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In Silico Identification and Expression Analysis of Nuclear Factor Y (Nf-Y) Transcription Factors in Cucumber

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Abstract: The nuclear factor Y (NF-Y) transcription factors (TFs) play vital regulatory roles in diverse developmental processes and responses to abiotic stresses in plants. However, the *NF-Y* genes remain largely unknown in cucumber. In this study, based on phylogenetic and protein structure analyses, we identified 27 *CsaNF-Y* members of this gene family in the cucumber genome, including 7 *NF-YAs*, 13 *NF-YBs*, and 7 *NF-YCs*. Their chromosome locations, gene structures, conserved domains, gene duplication, and promoter regions containing stress- and hormone-responsive *cis*-elements were also analyzed. As reported earlier, RNA-seq data showed that the expression of some *CsaNF-Y* genes was tissue-specific and varied during fruit development. The qRT-PCR results showed that all the detected *CsaNF-Y* genes were differentially regulated by drought and salt stress. Taken together, our findings provide a comprehensive understanding of *CsaNF-Y* genes in the development and abiotic stress response of cucumber and lay the foundation for future crop improvement.

Keywords: cucumber; nuclear factor-Y (NF-Y); gene family; expression analysis; abiotic stress

1. Introduction

Nuclear factor Y (NF-Y) transcription factors (TFs), also known as heme activator protein (HAP) or CCAAT-binding factor (CBF), are present in nearly all eukaryotes [1–3]. NF-Y TFs consist of three distinct subunits: NF-YA (HAP2 or CBF-B), NF-YB (HAP3 or CBF-A), and NF-YC (HAP5 or CBF-C), which are characterized by their conserved domains and sequence lengths [4,5]. Each of NF-Y TFs contain highly evolutionarily conserved domains in their central regions for DNA binding and NF-Y subunit interaction to form heterotrimeric complexes. In mammals and plants, NF-YB and NF-YC can form a dimer in the cytoplasm, and then this dimer is transferred into the nucleus to interact with NF-YA to form a NF-YA/NF-YB/NF-YC heterotrimer complex, which could bind to the CCAAT box in eukaryotic promoter regions to activate or inhibit the expression of downstream target genes [2,6,7].

Among yeast and mammals, each subunit of the NF-YA, NF-YB, and NF-YC proteins is encoded by a single gene, while each subunit of NF-Y protein is encoded by a multigene family in plants [8,9], suggesting that all the three types of *NF-Y* genes have undergone multiple duplications in plants. For example, 36 and 34 *NF-Y* genes were identified in *Arabidopsis* and rice (over 10 genes for each subunit), respectively [10,11]. In recent years, the *NF-Y* gene family has been identified and characterized in

a number of plant species, including 23 *NF-Y* genes in barley (*Hordeum vulgare*) [12], 24 in *Citrus grandis* [13], 24 in peach (*Prunus persica*) [14], 25 in castor bean (*Ricinus communis*) [15], 35 in tea plant (*Camellia sinensis*) [16], 42 in sorghum (*Sorghum bicolor*) [17], 44 in banana (*Musa acuminata*) [18], and 51 in cassava (*Manihot esculenta*) [19]. Many reports have shown that NF-Y TFs are involved in various physiological and biochemical processes of plants, such as flowering [20,21], seed germination [22], hypocotyl elongation [23], photomorphogenesis [24], root development [25,26], and grain filling and endosperm development [27–29]. In addition, the NF-Y TFs have been found to play critical roles in responses to a variety of biotic and abiotic stresses [2,5,19], and the biological functions of some NF-Y TFs in regulating plants' responses to various abiotic stresses have been studied in detail. For example, maize ZmNF-YA3 was found to have dual functions in photoperiod-dependent flowering and drought and high-temperature tolerance through binding to the promoter of FLOWERING LOCUS T-like12 (FT-like12) and interacting with several important TFs in JA- and ABA-associated signaling pathways [30]. A wheat nuclear factor Y (NF-Y) B subfamily gene *TaNF-YB3;l* can also confer tolerance to drought stress through the modulation of an ABA-signaling pathway [31].

Cucumber (*Cucumis sativus* L.) is one of the worldwide popular vegetable crops, and belongs to the genus *Cucumis* in *Cucurbitaceae*, which is prone to be threatened by various abiotic stresses [32]. Therefore, identification and characterization of genes related to stress resistance in cucumber would be of great significance to the improvement of resistance, yield, and quality of cucumber plants in future cucumber planting. In this study, we performed the genome-wide identification and systematic analysis of NF-Y TFs in cucumber, including genome distributions, gene and protein structures, phylogenetic relationships, and promoter sequences. Moreover, the expression patterns of the *CsaNF-Y* genes in various tissues and fruit development stages, as well as in response to various abiotic stress treatments were also determined. The results will provide a basis for further revealing the biological roles of *CsaNF-Y* genes in the development and stress responses of cucumber.

2. Materials and Methods

2.1. Plant Materials and Abiotic Stress Treatments

Cucumber (*Cucumis sativus* L. var. *sativus* cv. 9930) was used in this study. Two-week-old cucumber seedlings were subjected to abiotic stress treatments including drought and salt as described in our previous study [32]. In brief, the seedlings were treated with 10% PEG-6000 (Sigma–Aldrich, Shanghai, China) and 200 mM NaCl to induce drought stress and salt stress, respectively. The leaf samples were collected at different time points (0, 6, 12, and 24 h), and each sample was harvested from over 10 plants with three biological replicates. All of the samples were immediately frozen in liquid nitrogen, and stored at −80 °C until use.

2.2. Identification and Protein Property Analysis of NF-Y Members in Cucumber

The Hidden Markov model (HMM) of the NF-Y conserved domains (PF02045 and PF00808) were downloaded from Pfam database (http://pfam.xfam.org/), and then used as Blast queries to search against the cucumber proteome (version 2.0, http://cucurbitgenomics.org/organism/2) using HMMER software (http://hmmer.org) with an E-value of $1e^{-5}$. In addition, the amino acid sequences of the *Arabidopsis* NF-Y proteins were downloaded from TAIR (http://www.arabidopsis.org/) based on a previous report [10], and also used as query sequences for Blast searching against the cucumber proteome. After removal of the redundant sequences, the remaining sequences were checked by the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/) to scan the complete NF-Y domains. The protein properties of NF-Y members, such as number of amino acids, molecular weight (MW), isoelectric points (pI), and grand average of hydropathicity (GRAVY), were examined with the ProtParam program (http://web.expasy.org/protparam).

2.3. Chromosomal Location, Sequence Alignment, and Phylogenetic Analysis

The chromosomal locations of the cucumber *NF-Y* genes were obtained in the cucumber genome database (http://cucurbitgenomics.org/organism/2), and drawn with the MapChart software (https://www.wur.nl/en/show/Mapchart.htm) as previously described [33]. Duplication analysis was conducted using MCScanX software (http://chibba.pgml.uga.edu/mcscan2), and tandem and segmental duplication events were determined based on the previous description [34]. For sequence alignment of cucumber NF-Y proteins, their amino acid sequences were aligned using MAFFT (https://www.ebi.ac.uk/Tools/msa/mafft/) with default settings and visualized with GeneDoc. Moreover, multiple sequence alignments of NF-Y proteins from cucumber, *Arabidopsis thaliana*, and rice were also carried out with MAFFT, and a phylogenetic tree was constructed using the neighbor-joining (NJ) algorithm in MEGA 7.0 (https://www.megasoftware.net) with the bootstrap of 1000 replicates [35].

2.4. Gene Structure and Promoter Region Analysis

For gene structure analysis, the mRNA sequences and corresponding genomic DNA (gDNA) sequences of cucumber *NF-Y* genes were retrieved from the cucumber genome database, and the gene structures were determined by comparing the mRNA and gDNA sequences using GSDS tool (http://gsds.cbi.pku.edu.cn/). For promoter region analysis, the *cis*-elements in the 2.0 kb upstream region from translation start code ATG of each cucumber *NF-Y* gene were investigated using the PlantCARE tool (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

2.5. Expression Analysis of Cucumber NF-Y Genes With RNA-seq Data

For analysis of the cucumber *NF-Y* genes in different tissues, the RNA-seq data from ten different tissues (unexpanded ovary, fertilized ovary, unfertilized ovary, root, stem, leaf, male flower, female flower, tendril, and basal tendril) were retrieved in Sequence Read Archive under BioProject PRJNA80169 [36]. The expression of the cucumber *NF-Y* genes was analyzed based on these data, and the expression values were indicated as the RPKM values. To analyze the cucumber *NF-Y* genes during fruit ripening, the RPKM values of each *NF-Y* gene in leaves and fruits (10 days after pollination, 10 DAP; 20 DAP; 30 DAP; and 40 DAP) were obtained in the fruitENCODE database (http://www.epigenome.cuhk.edu.hk/encode.html). The expression values were log2-transformed and the heat maps were created with the TBtools software [37].

2.6. RNA Extraction and Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated using the Eastep Super Total RNA Extraction Kit (Promega, Madison, WA, USA) according to the manufacturer's protocols. The genomic DNA contamination was eliminated by RNase-free DNase I (TianGen, Beijing, China), and the first-strand cDNA was obtained using the M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The qRT-PCR was performed using the TB Green Premix Ex TaqII Kit (TaKaRa Biotechnology, Dalian, China) and the amplification program was set as previously described [32]. Three biological replicates were performed to test the expression of all the selected genes. Their relative transcript levels were calculated with the $2^{-\Delta\Delta CT}$ method by using *CsACT3* as an internal reference [38]. The qRT-PCR data were analyzed with one-way analysis of variance using Tukey's "Honest Significant Difference" method, and the significance was set at $P < 0.05$. Gene-specific primers used for qRT-PCR are presented in Table S1.

3. Results

3.1. Genome-Wide Identification of the NF-Y Gene Family in Cucumber

After the removal of redundant sequences and conserved domain analysis, 27 *NF-Y* genes were identified in the cucumber genome, including 7 *NF-YAs*, 13 *NF-YBs*, and 7 *NF-YCs*, which were named in accordance with their positions and relative distance on cucumber chromosomes (Table 1).

The gDNA and CDS lengths of the identified *CsaNF-Y* genes ranged from 357 bp (*CsaNF-YB3*) to 5290 bp (*CsaNF-YA4*), and 357 bp (*CsaNF-YB3*) to 1035 bp (*CsaNF-YA4*), respectively. Besides, these genes encoded proteins ranging from 118 (CsaNF-YB3) to 344 (CsaNF-YA4) amino acids in length, and the MWs and pIs values of CsaNF-Y proteins varied from 13.09 to 38.98 kDa and 4.68 to 9.53, respectively (Table 1). The grand average of hydropathicity (GRAVY) values of the CsaNF-Y proteins were calculated to be lower than 0 except for CsaNF-YC2 (Table 1), suggesting that they are hydrophilic.

3.2. Phylogenetic Analysis, Chromosomal Location, and Gene Duplication of the NF-Y Gene Family

To further understand the phylogenetic relationships of NF-Y proteins between cucumber and other plant species, a phylogenetic tree was generated with the NF-Y amino acid sequences from cucumber, *Arabidopsis* [10,39], and rice [11]. As shown in the phylogenetic tree (Figure 1), these NF-Y proteins could be classified into three subfamilies named as NF-YA, NF-YB, and NF-YC, which was in accordance with our identification results. In addition, cucumber NF-Ys displayed closer relationships with AtNF-Ys than with OsNF-Ys (Figure 1).

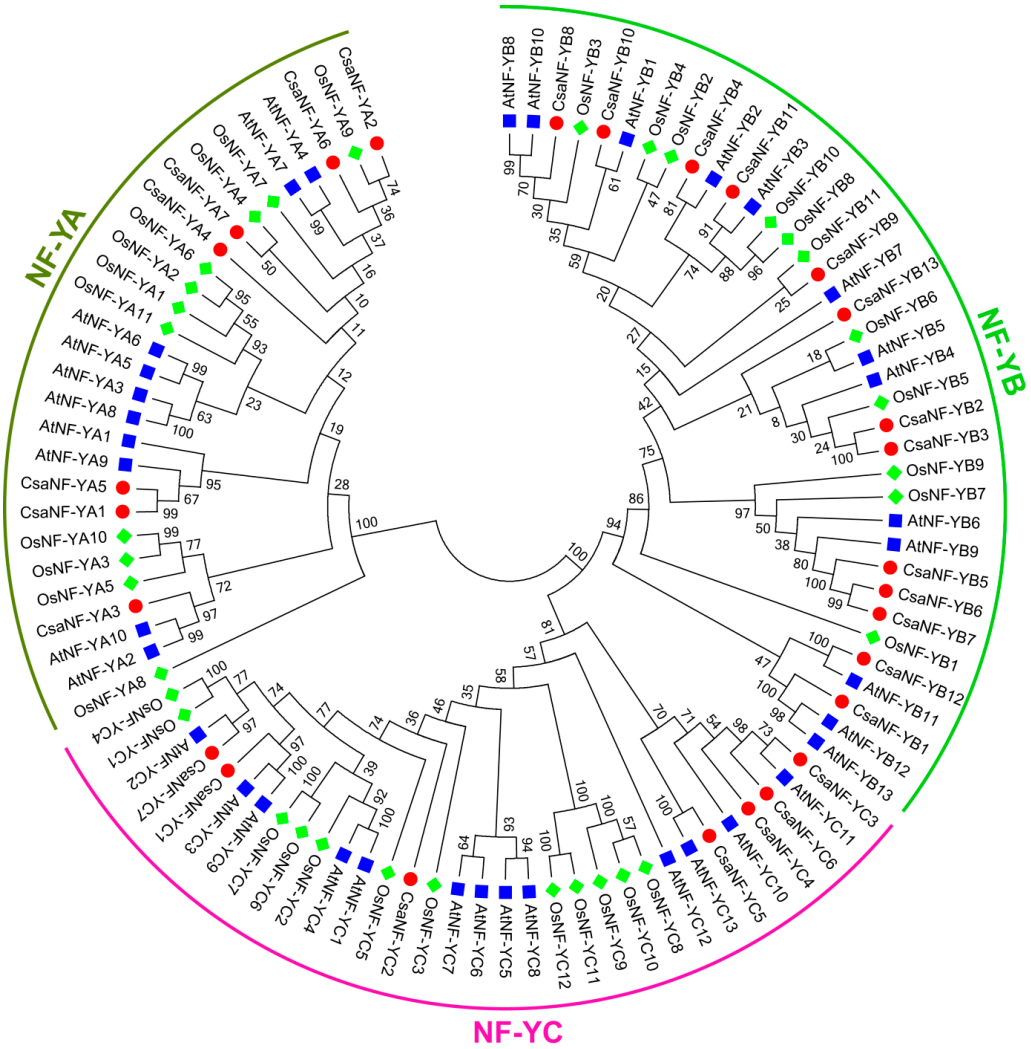

Figure 1. Phylogenetic analysis of *NF-Y* gene family among cucumber, *Arabidopsis*, and rice. Full-length NF-Y proteins from cucumber, *Arabidopsis*, and rice were aligned using MAFFT, and the phylogenetic tree was created with MEGA 7.0 by the neighbor joining (NJ) method with 1000 bootstrap replicates. The protein IDs from *Arabidopsis* and rice are listed in Table S2.

Table 1. Identification and characterization of nuclear factor Y (*NF-Y*) family genes in cucumber.

Nomenclature	Locus	Chromosomal Position	gDNA (bp)	CDS (bp)	Protein			
					Length (aa)	MW (KDa)	pI	GRAVY
CsaNF-YA1	Csa1G613580	Chr1: 24112521–24117512	3199	810	269	29.52	6.56	−0.984
CsaNF−YA2	Csa3G644810	Chr3: 25263223–25264735	1513	444	147	16.91	9.00	−0.122
CsaNF-YA3	Csa3G782710	Chr3: 30348958–30352913	3395	975	324	35.46	9.40	−0.436
CsaNF-YA4	Csa4G159320	Chr4: 8340821–8346110	5290	1035	344	38.98	8.63	−0.489
CsaNF-YA5	Csa6G439960	Chr6: 20632195–20637169	3212	1026	341	37.10	6.53	−0.895
CsaNF-YA6	Csa6G525440	Chr6: 28505741–28509554	3814	609	202	22.53	8.58	−1.032
CsaNF-YA7	Csa7G428130	Chr7: 16427189–16430567	2325	957	318	35.57	6.96	−0.733
CsaNF-YB1	Csa1G015800	Chr1: 2159806–2163445	3332	471	156	17.47	4.68	−0.547
CsaNF-YB2	Csa1G569510	Chr1: 21018573–21018944	372	372	123	14.11	6.10	−0.903
CsaNF-YB3	Csa1G569530	Chr1: 21023622–21023978	357	357	118	13.63	5.29	−0.815
CsaNF-YB4	Csa3G047800	Chr3: 3332696–3333441	555	555	184	19.54	5.46	−0.630
CsaNF-YB5	Csa3G048940	Chr3: 3452386–3453255	870	411	136	15.10	5.51	−0.680
CsaNF-YB6	Csa3G049440	Chr3: 3458401–3459120	720	720	239	26.50	5.29	−0.495
CsaNF-YB7	Csa3G055940	Chr3: 3469618–3470370	753	666	221	24.78	5.04	−0.533
CsaNF-YB8	Csa3G736780	Chr3: 28408903–28412596	3242	522	173	18.76	6.09	−0.758
CsaNF-YB9	Csa4G037610	Chr4: 3160771–3161568	722	678	225	24.73	7.76	−0.638
CsaNF-YB10	Csa5G175930	Chr5: 7498740–7502620	3881	528	175	19.17	5.26	−0.709
CsaNF-YB11	Csa6G112490	Chr6: 7775649–7777337	925	606	201	20.62	6.31	−0.636
CsaNF-YB12	Csa6G425700	Chr6: 19919807–19922480	2190	483	160	18.05	4.77	−1.087
CsaNF-YB13	Csa7G395250	Chr7: 15134980–15135499	520	483	160	17.37	5.03	−0.781
CsaNF-YC1	Csa1G570150	Chr1: 21086208–21089499	1214	780	259	28.70	5.89	−0.518
CsaNF-YC2	Csa4G049050	Chr4: 3878004–3878711	513	360	119	13.09	7.76	0.035
CsaNF-YC3	Csa5G583350	Chr5: 20754210–20758105	3896	852	283	31.66	5.14	−0.918
CsaNF-YC4	Csa6G294440	Chr6: 14150180–14153107	1843	573	190	21.31	9.53	−1.115
CsaNF-YC5	Csa6G365140	Chr6: 16501105–16501524	420	420	139	15.72	9.01	−0.658
CsaNF-YC6	Csa6G426970	Chr6: 20154306–20158203	3898	843	280	31.23	4.90	−0.816
CsaNF-YC7	Csa7G051420	Chr7: 3270343–3272852	2510	801	266	29.96	5.96	−0.705

All the *CsaNF-Y* genes were distributed throughout all seven chromosomes of cucumber, except for chromosome 2 (Figure 2). Chromosomes 3 and 6 harbored the largest number of *CsaNF-Y* genes (seven genes each), followed by chromosome 1 (five genes), and two chromosomes (chromosomes 4 and 7) each contained three *CsaNF-Y* genes, while chromosome 5 harbored the fewest *CsaNF-Y* genes (two genes) (Figure 2).

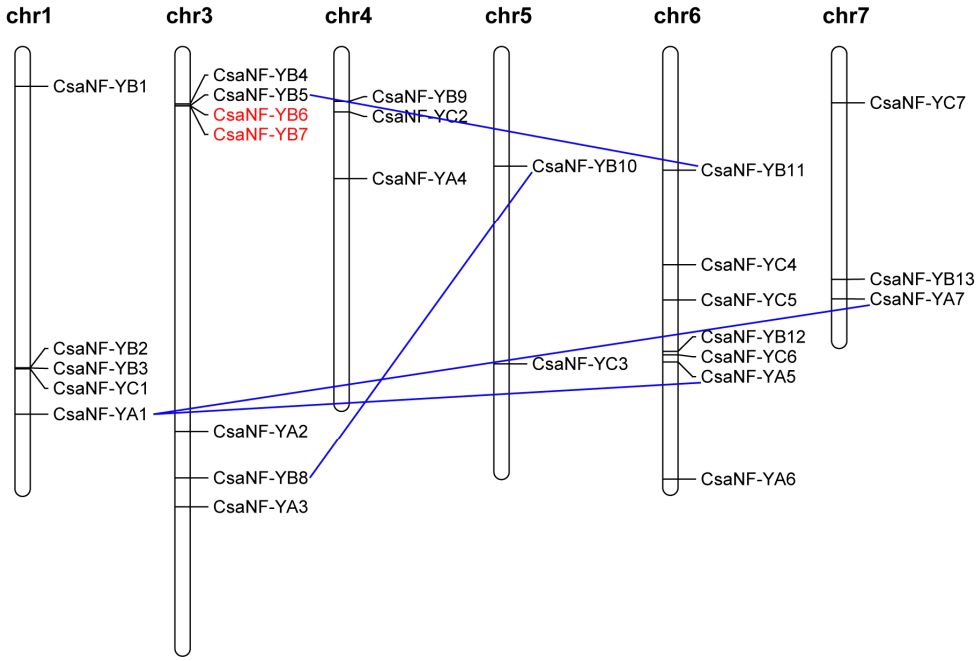

Figure 2. Chromosomal distribution of cucumber *NF-Y* genes. The chromosome number is indicated at the top, and chromosome 2 harbors no *CsaNF-Y* genes.

To identify potential segmental and tandem duplications within the cucumber genome, we assessed the duplication events among the identified *CsaNF-Y* genes using the criteria from a previous study [34]. Tandem duplication was detected for one pair of *CsaNF-Y* genes, *CsaNF-YB6* and *CsaNF-YB7*, whereas segmental duplication was found for four pairs of genes, including *CsaNF-YA1* and *CsaNF-YA5*, *CsaNF-YA1* and *CsaNF-YA7*, *CsaNF-YB5* and *CsaNF-YB11*, and *CsaNF-YB8* and *CsaNF-YB10* (Figure 2).

3.3. Conserved Domain Analysis of CsaNF-Y Proteins

Full-length CsaNF-YAs, CsaNF-YBs, and CsaNF-YCs were aligned by MAFFT to determine the evolutionarily conserved domains for DNA-binding and NF-Y subunit interaction. The multiple alignment results revealed that a heterodimerization domain (for interaction with other subunit of NF-Y) and a DNA-binding domain (for recognition of CCAAT binding sites) were commonly found in CsaNF-Y proteins, and these domains of CsaNF-YAs were found to be much more conserved than those of CsaNF-YBs and CsaNF-YCs (Figure 3). The CsaNF-YA proteins contained two highly conserved sub-domains, one domain for interaction with NF-YB/C and one domain for DNA binding, which were separated by a relatively conserved linker (Figure 3A). In addition, three histidine (H) and three arginine (R) residues were present in DNA binding domain of the majority of the CsaNF-YAs, while CsaNF-YA2 had obviously different residues from other six CsaNF-YA members in DNA binding domain (Figure 3A). Furthermore, the core histone structures including the HFM (histone fold motif) of the core histone H2B and H2A were present in CsaNF-YB and CsaNF-YC proteins, respectively (Figure 3B,C). CsaNF-YB proteins contained a DNA-binding domain, a NF-YA interaction domain, and a NF-YC interaction domain, and the region of the NF-YC interaction domain was so long that it overlapped with the NF-YA interaction and DNA-binding domains (Figure 3B). Most CsaNF-YC proteins contained one DNA-binding domain, two NF-YA interaction domains and one

NF-YB interaction domain. The two NF-YA interaction domains were separated by the NF-YB interaction domain, and the DNA-binding domain was embedded in the first NF-YA interaction domain (Figure 3C).

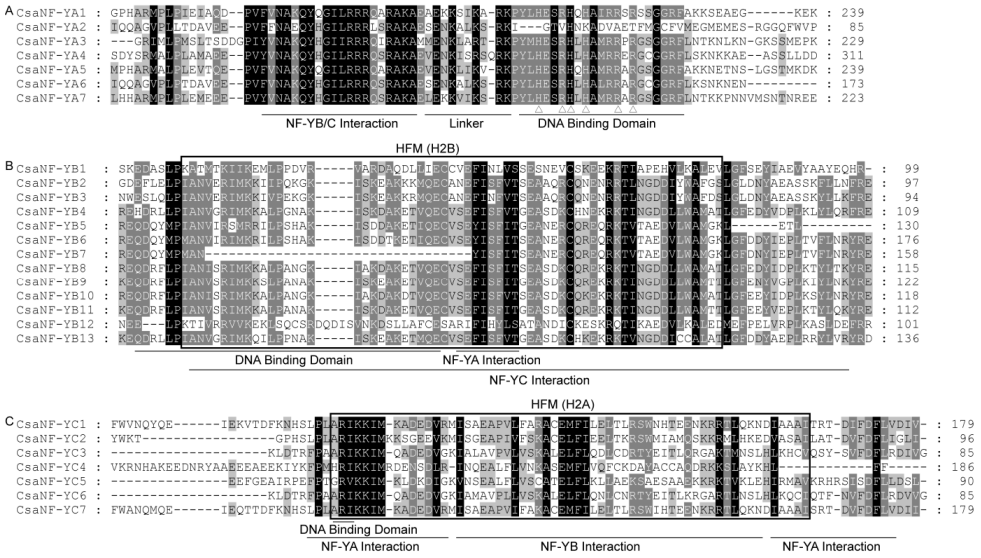

Figure 3. Multiple alignments of CsaNF-Y protein sequences. (**A**) Multiple alignments of CsaNF-YA protein sequences. (**B**) Multiple alignments of CsaNF-YB protein sequences. (**C**) Multiple alignments of CsaNF-YC protein sequences. The evolutionarily conserved domains including the DNA-binding domains and the NF-Y subunit interaction domains are underlined. The histone-fold motif (HFM) of the core histones (H2A and H2B) are boxed. Three histidine (H) and three arginine (R) residues in the DNA binding domain of the CsaNF-YA proteins are marked with triangles.

3.4. Gene Structure Analysis of CsaNF-Y Genes

To determine the exon–intron structures of the *CsaNF-Y* genes, we compared the mRNA sequences with the corresponding gDNA sequences by GSDS tool. As a result, the intron numbers of *CsaNF-Y* genes varied from 0 to 5 (Figure 4). Most *CsaNF-YA* genes contained four introns, with the exception of *CsaNF-YA2* and *CsaNF-YA4*, which had two and five introns, respectively. Among the *CsaNF-YB* genes, three contained four introns (*CsaNF-YB1*, *CsaNF-YB8*, and *CsaNF-YB10*), one contained two introns (*CsaNF-YB12*), two contained only one intron (*CsaNF-YB5* and *CsaNF-YB7*), while seven other *CsaNF-YB* genes were intronless (Figure 4). As for the *CsaNF-YC* genes, the largest number of introns was found for *CsaNF-YC3* and *CsaNF-YC6* (five introns); *CsaNF-YC7* and *CsaNF-YC4* contained one and two introns, respectively; while *CsaNF-YC1*, *CsaNF-YC2*, and *CsaNF-YC5* had no intron (Figure 4).

3.5. Bioinformatics Analysis of Putative CsaNF-Y Promoters

To investigate the potential functions of *CsaNF-Y* genes, 2.0-kb promoter region of each *CsaNF-Y* gene was analyzed by PlantCARE. A total of 15 *cis*-elements were found in the promoters of the *CsaNF-Y* genes, including six stress-responsive elements and nine hormone-responsive elements (Figure S1). Amongst these *cis*-elements, ARE (anaerobic induction element) and ERE (ethylene-responsive element) were the most abundant stress-responsive element and hormone-responsive element, being present in 24 and 21 *CsaNF-Y* genes, respectively. In addition, five other stress-responsive elements, including MBS, LTR, W-box, WUN-motif, and TC-rich repeats, were also present in a series of *CsaNF-Y* genes (Figure S1), indicating their important roles in responses to various stresses. Besides ERE, five kinds of other hormone-responsive *cis*-elements, including abscisic acid (ABA)-responsive element (ABRE), methyl jasmonate (MeJA)-responsive element (CGTCA-motif), salicylic acid (SA)-responsive elements (TCA-element), auxin-responsive elements (AuxRR-core and TGA-element), and gibberellin-responsive elements (P-box, GARE-motif, and TATC-box), were found in the promoter regions of 16, 12, 11, 12, and

14 *CsaNF-Y* genes, respectively (Figure S1), indicating that the *CsaNF-Y* genes may also play important roles in hormone responses.

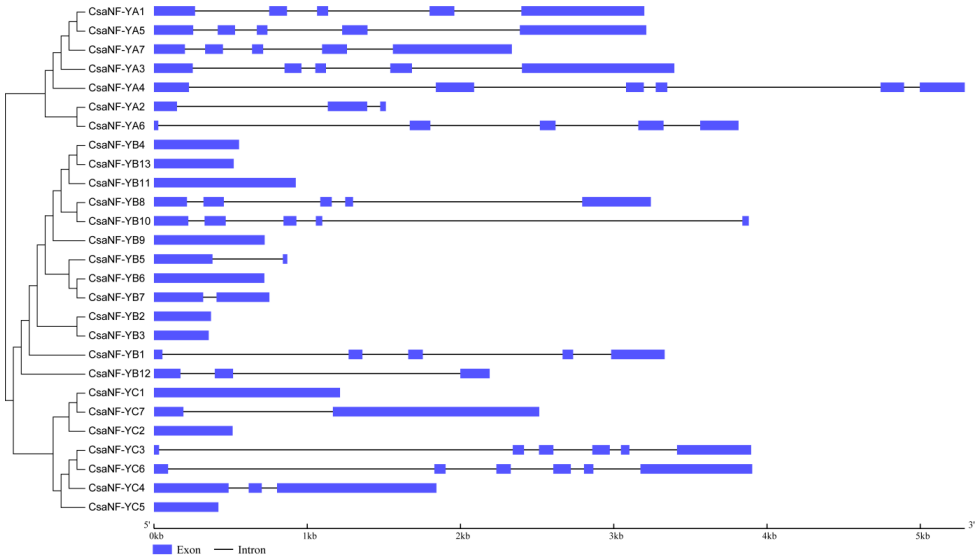

Figure 4. Gene structure analysis of *CsaNF-Y* genes according to the phylogenetic relationship. The blue boxes and black lines indicate exons and introns, respectively, and their lengths can be estimated by the scale bar at the bottom.

3.6. Expression Profiles of CsaNF-Y Genes in Various Tissues and Organs

To gain insights into the tissue expression profiles of *CsaNF-Y* genes, we analyzed their expression levels in ten tissues (unexpanded ovary, fertilized ovary, unfertilized ovary, root, stem, leaf, male flower, female flower, tendril, and basal tendril) using publicly available RNA-seq data [36]. According to the transcriptomic dataset, five genes (*CsaNF-YB2*, *CsaNF-YB3*, *CsaNF-YB5*, *CsaNF-YB6*, and *CsaNF-YB7*) showed RPKM values lower than 1.0 in all the tested tissues, while other 22 *CsaNF-Y* genes were expressed in at least one of the tested tissues (Figure S2). Several genes, such as *CsaNF-YB1*, *CsaNF-YB8*, *CsaNF-YC1*, and *CsaNF-YC7*, had relatively higher expression levels in all detected tissues (Figure S2), suggesting that they might play essential roles in plant growth. In addition, some *CsaNF-Y* genes exhibited tissue-specific expression patterns, suggesting that these genes possibly play certain roles in organ development of cucumber. For example, *CsaNF-YA3* and *CsaNF-YB13* were more abundantly expressed in roots, and *CsaNF-YB1* and *CsaNF-YC7* had higher expression in unfertilized ovaries. In addition, *CsaNF-YB9* and *CsaNF-YC2* were highly expressed in male and female flowers, respectively (Figure S2).

To better examine the functions of *CsaNF-Y* genes in the growth and development of cucumber, their expression profiles during fruit development were analyzed according to a previous study [40]. The RPKM values of 10 *CsaNF-Y* genes (*CsaNF-YA2*, *CsaNF-YA4*, *CsaNF-YB2*, *CsaNF-YB3*, *CsaNF-YB5*, *CsaNF-YB6*, *CsaNF-YB7*, *CsaNF-YB13*, *CsaNF-YC2*, and *CsaNF-YC6*) were 0 or lower than 1.0 during fruit development, suggesting that they are not expressed in fruit (Figure S3). The expression levels of several *CsaNF-Y* genes, such as *CsNF-YA1*, *CsNF-YA3*, *CsNF-YA6*, and *CsNF-YC7*, gradually increased during fruit development and reached the maximum at 40 DPA, indicating their vital roles in fruit ripening. *CsNF-YA5* and *CsNF-YA7* exhibited up-regulated expression at the onset of fruit development (20 DPA or 30 DPA), but their expression levels were significantly down-regulated at 40 DPA (Figure S3). In contrast, *CsaNF-YB4*, *CsaNF-YC4* and *CsaNF-YC5* showed significant decreases in expression during fruit development (Figure S3), suggesting their possible roles in suppressing the ripening of fruit.

3.7. Expression Patterns of CsaNF-Y Genes in Response To Various Abiotic Stresses

To study the roles of *CsaNF-Y* genes in abiotic stress response, the expression profiles of six selected *CsaNF-Y* genes (2 each in distinct subunits) in response to drought and salt stresses were assessed using qRT-PCR. All the six *CsaNF-Y* genes displayed relatively high expression in various tissues and organs (Figures S2 and S3). Upon the drought stress, the expression of four *CsaNF-Y* genes (*CsaNF-YA5*, *CsaNF-YA6*, *CsaNF-YB4*, and *CsaNF-YC7*) increased to varying degrees, while that of *CsaNF-YC3* decreased (Figure 5). The expression levels of the up-regulated *CsaNF-Y* genes peaked at 12 h, with the exception of *CsaNF-YB4*, whose expression reached the peak at 6 h (Figure 5). Under salt stress conditions, all of the tested *CsaNF-Y* genes were significantly up-regulated (Figure 6). The expression levels of *CsaNF-YA5* and *CsaNF-YB11* significantly increased and reached the highest values at 6 h, while those of *CsaNF-YB4*, *CsaNF-YC3*, and *CsaNF-YC7* reached the maximum values until 24 h (Figure 6). Notably, *CsaNF-YA6* was the most observably induced gene, and its expression sharply increased at 12 h and displayed a 23.2-fold change under salt stress (Figure 6).

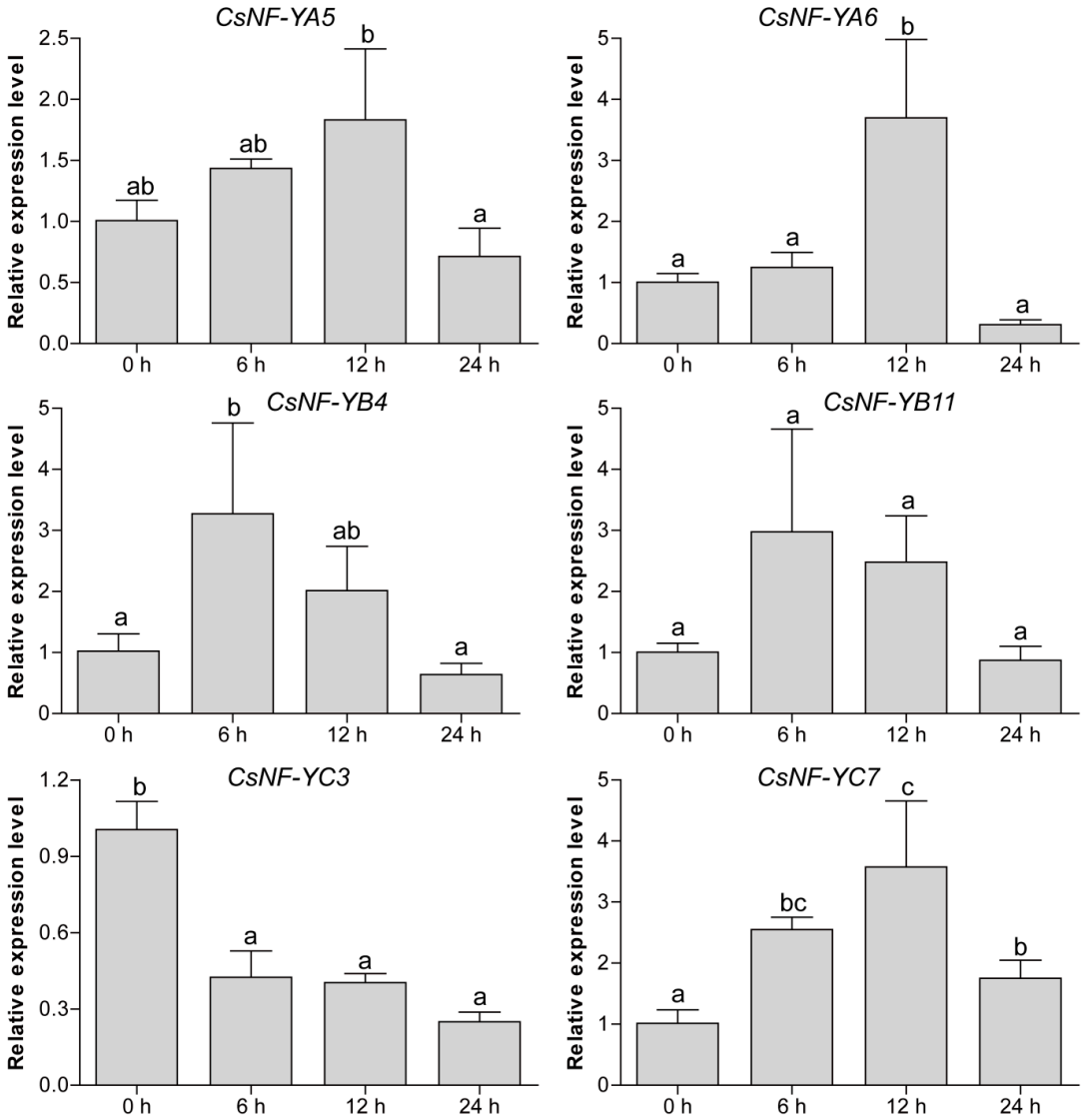

Figure 5. Expression analysis of six selected *CsaNF-Y* genes in response to drought stress using qRT-PCR. Vertical bars indicate the standard error of the mean, and different letters are significantly different.

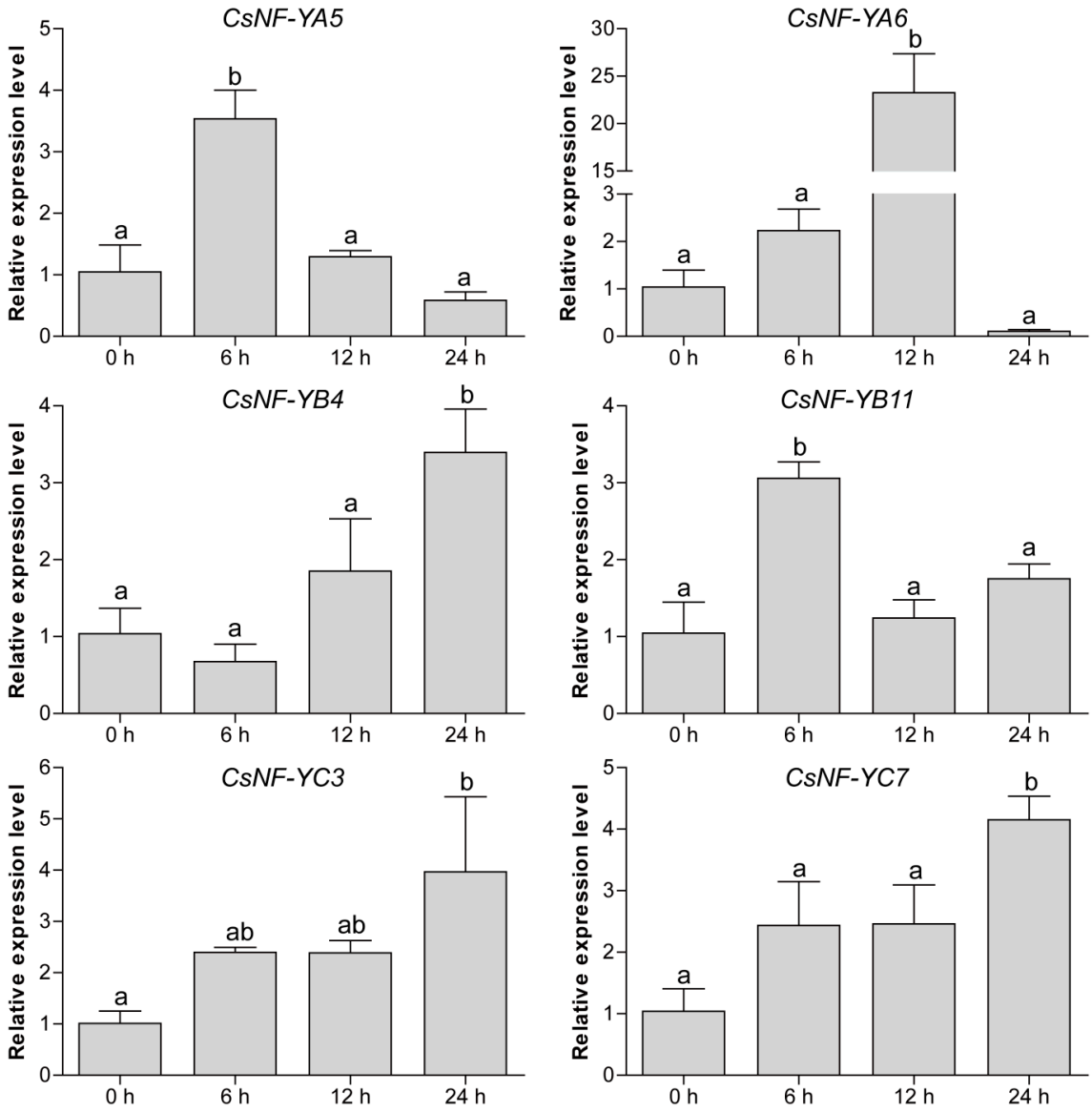

Figure 6. qRT-PCR expression analysis of six selected *CsaNF-Y* genes in response to salt stress. Vertical bars indicate the standard error of the mean, and different letters are significantly different.

4. Discussion

In this study, genome-wide identification and characterization of *NF-Y* gene family were carried out, and a total of 27 *CsaNF-Y* genes (including 7 *NF-YAs*, 13 *NF-YBs*, and 7 *NF-YCs*) were identified in cucumber (Table 1). Similar to that in other plants, the NF-Y subunit in cucumber also exists in multiple copies (Table 1; Figure 1), suggesting that the cucumber *NF-Y* gene family has undergone a number of duplication events. In the present study, eight segmentally duplicated genes and two tandemly duplicated genes were identified in the cucumber genome (Figure 2). In previous research, a total of 15 and 4 *OsNF-Y* genes could be assigned as segmentally and tandemly duplicated genes in rice [11], 12 and 9 *SiNF-Y* genes made up to six segmental duplication events and four tandem duplication events, respectively [41]. These results indicate that both of segmental and tandem duplications play a crucial role in the expansion of *NF-Y* genes across different plants.

The phylogenetic analysis suggested that the NF-Y proteins from cucumber, *Arabidopsis*, and rice could be classified into three subfamilies named as NF-YA, NF-YB, and NF-YC, and NF-Y proteins from cucumber had closer phylogenetic relationship with those from *Arabidopsis* than with

those from rice (Figure 1). In addition, the multiple sequence alignments revealed that nearly all CsaNF-Y proteins possess evolutionarily conserved domains for DNA binding and heterotrimerization (Figure 3), which is in agreement with the NF-Y protein characteristics [14,16,41,42]. Previous studies have shown that *NF-YA* genes always display a highly structured intron–exon organization and the majority of them have 3–6 introns, while the *NF-YB* and *NF-YC* genes exhibit variable intron/exon organizations [15,16,41]. In this study, *CsaNF-YA* genes contained 2–5 introns, and multiple intron/exon organizations were observed for *NF-YB* and *NF-YC* genes (Figure 4), which is in agreement with previous studies, revealing the conserved features of the evolution of these subunits in plants. In addition, the *CsaNF-YBs* (7/13) and *CsaNF-YCs* (3/7) were prevalently lack of introns (Figure 4), which is a universal feature of *NF-YB* and *NF-YC* genes in other plant species, such as peach, in which 6 out of 12 *NF-YBs* and 4 out of 6 *NF-YCs* have no introns [14]; tea plant, in which 7 out of 15 *NF-YBs* and 6 out of 10 *NF-YCs* have no introns [16]; castor bean, in which 8 out of 12 *NF-YBs* and 3 out of 7 *NF-YCs* have no introns [15]; chickpea, in which 11 out of 21 *NF-YBs* and 7 out of 11 *NF-YCs* have no introns [43]; and banana, in which 12 out of 16 *NF-YBs* and 12 out of 14 *NF-YCs* have no introns [18]. The similar conserved domain arrangements and intron/exon organizations in each subunit indicate that *NF-Y* genes are highly conserved during the evolution of plants, which may contribute to their crucial biological functions.

It has been demonstrated that *NF-Y* genes have diverse expression patterns in different tissues of plants. For example, five *RcNF-Y* genes were highly and specifically expressed in the early and later stages of developing endosperm in castor bean [15]. In walnut, 24 out of 33 *JrNF-Y* genes displayed higher expression levels in female flower buds than in leaf buds, suggesting that these genes play direct or indirect roles in the process of flower bud development [42]. In this study, nearly all *CsaNF-YA* genes were highly expressed in ovaries at different stages (Figure S2), indicating that they might participate in the regulation of ovary development in cucumber. In addition, some *CsaNF-Y* genes showed high abundance of expression in flowers (Figure S2), suggesting their importance in the control of flowering. Amongst them, *CsaNF-YB4* and *CsaNF-YB11* are orthologous to *AtNF-YB2* and *AtNF-YB3*, both of which have been identified as regulators that control photoperiod-dependent flowering time by interacting with other AtNF-Y subunit proteins [44]. In addition, OsNF-YB8 and OsNF-YB10 were clustered with CsaNF-YB4, CsaNF-YB11, AtNF-YB2, and AtNF-YB3 (Figure 1), and overexpression of *OsNF-YB8* and *OsNF-YB10* could complement the late flowering phenotype of *Arabidopsis nf-yb2 nf-yb3* mutants [21]. *CsaNF-YC1* is orthologous to *AtNF-YC3* and *AtNF-YC9*, which were found to have overlapping functions in CONSTANS-mediated floral promotion [24,45]. It should be noted that *CsaNF-YB13* was specifically expressed in root (Figure S2), indicating its particular role in root development. Furthermore, the expression levels of several *CsaNF-Y* genes, such as *CsNF-YA1*, *CsNF-YA3*, *CsNF-YA6*, and *CsNF-YC7*, gradually increased throughout the fruit development stage, while some other *CsaNF-Y* genes, such as *CsaNF-YB4*, *CsaNF-YB11*, *CsaNF-YC4*, and *CsaNF-YC5*, showed significant decreases in expression during fruit development (Figure S3), suggesting that they might participate in the regulation of fruit development, and have the potential to improve the fruit yield of cucumber.

Recently, numerous reports have revealed the important functions of *NF-Y* genes in mediating abiotic stress tolerance in plants [2,46]. In this study, six stress-responsive elements and nine hormone-responsive elements were observed in the promoters of *CsaNF-Y* genes (Figure S1), suggesting that some of the *CsaNF-Y* genes may also participate in stress responses. We then determined the expression of *NF-Y* genes under drought and salt stresses by qRT-PCR. Under the two stress conditions, four *CsaNF-Y* genes (*CsaNF-YA5*, *CsaNF-YA6*, *CsaNF-YB4*, and *CsaNF-YC7*) were up-regulated at certain time points, suggesting that these genes may play positive roles in response to these abiotic stresses (Figures 5 and 6). The possible roles of these *CsaNF-Y* genes in regulating stress tolerance should be further determined through genetic transformation. In a previous study, *Picea wilsonii PwNF-YB3* transcript was induced by salinity and PEG treatments, and overexpression of *PwNF-YB3* resulted in enhanced drought and salt tolerance in transgenic *Arabidopsis* plants [47]. Similarly, bermudagrass

CdtNF-YC1 was up-regulated under salt, drought, and ABA stresses, and its overexpression conferred tolerance to drought and salt stress in transgenic rice and seashore paspalum plants [48,49]. Therefore, the four *CsaNF-Y* genes (*CsaNF-YA5*, *CsaNF-YA6*, *CsaNF-YB4*, and *CsaNF-YC7*) may have the potential roles in the resistance to drought and salt stress. In addition, *CsaNF-YB11* was induced by salt stress but not by drought stress (Figures 5 and 6), implying its special role in response to salt stress. *CsaNF-YB4* and *CsaNF-YB11* are homologous with *AtNF-YB2* and *AtNF-YB3* (Figure 1), which were reported to specifically enhance drought and heat stress tolerance in *Arabidopsis* through specifically activating the drought-inducible and heat-inducible genes, respectively [50]. Moreover, *CsaNF-YC3* was induced by salt stress but suppressed by drought stress (Figures 5 and 6), indicating that it may have opposite functions in response to salt and drought stress. In a previous study, overexpression of *TaNF-YA10-1* in *Arabidopsis* significantly increased the sensitivity of the plant to salinity, but the transgenic plants displayed enhanced drought tolerance [51]. Therefore, the *CsaNF-Ys* are crucial candidate genes for genetic improvement of drought and salt stress tolerance in cucumber.

5. Conclusions

In this study, a total of 27 *NF-Y* family genes including 7 *NF-YAs*, 13 *NF-YBs*, and 7 *NF-YCs* were identified in the whole genome of cucumber. A comprehensive analysis of these *NF-Y* genes was preformed, including phylogenetic relationships, genomic organizations, gene duplication events, conserved domains, gene structures, and promoter regions. In addition, RNA-seq and qRT-PCR assays were employed to investigate the possible roles of *CsaNF-Y* genes in fruit development and abiotic stress response. The in silico expression results highlighted the importance of *CsaNF-Y* genes in fruit development, and modulation of the expression levels of *CsaNF-Y* genes in response to drought and salt stresses indicates their key roles in stress tolerance. Our results provide the relevant information for further investigation of the biological functions of *NF-Y* genes and improving cucumber agricultural traits in the future.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/2/236/s1: Table S1: The gene-specific primers used for qRT-PCR, Table S2: NF-Y IDs from *Arabidopsis* and rice, Figure S1: Analysis of stress-responsive and hormone-responsive *cis*-elements in *CsaNF-Y* genes. The numbers of *cis*-elements in the promoter region of each *CsaNF-Y* gene are boxed and colored, Figure S2: Expression profiles of *CsaNF-Y* genes in various cucumber tissues and organs. The vertical color scale from red to blue indicates the expression levels of *CsaNF-Y* genes in log2-transformed RPKM + 1 values, Figure S3: Expression profiles of *CsaNF-Y* genes during fruit development. The vertical color scale from red to blue indicates the expression levels of *CsaNF-Y* genes in log2-transformed RPKM + 1 values.

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