive correlations were found to exist in the expression profiles of BnNF-Ys not only within the same subfamily, but also between the three subfamilies. The expression profiles of BnNF-YA6 and BnNF-YA11, of BnNF-YA8 and BnNF-YB14, and of BnNF-YC4 and BnNF-YB12 were highly correlated under NaCl treatment (R = 0.93, 0.948 and 0.961, respectively. Fig. 4A). Under drought stress, the expression of BnNF-YA10 and -YB3, -YC4, or -YB12 were highly correlated and of -YC4 and -YC5 were highly correlated (R = 0.915, 0.927, 0.913, 0.905, and 0.981, respectively. Fig. 4B). Under ABA treatments, BnNF-YB3 and BnNF-YC5 showed very similar expression profiles (R = 0.99. Fig. 4C).

The Promoters of NF-Y Genes Contain Diverse Stress-responsive Elements

Since most BnNF-Y members were found to be regulated by abiotic stress, we sought to identify the promoter sequences of their corresponding genes and to identify stress-relative cis-elements. We cloned the promoters of 12 BnNF-YAs, 13 BnNF-YBs, and 3 BnNF-YCs that were responsive to one or more of the three treatments used above (i.e., NaCl, PEG6000, and ABA). Using a BLASTN search and genome walking, we successfully acquired all
the promoter sequences, most of which consisted of around 1000 bp upstream of the ATG start codon. However, we were unable to acquire the promoter sequences of two BnNF-YA members (BnNF-YA4/5 and BnNF-YA6) and two BnNF-YB members (BnNF-YB1 and BnNF-YB8) longer than 1000 bp. We then identified cis-elements in these promoters using PLACE and PlantCARE software (Fig. 5), focusing on stress-related cis-elements, such as ABRE, CBF/DRE, MYB, and MYC. The promoters of each of the above-mentioned BnNF-Ys included at least two types of cis-elements, with the exception of the truncated BnNF-YA4/5 promoter sequences. All of the promoters, except for the promoter of BnNF-YA4/5, harbored MYB and MYC elements. More than half of the BnNF-YA and BnNF-YC promoters harbored at least one ABRE element, while only a few BnNF-Ys had DRE elements. We also constructed Arabidopsis cis-elements in the homologous NF-Y family and compared the arrangement of cis-elements in canola and Arabidopsis. Most canola NF-Y members had analogous cis-elements in Arabidopsis, but the number and arrangement of cis-elements differed (Fig. S5).

Canola promoter sequences showed greater similarities with those of B. rapa than with those of Arabidopsis thaliana

We previously showed that canola NF-Ys were more similar to those of the dicot Arabidopsis than to those of the monocot Oryza sativa (rice) based on putative amino acid sequences. In this study, we also used the promoter sequences to further explore the phylogenetic relationship amongst members of this family. All canola promoters grouped with promoters of their own homologs and formed clades with the corresponding subfamily members in the other species (Fig. 6). The promoter sequence of each canola NF-Y usually had the highest level of identity with B. rapa, and the lowest with Arabidopsis, with the exceptions of BnNF-YA6, BnNF-YB2 and BnNF-YB8. The promoter regions of these three BnNF-Ys were more similar to those of B. oleracea than to those of B. rapa.
Figure 3. Expression pattern of the BnNF-Y genes in the leaves and roots of plants exposed to 100 μM ABA. Transcript levels of BnNF-Y (A), BnNF-YB (B), and BnNF-YC (C) genes after exposure to 100 μM ABA. Three-week-old canola plantlets were treated with 100 μM ABA for 1 h and 3 h. Total RNA was extracted from leaves and roots for quantitative PCR (qRT-PCR) analysis. Transcript levels of each BnNF-Y were first normalized to those of the housekeeping gene 18S and then compared to levels at each time point in the control. Each data point represents the mean ± SE of three independent experiments. Significant differences between treated samples and untreated controls (same tissue only) are indicated by a single (P<0.05) or double (P<0.01) asterisk, according to Dunnett’s method of one-way ANOVA in SPSS. Expression levels in untreated samples (CK, 1-h or 3-h leaf and root samples) were arbitrarily set to 1.0. doi:10.1371/journal.pone.0111354.g003

Figure 4. Correlation in expression levels of the three BnNF-Y subfamily members. Correlated gene expression under salinity (A); drought (B); and ABA (C) treatments. Relative expression levels including 8 data points (relative NF-Y gene expression levels with and without treatments at 1 h or 3 h compared to the same 0-h samples) are used for each gene according to Fig. S1, S2, and S3. These data were fitted using linear regression analysis. doi:10.1371/journal.pone.0111354.g004
Arabidopsis treatment, and GmNF-YA3 showed a high level of similarity with YA homolog, increased ABA sensitivity during seed germination [32]. One NF-YA3, whereas the corresponding plants more sensitive than the control to a 200 mM NaCl overexpression of PEG, and ABA treatments. It was previously found that transcripts of YA subfamily [15]. Quantitative RT-PCR analysis indicated that in salinity, drought, or ABA treatments, as most previously found to be activated by photooxidative stress [21]. This gene from soybean was also shown to be involved in the stress response [21,22]. Canola GmNF-YA3, 5, 6, and 8. Of the reported members of the plant NF-YA subfamily, the transcript levels of several members (including NF-YA1, 5, 6, and 9) were affected by ABA, implying that these genes regulate the plant’s response to abiotic stress via an ABA-dependent pathway. This may also be the case for BnNF-YA1, BnNF-YA7, and BnNF-YA9, which exhibit similar expression patterns under salinity, drought, or ABA stress.

Arabidopsis NF-YA1 and its maize homologue ZmNF-YB2 were shown to enhance drought resistance, and maize plants overexpressing ZmNF-YB2 performed well in the field [11]. Barley HvNF-YB5, similar to Arabidopsis NF-YB1, showed around a 70-fold expression increase under salinity treatment [20]. *Triticum aestivum* (wheat) TaNF-YB2, homologous to Arabidopsis NF-YB1, was also upregulated in response to drought stress in the leaves [18]. BnNF-YB1, which is homologous to Arabidopsis NF-YB1, was induced only by drought in our study. Arabidopsis NF-YB2 was first found to be up-regulated by NaCl, mannitol, or cold (4°C) treatment [17]. Interestingly, NF-YB2 was identified as the top third gene to respond to osmotic stress. The expression of poplar NF-YB7 increased during a 15-d PEG or ABA treatment [33]. Transgenic Arabidopsis plants overexpressing poplar NF-YB7 exhibited an increased germination rate, root length, and photosynthetic rate under drought stress. Poplar NF-YB7 showed a high level of amino acid sequence identity with Arabidopsis NF-YB3, while Arabidopsis nsf-y3 mutant plants were more sensitive than the wild type to environmental stimuli. Canola BnNF-YB2 and BnNF-YB3, similar to Arabidopsis NF-YB2, were found to be responsive to NaCl and ABA treatments in this study. The expression of several poplar NF-YB5, such as NF-YB6, NF-YB11, and NF-YB13, was altered in the leaves of plants treated with PEG-6000 [19], while the expression of BnNF-YB7 and BnNF-YB11 (high identity with Poplar NF-YB11 and NF-YB13 in terms of the conserved core domain) was also up-regulated in the leaves under PEG or NaCl treatment (Fig. 1 and Fig. 2).

Previous studies revealed that a few NF-YC members were involved in the stress response [21,22]. One NF-YC member, NF-YC2, from both Arabidopsis and *Nicotiana tabacum* (tobacco), was found to be activated by photooxidative stress [21]. This gene

![Figure 5. Diagrammatic representation of stress-related elements in the BnNF-Y promoters. Elements in the BnNF-YA (A), BnNF-YB (B), and BnNF-YC (C) promoters. Symbols above the top line indicate elements that are in the forward orientation, and those below the bottom line indicate elements in the reverse orientation. MYCC(CANNTG) sequences are between the double lines, to indicate their palindromic nature. Numbers are the distance in nucleotides between the sequences and ATG start codon.](image)

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responded to multiple stresses, including heat, cold, and drought. In the same study, several Arabidopsis NF-YC members were shown to be induced by abiotic stress. The transcript levels of Arabidopsis NF-YC3 and NF-YC4 were increased under drought stress, while NF-YC4 was also induced by cold. Furthermore, Arabidopsis NF-YC2 formed a complex with NF-YB3, bZIP28, and NF-YA4 and was shown to play a role during endoplasmic reticulum stress [30]. PwHAP5 (Picea wilsonii) was up-regulated by NaCl, dehydration, and ABA treatment [22]. Ectopic expression of PwHAP5, which is homologous to Arabidopsis

Figure 6. Phylogenetic trees of Brassica and Arabidopsis NF-Y families based on the nucleotide sequences in the promoter regions. The phylogenetic trees were constructed by the neighbor-joining method implemented by MEGA software, version 4.1. The numbers at each branch point represent the bootstrap scores (1,000 replicates). A branch with a bootstrap score of below 50 was usually considered unreliable. a Phylogenetic tree of Brassica and Arabidopsis NF-YA promoters. Due to low levels of nucleotide similarity, BnNF-YA4 homologues in B. oleracea were not included here. b Phylogenetic tree of Brassica and Arabidopsis NF-YB promoters. Due to low levels of nucleotide similarity, BnNF-YB14 homologues in B. oleracea were not included here. c Phylogenetic tree of Brassica and Arabidopsis NF-YC promoters. Bol and Bra represent B. oleracea and B. rapa, respectively. The numbers after the species abbreviation correspond to the individual gene name. Most promoters were at least 1000 bp in length, except for those of BnNF-YA1 (595 bp), BnNF-YA4/5 (577), BnNF-YA6 (806), BnNF-YA10 (892), BnNF-YB1 (519), BnNF-YB7 (988), BnNF-YB8 (391), and BnNF-YB13 (867) and their homologues in B. oleracea, B. rapa, and Arabidopsis.

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NF-YC2, improved salinity tolerance in plants. In our previous study, we showed that BnNF-YC2 and BnNF-YC3 grouped with NF-YC2 and NF-YC3 [9] and, like these homologs in Arabidopsis, responded to salinity and drought stress.

Since plants have so many NF-Ys, functional redundancy seems inevitable. Arabidopsis NF-YB2 and NF-YB3 function additively in the long-day flowering pathway [34]. LEAFY COTYLEDON1 (LEC1) and LEAFY COTYLEDON1-LIKE (L1L) were shown to be involved in embryo development [53,56]. Many of the BnNF-Ys characterized in our study were responsive to salinity, drought, or ABA treatment. Some of these members in the same subfamily were clustered in the same phylogenetic clade based on their promoter sequences, such as BnNF-YA1 and BnNF-YA2 (Fig. 6). Further evidence is needed to confirm the roles of these proteins during the abiotic stress response. It would also be interesting to explore whether these proteins from the three subfamilies combine to form trimeric complexes, as reported in yeast and animal systems [37]. It is not known whether the NF-Y complex always has fixed components or whether the components differ under different conditions. Even though extensive correlations in the expression patterns of TaNF-Y subfamily members were identified [18], no three members from the three different BnNF-Y subfamilies exhibited the same expression pattern under all three treatments in our study. This phenomenon implies that the canola BnNF-Y complex does not always consist of the same monomers under different conditions.

The plant’s response to abiotic stress involves the transcriptional regulation of genes via their cis-regulatory elements. ABRE, MYB, and MYC elements are known to be involved in the ABA-dependent stress pathway, while the DRE element plays a role in the ABA-independent pathway [38–40]. Two ABRE elements were identified in the promoter region of Arabidopsis drought-responsive NF-YA5, which functioned in an ABA-dependent manner [13]. Interestingly, GmNF-YA3, a homolog of NF-YA5, harbored an additional DRE cis-acting element in its promoter, which suggests that soybean NF-YA3 may be involved in both pathways [16]. A previous study of the global expression patterns of rice plants subjected to various abiotic stresses identified more than 5 ABRE elements and were strongly up-regulated by ABA or drought treatment, had few DRE or ABRE elements, but several MYB or MYC elements, suggesting that MYB or MYC play roles in the abiotic stress response.

According to the well-known triangle theory [42,43], canola, an allopolyploid, originated from the hybridization of B. rapa (the A genome) and B. oleracea (the C genome), while all Brassica species basically arose from common Arabidopsis ancestors. Based on our NF-Y stress-related cis-element analysis and promoter sequence alignments, the upstream regulatory regions of NF-Y sequences of canola were found to be similar to those of Arabidopsis (Fig. 5 and 6). Through extensive comparisons based on nucleotide sequences, homoeologous segments conserved in canola and Arabidopsis were found to exhibit perfect collinearity [44]. Our study revealed that the promoters of canola NF-Ys were more similar to those of B. rapa than to B. oleracea. Canola (B. napus) was proposed to have multiple origins, and natural canola species were found to be more closely related to B. rapa species than to B. oleracea species, according to an Restriction Fragment Length Polymorphisms (RFLP) analysis of nuclear, chloroplastic, and mitochondrial DNA [45]. A recent study found that two canola self-incompatibility genes (S-locus glycoproteins, SLGs) and an S-locus receptor kinase (SRK) had higher levels of amino acid sequence identity with their B. rapa homologues than with those from B. oleracea [46], supporting the notion that a subset of canola sequences are more closely related to B. rapa than to B. oleracea.

In conclusion, this study represents an extensive evaluation of BnNF-Y family members under salinity, drought, or ABA stress. The results presented here offer a useful foundation for further studies of BnNF-Y proteins under abiotic stress conditions. Several BnNF-Y members in each subfamily showed similar expression patterns, indicating that these genes may have redundant functions. Members of different families were found to have similar expression patterns, suggesting that BnNF-Ys form a heterocomplex. Our BnNF-Y promoter analysis shows that multiple BnNF-Y members contain abiotic stress-responsive elements and provides clues as to the evolution of BnNF-Ys in Brassica species.

Supporting Information

Figure S1 Expression pattern of BnNF-Y genes in the leaves and roots of plants subjected to salinity stress for 1 or 3 h. The expression of BnNF-YA (A), BnNF-YB (B), and BnNF-YC (C) in the leaves and roots of plants exposed to 150 mM NaCl for the indicated periods of time. The transcript levels of each BnNF-Y gene were first normalized to those of the housekeeping gene 18S and then compared to the control (0-h level in the leaf). The expression levels of untreated samples (C, 0-h leaf samples) were arbitrarily set to 1.0. L, leaves; R, roots. CK, no treatment; Salt, NaCl treatment. Significant differences between different samples and 0-h samples (same tissue only) are indicated by a single (P<0.05) or double (P<0.01) asterisk, according to Dunnett’s method of one-way ANOVA in SPSS.

(DOC)

Figure S2 Expression pattern of BnNF-Y genes exposed to osmotic stress. The expression of BnNF-YA (A), BnNF-YB (B), and BnNF-YC (C) genes in the leaves of plants exposed to treatment with 15% (w/v) PEG-6000 for the indicated periods. The transcript levels of each BnNF-Y gene were first normalized to those of the housekeeping gene 18S and then compared to the levels in the 0-h leaf control. Expression levels in untreated samples (C, 0-h leaf samples) were arbitrarily set to 1.0. L, leaves; R, roots. CK, no treatment; Drought, PEG6000 treatment. Significant differences between different samples and 0-h samples (same tissue only) are indicated by a single (P<0.05) or double (P<0.01) asterisk, according to Dunnett’s method of one-way ANOVA in SPSS.

(DOC)

Figure S3 Expression pattern of the BnNF-Y genes after exposure to 100 μM ABA. The expression of BnNF-YA (A), BnNF-YB (B), and BnNF-YC (C) genes in the leaves and roots of plants exposed to 100 μM ABA. Transcript levels of each BnNF-Y were first normalized to those of the housekeeping gene 18S and then compared to levels at 0 h in the control (untreated) leaves. Expression levels in untreated samples (CK, 0-h leave samples) were arbitrarily set to 1.0. L, leaves; R, roots. CK, no treatment; ABA, ABA treatment. Significant differences between different samples and 0-h samples (same tissue only) are indicated by a
single (P<0.05) or double (P<0.01) asterisk, according to Dunnett’s method of one-way ANOVA in SPSS.

(DOC)

Figure S4  Semi-quantitative RT-PCR analysis of BnNF-Y expression in control leaves and roots. RT-PCR analysis was performed on untreated leaf (L) and root (R) tissue samples. The 18S housekeeping gene and BnNF-Ys were amplified for 28 cycles and 35 cycles, respectively.

(DOC)

Figure S5  Diagrammatic representation of NF-Y promoter regions. Arabidopsis NF-YA (A), NF-YB (B), and NF-YC (C) promoter regions. Symbols above the top line indicate elements in the forward orientation, and those below the bottom line are in the reverse orientation. MYC(CANNTG) sequences are in the forward orientation, and those below the bottom line are in the reverse orientation. MYC(CANNTG) sequences are between the double lines, to indicate their palindromic nature.

(DOC)

Table S1  Primers for BnNF-Y promoters. F indicates the PCR forward primer and R indicates the PCR reverse primer.

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Particularly, SP1, SP2, SP3 are three specific primers for Genome Walking.

(DOC)

Table S2  Relative water content (RWC) of drought-stressed plants. Plants were treated with 10%, 15% and 20% (w/v) PEG6000 solution for 24 h.

(DOC)

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Author Contributions

Conceived and designed the experiments: ML, LX GZ. Performed the experiments: ZL QT LX. Analyzed the data: LX ZL ML. Contributed reagents/materials/analysis tools: RX XY. Contributed to the writing of the manuscript: LX ML ZL.
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