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IN SILICO ANALYSIS AND FUNCTIONAL VALIDATION OF *SINF-YC* GENES IN FOXTAIL MILLET (*SETARIA ITALICA* (L.)

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Abstract

Nuclear transcription factor YCs (NF-YCs) are universally involved in plant development and abiotic stress response. As a typical C_4 crop, foxtail millet has significant research value for studies of functional genes and mechanisms of plant stress resistance. To further investigate the correlation between the structure and function of the *SiNF-YC* genes in foxtail millet (*Setaria italica*), bioinformatic analysis of the *SiNF-YCs* on the subcellular localization, physicochemical properties, and *cis* acting elements were performed in this study. The results showed that a total of 12 *SiNF-YC* subfamily genes that distributed on chromosomes 1-9 were identified from the foxtail millet genome, which encoding 129-469 amino acids. The subcellular localization prediction results showed that the proteins encoded by this gene subfamily are mainly located in the nucleus, with a few being extracellular secreted. The multiple *cis*-elements related to drought resistance and light response are contained in the gene promoter region. Through haplotype analysis on the phenotypes of drought treated and heading dates that from five different regions, obvious haplotypes were identified except for *SiNF-YC8* and *SiNF-YC14*. At last, drought regulation related gene *SiNF-YC5* and heading date regulation related gene *SiNF-YC10* were selected to explore superior gene haplotypes, laying a foundation for further exploring the functional genes involved in drought resistance and development within this gene family.

Introduction

Nuclear factor *NF-Y* is a type of transcription factor that is commonly present in eukaryotes, with a wide range of functions, especially in stress response. Changing the expression level of *NF-Y* genes can improve plant growth and development, enhance adaptability to nutrient stress environments, and enhance resistance to various stresses (Liu *et al*. 2018, Swain *et al*. 2016). The *NF-Y* includes three subunits: *NF-YA*, *NF-YB*, and *NF-YC*. The *NF-Y* transcription factors in plants consists of multiple genes encoding each subunit, thus a large number of heterotrimeric combinations exist (Tuncher *et al*. 2005, Luo *et al*. 2018).

A total of 10 *NF-YA* subunits, 14 *NF-YB* subunits, and 12 *NF-YC* subunits were identified in foxtail millet (Feng *et al*. 2015). The protein molecule of *NF-YCs* are usually between *NF-YA* and *NF-YB*, besides, these genes were involved in the regulation of flowering time, heading date, and stress response (Li *et al*. 2019). In response to stress, *AtNF-YC1* can regulate the response of *Arabidopsis* to cold stress (Kumimoto *et al*. 2010), *OsNF-YC5* regulates salt tolerance negatively in rice and *ZmNF-YC12* regulates drought resistance positively in *Zea mays* (Yan *et al*. 2024). Besides, over-expression of the *GmNF-YC9* in *Arabidopsis* enhances the resistance of transgenic plants to drought and high salt (Li *et al*. 2017), and over-expression *StNF-YC9* increased drought tolerance in potato (Li *et al*. 2021). In foxtail millet, *SiNF-YC2* has a promoting effect on heading

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date and salt tolerance (Niu *et al.* 2023). Otherwise, *AtNF-YC3*, *AtNF-YC4*, and *AtNF-YC9* are regulated by light signals and play an important role in the photoperiod-dependent flowering regulation (Kumimoto *et al.* 2010); *OsNF-YC2*, *OsNF-YC4* and *OsNF-YC6* are involved in regulating heading and flowering stage in rice; *OsNF-YC8* and *OsNF-YC12* are related to endosperm development (Ceribelli *et al.* 2008).

Very few systematic analysis and research on the function of *SiNF-YC* genes were reported in foxtail millet. Through bioinformatics analyses on the physicochemical properties, promoter elements, and expression of *SiNF-YC* genes, combining with further haplotype analysis that identified from drought treatment in connection with heading date phenotype in different regions, gene expression characteristics and potential functions were obtained preliminary in the present study. The results can provide more directions for the improvement of agronomic traits and molecular breeding of crops.

Materials and Methods

Data of the genomic sequence, CDS sequence, and amino acid sequence of *Setaria italica* (L.) were downloaded from the Pytozome database（(https://phytozome-next.jgi.doe.gov/)）. The raw data of 916 varieties of foxtail millet worldwide collection (secondary study accession number ERP002070) used in the haplotype analysis were provided by the Crop Science Research Institute of the Chinese Academy of Agricultural Science (Jia *et al.* 2013, He *et al.* 2024) and Crop Germplasm Resources Center, Shanxi Agricultural University including data from heading date in four regions: Anyang, Xinjiang, Yulin Shaanxi, and Qiqihar, as well as statistic data after drought treatment, which featured by four indicators: embryonic sheath length, embryonic root length, lateral root number, and germination rate.

The molecular weight and theoretical isoelectric point of proteins encoded by the *SiNF-YCs* were predicted through the Expasy tool (https://www.expasy.org/). The subcellular localization of the *SiNF-YCs* in foxtail millet were predicted through the SoftBerry website (http://www.softberry.com/). The *cis*-elements were predicted through PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), and visualized via Basic Biosequence View tool in TBtools software (Quinlan *et al.* 2010).

The method of haplotypic analysis was referred to Zhang *et al*.(2023). The haplotype analysis can be divided into two parts. In the first part, the Ubuntu system was used to correlate the gene files and phenotypic data. The second part is based on RStudio software: Shapiro.test instruction was used for normality test; Bartlet.test instruction was used to test the homogeneity of variance; ANOVA<-aov and summary (ANOVA) instructions were used for one-way analysis of variance; LSD method was used for multiple comparisons, then the haplotype typing were output. After selection, the major haplotypes exhibiting significant differences were retained for statistic.

Results and Discussion

A total of 12 *NF-YC* subfamily genes were identified from the foxtail millet genome and the gene ID numbers were listed in the Table1. Bioinformatics analysis showed that most *SiNF-YC* genes in the family do not contain or have fewer introns. *SiNF-YC9*, *SiNF-YC10* and *SiNF-YC11* contain 2, 2 and 1 intron respectively. This might be due to the strong selection pressure experienced by the *NF-Y* gene family during evolution, because a smaller number of introns conduce to the stronger adaptability (Ding *et al.* 2017).

The sequence length of the *NF-YCs* was found to range from 387 to 5217 bp, genes with the longest and shortest open reading frame were *SiNF-YC9* and *SiNF-YC14*. Besides, the longest and shortest amino acids were 469 and 129 which encoded by *SiNF-YC12* and *SiNF-YC14* respectively. The molecular weight of NF-YC proteins ranged from 14.19 ku (SiNF-YC14) to 51.59 ku (SiNF-YC12). The theoretical isoelectric points of proteins encoded by 12 members ranged from 4.89 (SiNF-YC8) to 9.58 (SiNF-YC4). The subcellular localization of *SiNF-YCs* predicted by SoftBerry website showed that *SiNF-YC1*, *SiNF-YC2*, *SiNF-YC4*, *SiNF-YC5*, *SiNF-YC6*, and *SiNF-YC14* mainly located in the nucleus, while *SiNF-YC8*, *SiNF-YC9*, *SiNF-YC10*, *SiNF-YC11*, *SiNF-YC12*, and *SiNF-YC13* were extracellular secreted mainly (Table 2).

To investigate the regulatory mechanism of the *SiNF-YCs* during heading date and drought resistance in foxtail millet, the promoter sequence of the *SiNF-YCs* were analyzed. Among those genes, three types of abiotic stress related *cis*-elements involved in drought stress, including MYB binding sites that involved in drought induction, and *cis*- elements participating in methyl jasmonate (MeJA) response, and defense and stress response. There are 9 *cis*-elements involved in light response regulation during heading date (Fig. 1). As a result, each member of the *SiNF-YC* gene family contains elements related to drought stress and light response during heading, indicating that *SiNF-YC* may be widely involved in drought resistance regulation and light response regulation pathways during heading.

To identify the superior haplotype of functional genes, 916 foxtail millet varieties after being processed by drought treatment combined with the genome type information were analyzed in the present study. Different haplotypes based on phenotypes (leaf length, leaf width, plant height, and radicle length) related to drought resistance in the *SiNF-YCs* are summarized in Table 3. Except for the *SiNF-YC8* and *SiNF-YC14*, other genes in this family have haplotypes at least for one phenotype (Table 3), which indicated that haplotypes probably related to the drought stress response.

In order to explore superior drought resistant haplotypes, *SiNF-YC5* was investigated as a sample. Phenotypic analysis of the materials before and after drought treatment revealed that plant height and leaf length become shorter after drought treatment. Among the four main haplotypes out of seven, H003 showed significant differences with other haplotypes in radicle length, leaf length, leaf width, and plant height before and after treatment, while the phenotype of H003 showed no significant difference after drought treated (Fig. 2A, Table 4). Additionally, in the *cis*-element sequence involved in plant defense and stress response at the position of -735 bp on the promoter (Table 5), the haplotype exhibited a C/A base mutation (Fig. 2B). This fact suggests that H003 is an excellent drought resistant haplotype. In addition, the expression levels of *SiNF-YC5* were measured between drought sensitive variety Ci603 and drought tolerant variety Ci409, the results showed that the expression levels decreased significantly in the leaves (Fig. 2C), it indicated that the *SiNF-YC5* is involved in drought response.

To explore the heading regulation related genes, the heading date data from 916 foxtail millet materials collected from different regions were used to analyze the haplotypes of *SiNF-YCs*. The haplotype numbers for each gene in four regions: Xinjiang in 2016, Anyang in 2016, Qiqihar in 2017, and Yulin in Shaanxi in 2020 are summarized in Table 6. Except for the *SiNF-YC14*, all other members in this gene family have haplotypes. Among them, more haplotype numbers in *SiNF-YC1*, *SiNF-YC4*, *SiNF-YC5*, *SiNF-YC10*, *SiNF-YC11*, and *SiNF-YC13* in the four regions, which indicate those genes may related to the regulation of heading date. Besides, the involvement of NF-YC transcription factors in regulating growth and development in *Arabidopsis* has been extensively studied (Zhang *et al*. 2023). NF-YC3/4/9 can interact with NF-YB2/3 and participate in regulating *Arabidopsis* flowering (Kumimoto *et al*. 2010). Overexpression of *ZmNF-YC14* in

Table 1. Basic Information on the *NF-YC* Gene Family in Foxtail Millet.

Gene name	Gene ID	Chromosomal Localization	Genomic Location	Number of Introns	Length of Open Reading Frame	Number of Amino Acids	Molecular Weights of Proteins/ku	Isoelectric Point
SiNF-YC1	Seita.1G071900	1	6578985-6582293	0	768	256	28.16	4.94
SiNF-YC2	Seita.4G268300	4	38568495-38573386	0	771	257	28.27	5.03
SiNF-YC4	Seita.2G085600	2	7644565-7645892	0	765	255	28.05	9.58
SiNF-YC5	Seita.9G468100	9	51135079-51138450	0	738	246	27.06	5.20
SiNF-YC6	Seita.6G196700	6	31833516-31833960	0	444	148	16.28	5.35
SiNF-YC8	Seita.5G130000	5	11161725-11162352	0	627	209	22.90	4.89
SiNF-YC9	Seita.3G194200	3	14746443-14752344	2	5217	343	37.73	5.09
SiNF- YC10	Seita.8G144800	8	28032955-28037286	2	3901	257	28.27	5.30
SiNF-YC11	Seita.8G085800	8	10304604~10305359	1	755	219	24.09	4.91
SiNF-YC12	Seita.3G338800	3	43338537~43340384	0	1407	469	51.59	5.17
SiNF-YC13	Seita.7G275200	7	32541667~32543223	0	1293	431	47.41	5.40
SiNF-YC14	Seita.3G010800	3	581977~582364	0	387	129	14.19	6.41

Table 2. Subcellular localization of NF-YCs based on bioinformatics prediction in Foxtail Millet.

Gene name	Nucleus	Membrane	Extracellular Secretion	Cytoplasm	Mitochondrion	Endoplasmic Reticulum Membrane	Peroxisome	Golgi Apparatus	Chloroplast	Vacuole
SiNF-YC1	9.07	0.58	0.08	0.14	0.00	0.00	0.04	0.00	0.00	0.09
SiNF-YC2	7.74	1.51	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.58
SiNF-YC4	8.62	0.82	0.02	0.00	0.00	0.00	0.08	0.00	0.07	0.38
SiNF-YC5	8.80	0.81	0.03	0.00	0.00	0.00	0.09	0.00	0.00	0.26
SiNF-YC6	9.05	0.68	0.01	0.08	0.00	0.00	0.07	0.00	0.00	0.11
SiNF-YC8	0.00	0.00	2.82	0.00	0.69	1.10	2.57	0.00	2.82	0.00
SiNF-YC9	0.00	0.00	2.81	1.25	0.06	0.38	2.44	0.91	2.15	0.00
SiNF-YC10	0.36	0.00	2.71	1.88	0.00	0.68	2.20	0.65	1.53	0.00
SiNF-YC11	0.07	0.00	2.70	0.00	0.27	1.52	2.36	0.39	2.69	0.00
SiNF-YC12	0.00	0.00	2.49	0.43	1.07	0.48	2.25	1.06	2.22	0.00
SiNF-YC13	0.00	0.00	2.92	0.00	0.83	0.84	2.48	0.00	2.92	0.00
SiNF-YC14	8.44	0.77	0.03	0.00	0.14	0.00	0.06	0.00	0.15	0.40

maize delay flowering period under long day conditions (Wang. 2020). Therefore, the foxtail millet *SiNF-YCs* are related to the regulation function of heading date, but the mechanism still needs further investigation through transgenic research.

Table 3. The haplotype numbers of *SiNF-YCs* based on four phenotypes after drought treatment.

Gene Name	Number of haplotypes (Leaf Length)	Number of haplotypes (Leaf Width)	Number of haplotypes (Plant Height)	Number of haplotypes (Radicle Length)
SiNF-YC1	5	2	3	5
SiNF-YC2	2	2	2	1
SiNF-YC4	6	2	3	3
SiNF-YC5	7	1	7	5
SiNF-YC6	3	2	3	2
SiNF-YC8	1	1	1	1
SiNF-YC9	3	2	3	1
SiNF-YC10	5	3	7	5
SiNF-YC11	7	3	5	3
SiNF-YC12	1	1	1	3
SiNF-YC13	3	3	3	2
SiNF-YC14	1	1	1	1

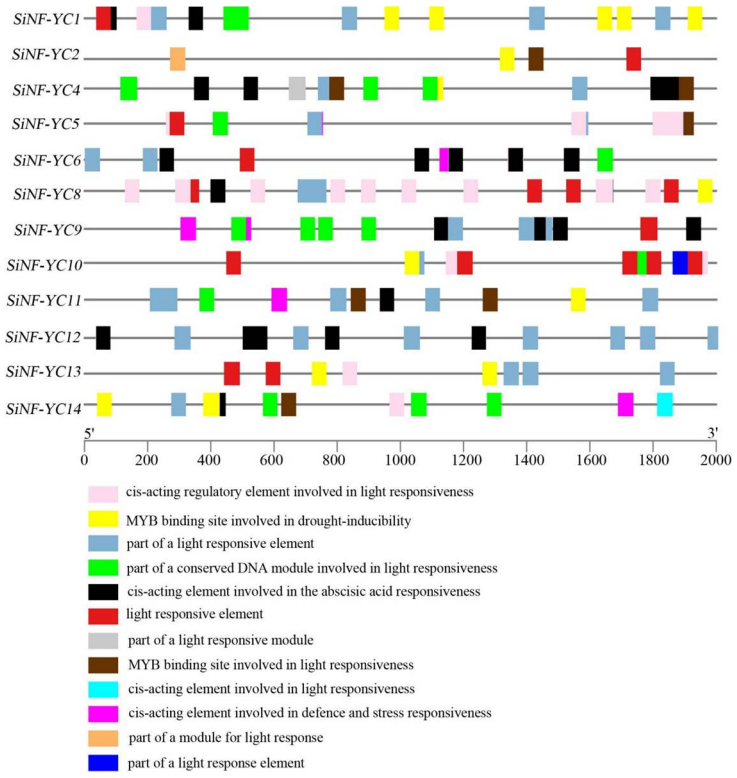

Fig. 1. Putative heading date and drought resistance related *cis*-elements in promoter of *SiNF-YCs* in foxtail millet.

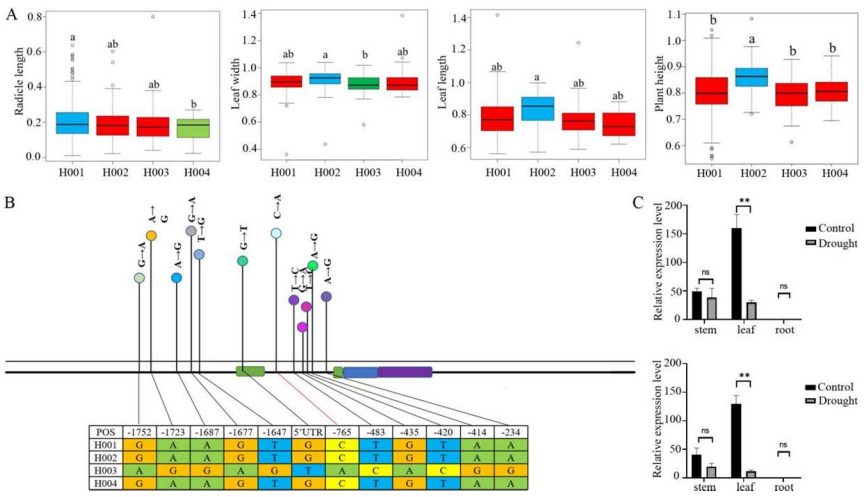

Fig. 2. Differential expression analysis and haplotype analysis of *SiNF-YC5* under drought stress. A: Phenotypic differences analysis of different haplotypes. B: Variable sites in genes and promoters of *SiNF-YC5*; different colored in the table represent different bases, the green, blue, yellow, and orange cells representing A, T, C, and G bases, respectively. -: Stands for deletion; +: Stands for insertion, and connecting lines represents the variation of key *cis*-elements; C: The expression level of *SiNF-YC5* in different foxtail millet varieties under drought stress; Ci409 is a drought resistant variety, while Ci603 is a drought sensitive variety; 'control' indicates normal treatment, 'drought' indicates drought treatment; '**' indicates the correlation coefficient is very significant (P<0.01).

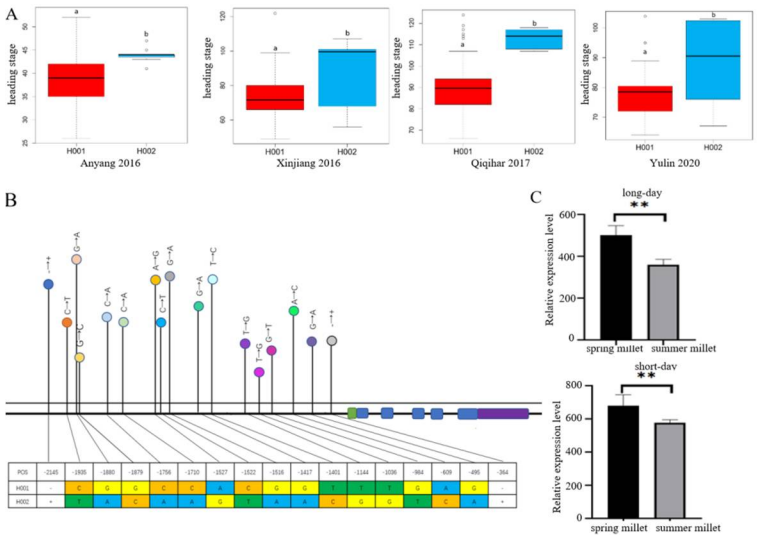

Fig. 3. Differential expression analysis and haplotype analysis of *SiNF-YC10* at heading date under different photoperiods. A: Haplotypes analysis of *SiNF-YC10* based on heading date in different regions; B: Variation sites in genes and promoters of *SiNF-YC10*; different colored in the table represent different bases, the blue, green, orange and yellow cells representing A, T, C, and G bases, respectively. -: Stands for deletion; +: Stands for insertion, and connecting lines represents the variation of key cis elements; C: Relative expression levels of *SiNF-YC10* in spring and summer foxtail millet under long and short day treatments, '**' indicates significant correlation (P<0.01).

Table 4. Phenotypic statistics of different haplotypes on *SiNF-YC5*.

Haplotype	Plant height	Radicle length	Leaf length	Leaf width	Accessions
H001	0.806±0.084 b	0.202±0.097 a	0.745±0.106b	0.893±0.072ab	508
H002	0.860±0.072 a	0.191±0.092 ab	0.739±0.095a	0.876±0.089a	108
H003	0.896±0.071 b	0.221±0.105 ab	0.839±0.111b	0.912±0.078b	62
H004	0.863±0.064 b	0.210±0.062 b	0.779±0.077b	0.897±0.102ab	33

The data shown in the table is the average value (± SD) of the samples. Different Lowercase letters in the same column show significant differences (P<0.05)

Table 5. Variations of key *cis*-elements in promoter of *SiNF-YC5*.

Variation type	C/A
Position	-765 bp
H001	C
H002	C
H003	A
H004	C

Table 6. Haplotype numbers of *SiNF-YCs* in foxtail millet at heading date.

Gene name	Number of Haplotypes (2016 Xinjiang)	Number of Haplotypes (2016 Anyang)	Number of Haplotypes (2017 Qiqihar)	Number of Haplotypes (2020 Yulin, Shaanxi)
SiNF-YC1	5	5	6	7
SiNF-YC2	3	3	2	3
SiNF-YC4	10	10	10	9
SiNF-YC5	7	7	12	14
SiNF-YC6	2	2	3	3
SiNF-YC8	2	2	2	1
SiNF-YC9	3	3	2	3
SiNF-YC10	5	5	6	4
SiNF-YC11	11	11	8	8
SiNF-YC12	2	2	2	4
SiNF-YC13	4	4	6	7
SiNF-YC14	1	1	1	1

Table 7. Phenotypic statistics of different haplotypes on *SiNF-YC10*.

Haplotype	2016 Xinjiang	2016 Anyang	2017 Qiqihar	2020 Yulin, Shaanxi
H001	73.165 ± 10.477	38.119 ± 4.938	88.832 ± 9.625	77.864 ± 8.326
H002	87 ± 18.182	43.917 ± 1.379	113 ± 4.422	88.875 ± 14.137

Table 8. Variations of key *cis* elements in promoter of *SiNF-YC10*.

Variation type	C/A	T/G
Position	-1710 bp	-1036
H001	C	T
H002	A	G

Further analysis on *SiNF-YC10* based on heading date data from different regions combined with gene sequence, result showed that there were two main haplotypes: H001 and H002 (Fig. 3A). The heading date of H001 was shorter than H002 in four regions: Anyang, Xinjiang, Qiqihar, and Yulin, Shaanxi (Table 7). Besides, the haplotype exhibited C/A and T/G base mutations in the *cis*-elements, which involved in plant photoresponse at the position of -1710 bp and -1036 bp in the promoter (Fig. 3B, Table 8). Additionally, the expression level of *SiNF-YC10* in spring foxtail millet is higher than that in summer foxtail millet under long and short day conditions, and the difference was significant (Fig. 3C), it seems that the *SiNF-YC10* is indeed related to heading date regulation.

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