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RESEARCH ARTICLE

Genome-wide identification and co-expression network analysis of nuclear factor-Y in barley revealed potential functions in salt stress

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Abstract Nuclear factor-Ys (*NF-Ys*) were previously shown to have important regulatory impacts in different developmental and physiological process. However, in barley the function of the NF-Y genes at system levels is not well known. To identify barley *NF-Ys*, Arabidopsis and wheat NF-Y protein sequences were retrieved and the BLAST program along with the hidden Markov model were used. Multiple sequence alignments of identified *NF-Ys* were constructed using ClustalW. Expression patterns of the *NF-Ys* at different physiological and developmental conditions were also surveyed based on microarray datasets in public databases and subsequently co-expression network were constructed. Validation of in silico expression analysis was performed by real-time qPCR under salt stress condition. In total, 23 barley *NF-Ys* (8 *NF-YA*, 11 *NF-YB* and 4 *NF-YC*) were identified. Based on the sequence homology, the subunits of the *NF-Y* complex were divided into three to five groups. Structural analysis highlighted the conserved domains of *HvNF-YA*, *HvNF-YB* and *HvNF-YC*. Co-expression network analysis indicated the potential functions of *HvNF-Ys* in photosynthesis, starch biosynthesis and osmotic stress tolerance. The results of qRT-PCR also confirmed the *HvNF-Ys* roles in adaptation responses of barley to salt stress. We identified some potential candidate genes which could be used for improvements of cereals tolerance to salinity stress.

Keywords Nuclear factor Y · Phylogeny · Co-expression network · qRT-PCR · Salt stress

Introduction

Nuclear factor Y (NF-Y) is a heterotrimeric transcription factor family that binds the CCAAT element (CBF) to regulate gene expression (Quach et al. 2015). It includes three subfamilies; NF-YA, NF-YB, and NF-YC (Mantovani 1999). Each of them shows high level of sequence conservation in their central regions (Mantovani 1999). This conserved central region of the NF-YA subunit contains interaction and DNA binding domains. Interaction domain is required for contact with the NF-YB, NF-YC heterodimer, whereas the DNA-binding domain is involved in the DNA-binding site recognition (Xing et al. 1994).

In plants, each of the *NF-YA*, *NF-YB* and *NF-YC* proteins are encoded by a larger gene family (Liang et al. 2012; Panahi et al. 2015; Malviya et al. 2016; Ren et al. 2016). *Arabidopsis* and wheat encodes 36 and 37 *NF-Y* subunits, respectively (Siefers et al. 2009). In rice

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genome at least 10, 12 and 8 genes encode *NF-YA*, *NF-YB* and *NF-YC* proteins, respectively (Thirumurugan et al. 2008) and rapeseed (*Brassica napus* L.) harbors 14, 14 and 5 *NF-YA*, *NF-YB*, and *NF-YC* genes, respectively (Xu et al. 2014).

Plant growth and development is influenced by various abiotic stresses such as salinity (Panahi et al. 2012). Salinity is one of the major factors limiting production of crop plants worldwide, therefore enhancement of plants tolerance to salt stress is one of the main goals of plant breeders and biotechnologist in recent decades. Underlying physiological and molecular mechanisms of salt tolerance remain to be elucidated and this is the major drawback for the enhancement of plants tolerance to salt stress condition (Panahi and Mohammadi 2018). Under salt stress, some physiological events including increased respiration rate, membrane instability, and failure in the maintenance of turgor pressure are observed (Babu et al. 2012). To ameliorate these effects, some processes are coordinated and triggered in plants to maintain cellular osmolality (Panahi et al. 2012). Transcription factors are mediated genetic networks rewiring in stress condition (Panahi et al. 2015). Therefore, identification of these factors not only will be provide insight to understanding the molecular mechanism of stress responses, but also it will be suitable for identification of candidate genes to improve the stress tolerance of crops by gene manipulation approaches.

The functions of *NF-Ys* in diverse developmental process such as embryogenesis, nitrogen fixing nodule development, flowering and photosynthesis have been suggested (Mu et al. 2013; Panahi et al. 2015). Moreover, increasing number of evidences advocate the role of transcription factor *NF-Ys* in osmotic stress responses. High throughput transcriptome profiling in leaves of wheat revealed that drought stress down regulates *TaNF-YA1* transcription (Stephenson et al. 2007). Additionally, it has been reported that over expression of *NFY-A3* and *NF-Y5* enhanced drought tolerance in soybean and *Arabidopsis*, respectively (Ni et al. 2013). Increased tolerance to drought stress was observed under overexpression of *AtNF-YB1* and *ZmNF-YB2* in *Arabidopsis* and *Zea mays*, respectively (Nelson et al. 2007).

Barley (*Hordeum vulgare*), member of the grass family is regarded as a salt-tolerant crop. Moreover as a diploid and inbreeding, it has been considered as a model for understanding the function and regulation of complex traits such as salt stress responses in *Triticeae* (I.B.G.S. 2012).

Although previous studies highlighted the importance of plant *NF-Y*s in wide range process, yet the molecular pathways of these transcription factors remains unclear. In this study, we characterized the function of three *NF-Y* families, their phylogenetic relationships, co-expression networks and expression under salt stress.

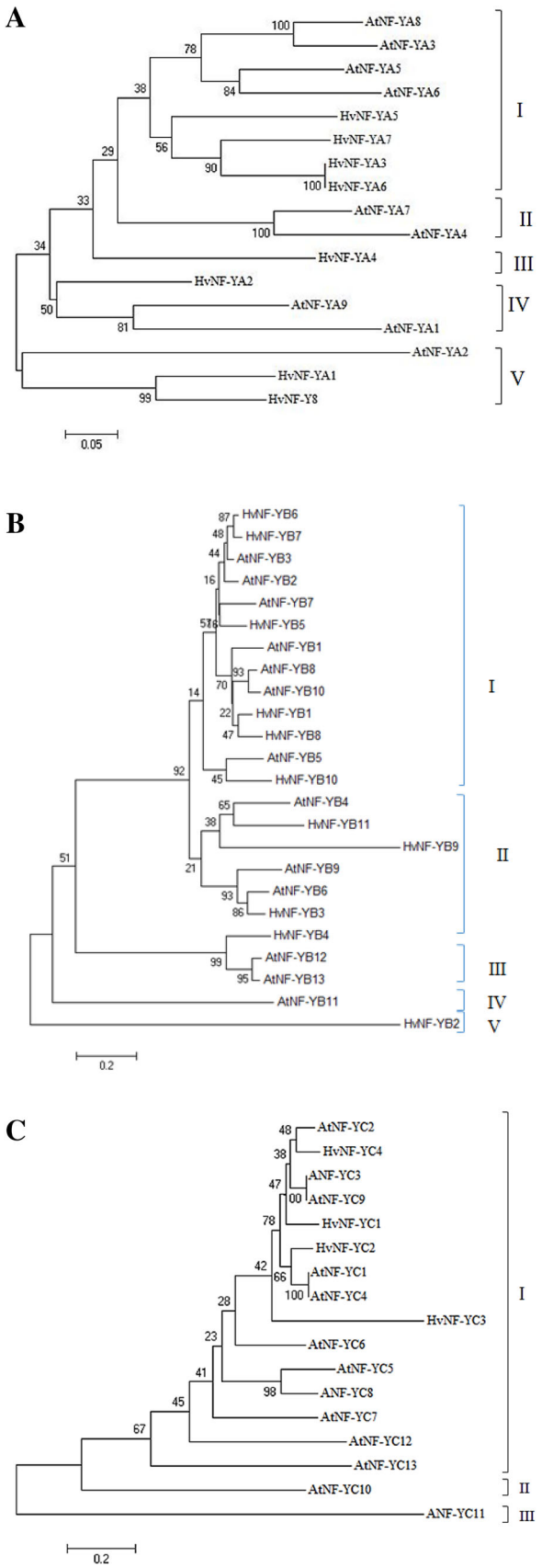
Materials and methods

Identification of barley NF-Y family members

To identify barley *NF-Ys*, Arabidopsis and wheat NF-Y protein sequences were retrieved and BLAST program with an *E*-value cut-off of E^{-10} were used to identify *HvNF-Y* using the *H. vulgare* genomes in ensemble Plants database (http://plants.ensembl.org/Hordeum_vulgare/Tools/Blast?db=core). In addition, hidden Markov model (HMM) for the *NF-Y* genes was constructed using HMMER package version 3.0 (Eddy 1998). MUSCLE algorithm was used

Table 1 List of identified NF-Ys in barley

NF-YC		NF-YB		NF-YA	
Gene ID	NF-Y name	Gene ID	NF-Y name	Gene ID	NF-Y name
MLOC_5869	NF-YA1	MLOC_5897	NF-YB1	BAJ98529	NF-YC1
MLOC_24655	NF-YA2	MLOC_70684	NF-YB2	BAK03493	NF-YC2
MLOC_36554	NF-YA3	MLOC_72428	NF-YB3	MLOC_11547	NF-YC3
MLOC_52315	NF-YA4	MLOC_36682	NF-YB4	MLOC_53118	NF-YC4
AK368372	NF-YA5	MLOC_36944	NF-YB5		
MLOC_62730	NF-YA6	MLOC_57782	NF-YB6		
MLOC_67781	NF-YA7	MLOC_75867	NF-YB7		
MLOC_76757	NF-YA8	MLOC_7755	NF-YB8		
		MLOC_79487	NF-YB9		
		AK357982	NF-YB10		
		MLOC_36879	NF-YB11		



◀**Fig. 1** The evolutional cladograms of the NF-Y proteins HvNF-YA (**a**), HvNF-YB (**b**), and HvNF-YC (**c**) genes generated by ClustalW using neighbor joining algorithm implemented in the MEGA software

for multiple alignments based on default parameters in Ugene software (Okonechnikov et al. 2012). Subsequently, the created HMM profiles were used to perform HMM search (http://hmmer.janelia.org/search/hmmsearch) against the Barley genome (I.B.G.S. 2012). By combination the results of two strategies, the final list of *HvNF-Y* genes were retrieved.

Multiple alignments and phylogenetic analysis

Multiple sequence alignments of identified *NF-Ys* in barley were constructed using ClustalW (Thompson et al. 2002). The Neighbor-Joining trees were constructed using evolutionary distance coefficient implemented in MEGA5.0 program (Tamura et al. 2011) with the 1000 bootstrap replications.

Expression pattern under different development stages and abiotic stresses condition

The expression data of the *HvNF-Y* gene family under different development stages (Array accession number BB3 in PLEXdb database) and abiotic stress including cold (short term cold stress and Long term stress, accession number GSE27822 and GSE27821 respectively), drought (GSE6990) and ABA (early and late responses, GSE10328) for Morex cultivar of barley was retrieved from the Gene Expression Omnibus (GEO). The expression pattern of all *NF-Y* genes was presented as heatmap with a color scale indicating log2 expression values. The heatmap was constructed using Euclidean distance and complete linkage algorithm implemented in heat mapper (http://www.heatmapper.ca/) server (Babicki et al. 2016).

Identification of correlated genes and network construction

High-throughput transcriptome data sets were retrieved from the PLEXdb and GEO databases. 1200 samples were used for the expression analysis and construction of the co expression-network. Raw data were normalized using the MAS 5.0 algorithm in R ("affy" package). Co-expression network were constructed based on weighted correlations algorithm in R ("WGCNA" package).The optimal threshold of the PCC was set at 0.75. Cytoscape software (version 2.8.3) was used to construct the co-expression networks. To predict the biological function of the *HvNF-Y* proteins, co-expressed genes were mapped into

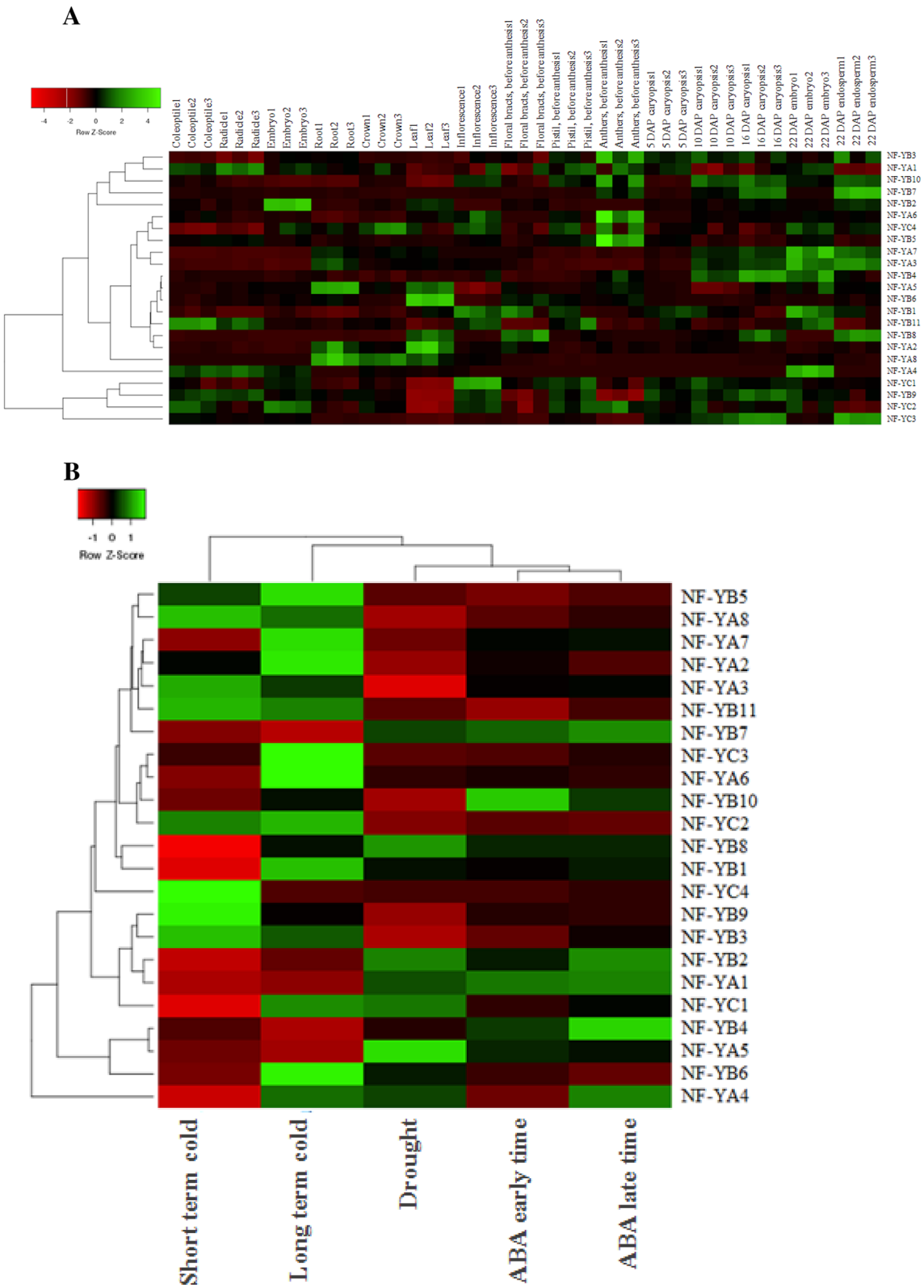


Fig. 2 Expression patterns of HvNF-Y genes in various barley tissues (**a**) and abiotic stresses (**b**). Hierarchical clustering displays the expression profiles of the 23 HvNF-Y genes. Cluster analyses were carried out with Euclidean distances and the hierarchical cluster method of the "complete linkage". The color bar at the base represents log2 expression values: green, low expression; black, medium expression; red, high expression

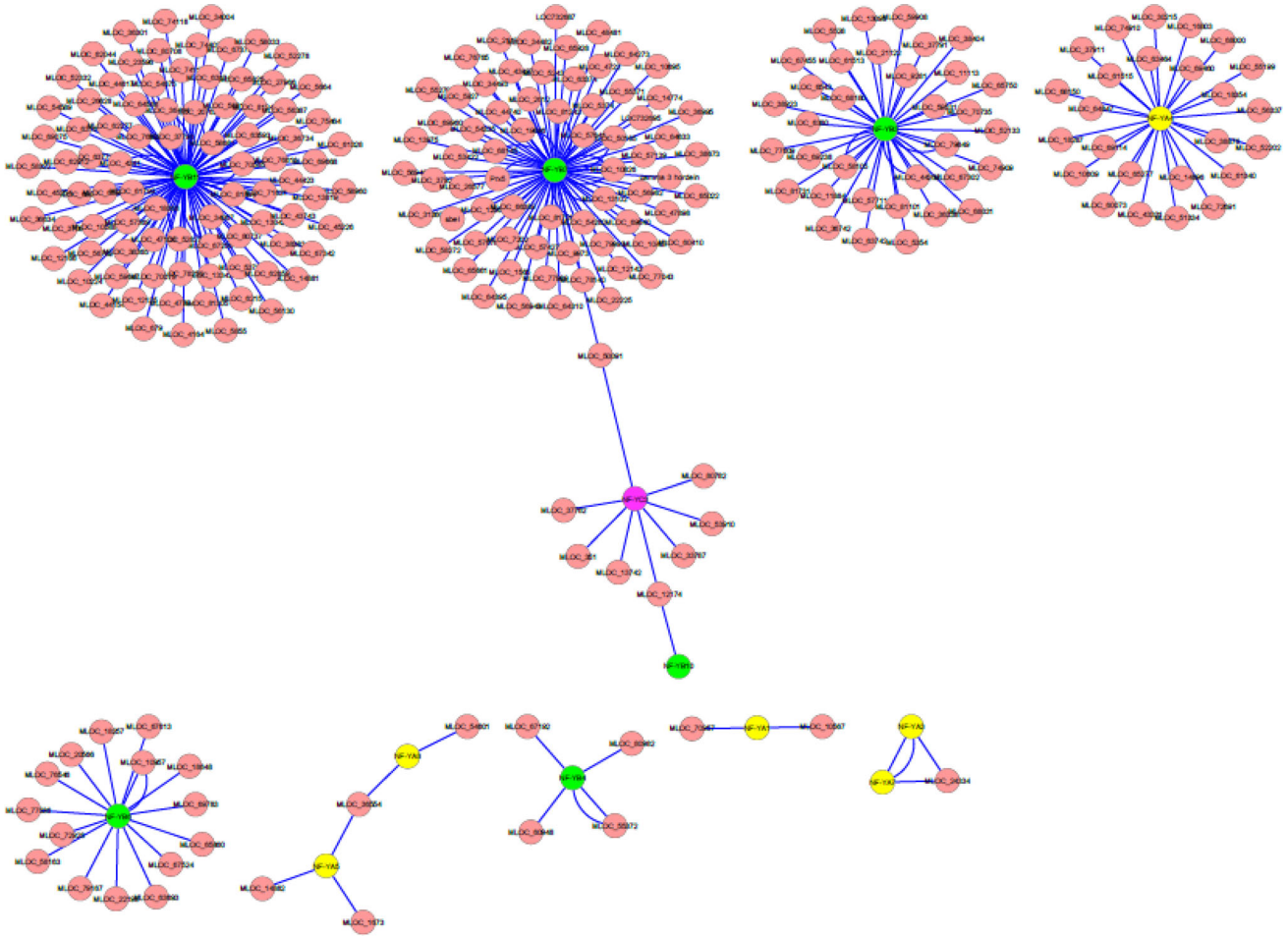


Fig. 3 Co-expression networks of *HvNF-Y* genes. Green, pink and yellow color nodes represent the NF-YB, NF-YC and NF-YA, respectively

biological pathways by the BLAST to the KEGG database (Kanehisa et al. 2014).

Plant material and stress treatment

Barley seeds (Morex cultivar) were obtained from Center of Excellence in Cereal Molecular Breeding, University of Tabriz, Tabriz, Iran. The seeds were sterilized with the 1.5% sodium hypochlorite solution for 3 min and transferred into pots containing sand. The seedlings were feed with 1/2 Hoagland's solution and 30-days-old seedlings (planted as about 8 individuals per pot) were exposed to salt stress by adding 300 mM NaCl to the Hoagland's solution. The treated and control plants (both shoots and roots) were sampled 6 h after salt stress treatment.

Total RNA isolation and cDNA synthesis

Total RNA was extracted using RNX^{TM} plus kit (Cinagen, Iran). The quality and quantity of extracted RNA were examined by agarose gel electrophoresis and NanoDrop spectrophotometer, respectively. For elimination of residual genomic DNA, RNA samples were treated with an RNase-Free DNaseI (Fermentas, Lituania). cDNA was synthesized using the PrimeScript RT-PCR Kit (TaKaRa, Japan), according to the manufacturer's instructions.

Gene expression analysis by quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was done on the Applied Biosystems 7500 device using the SYBR ExTaqTM Kit (TaKaRa, Japan). Each of 20 µl qRT-PCR reaction included 10 µl SYBR R PremixEx TaqTM (2 ×), 0.5 µl of each forward and reverse primers (10 µM), and 0.2 µl cDNA. Primer sequences of *HvNF-Ys* and housekeeping genes is presnetd in Supplementary Table 1. Gene expression data were normalized to 18S rRNA as housekeeping gene. Expression data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livack and Schmittgen 2001). To compare the gene expression between treated and normal conditions, Student's *t* test was performed.

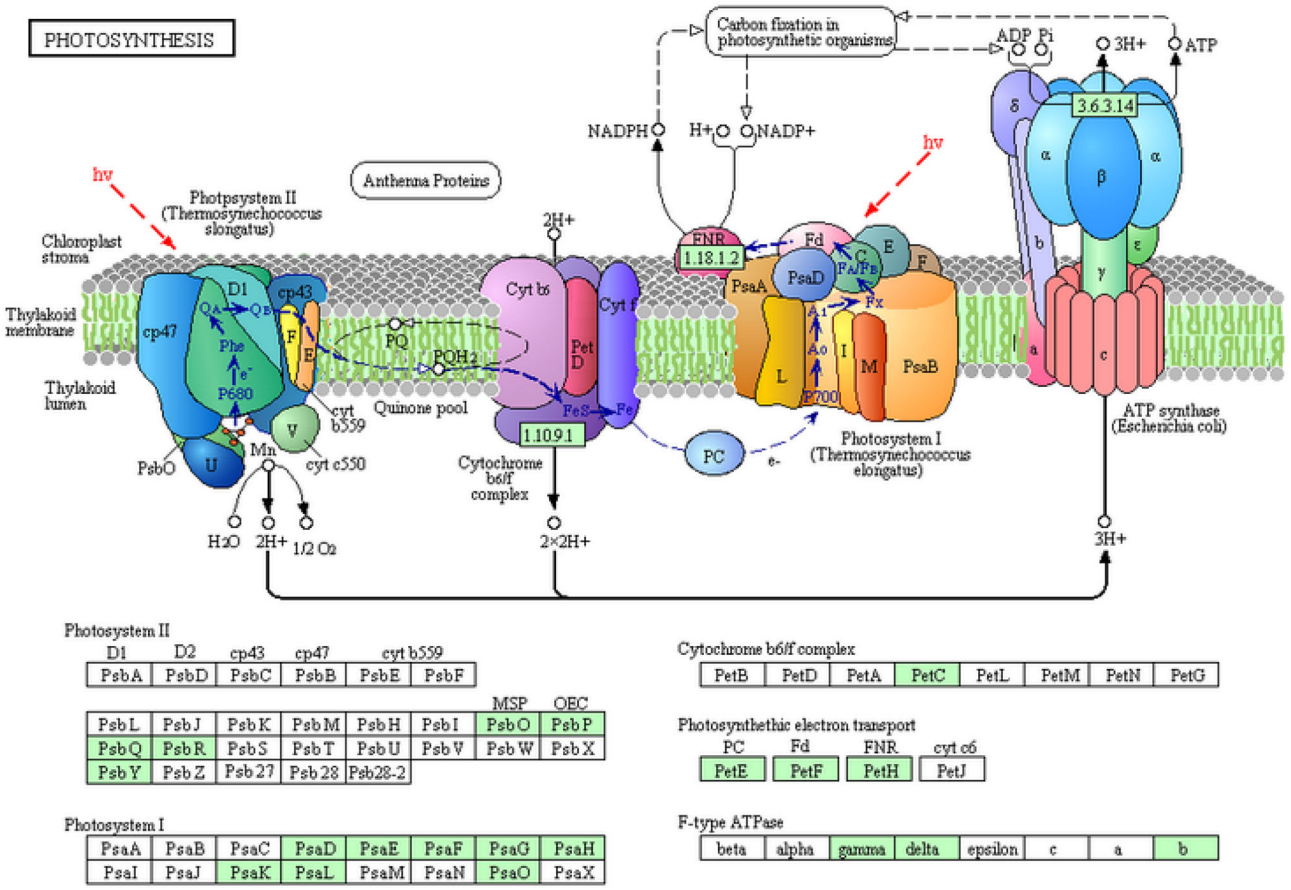


Fig. 4 Photosynthesis pathway, as adapted from the Kyoto Encyclopedia of Genes and Genomes (KEGG). Co-expressed genes identified are highlighted by green boxes

Results

Identification, multiple alignments and phylogenetic analyses of HvNF-Y genes

We used wheat and *Arabidopsis* NF-Y protein sequences as queries to search barley *NF-Y* genes. In total, 23 unique *NF-Y* sequences belonging to the three subgroups including 8 *NF-YA*, 11 *NF-YB* and 4 *NF-YC* genes were identified (Table 1). To understand the evolutionary relationship among individual *HvNF-Ys,* phylogenetic analysis was carried out (Fig. 1a–c). Based on the obtained tress, *HvNF-YAs* were clustered into five discrete clades (I–V). Clade I consisted of *HvNF-YA* 3, 5, 6 and 7 as well as *AtNF-YA* 3, 5, 6, 8 (Fig. 1a). The *HvNF-YB* subunit was clustered into five distinct groups (I–V) (Fig. 1b). In this grouping, *HvNF-YB* 1, 5, 7, 8 and 10 and correspondence *Arabidopsis NF-YBs* (*AtNF-YBs* 1, 5, 7, 8 and 10) were grouped in same clade. As shown in the Fig. 1c, all-*YCs* were grouped in the clade I.

Expression patterns of *HvNF-Y*s under different developmental stages and abiotic stresses condition

To examine the transcript accumulation of *HvNF-Y* genes in the entire barley life cycle, their expression profiles was analyzed at the 15 barley developmental stages. To infer the functional relationship of 23 *HvNF-Y* genes, hierarchical cluster was performed based on their expression levels (Fig. 2a). Based on expression patterns, the *HvNF-Y* genes were classified into three major groups.\ Groups A containing 5 genes including *HvNF-YB3*, *HvNF-YA1*, *HvNF-YB10*, *HvNF-YB7*and *HvNF-YB2*. Group B consisted of 13 genes which were further divided into three subgroups. In subgroup B1, *HvNF-YA6*, *HvNF-YC4* and *HvNF-YB5* showed high expression in the anthers before full anthesis. *HvNF-YA7* and *HvNF-YA3* showed high expression in embryos (22 day after pollination; Fig. 2a). *HvNF-YA8* showed high expression in the roots, crown and shoots, whereas *HvNF-YA2* showed high expression in the root and leafs. High expressions of *HvNF-YAs* were also detected in the coleoptile, radicals and embryos. Group C

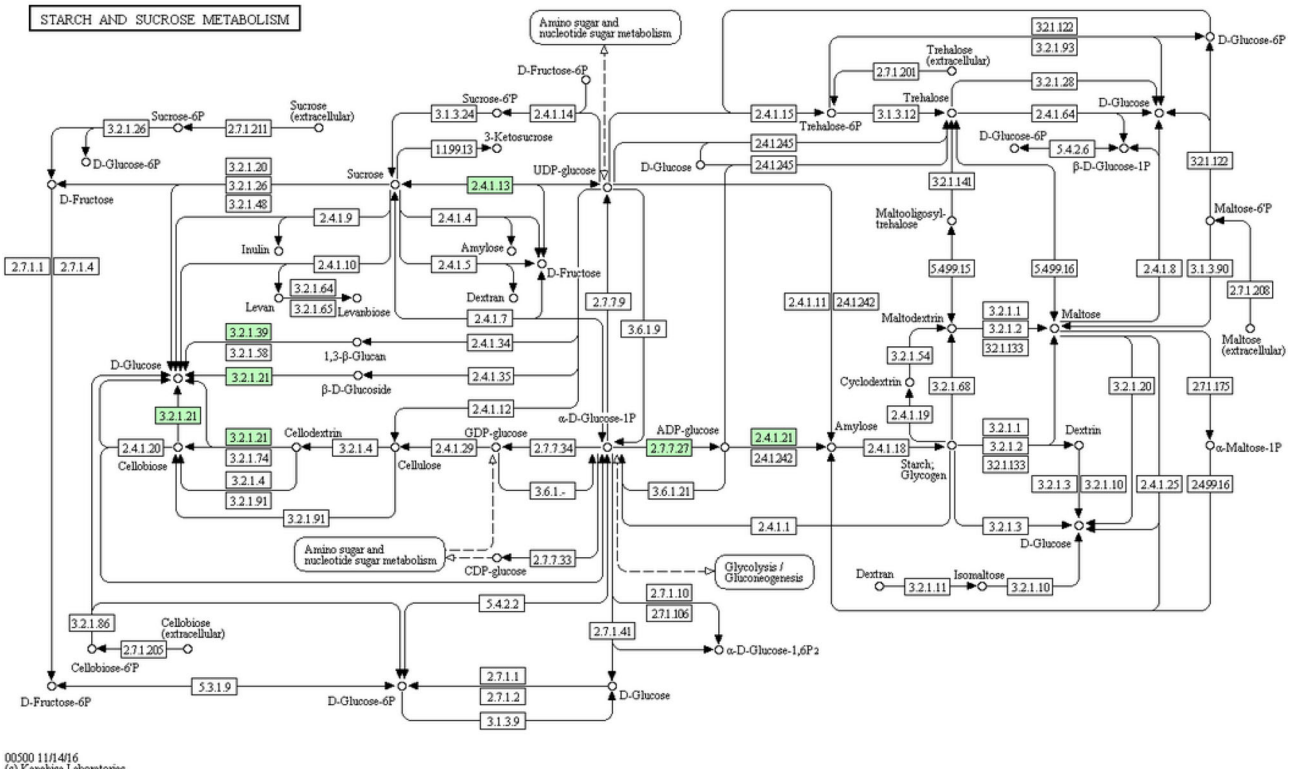


Fig. 5 Starch and Sucrose Metabolism pathway, adapted from the Kyoto Encyclopedia of Genes and Genomes (KEGG). Co-expressed genes identified in the present study highlighted by green boxes

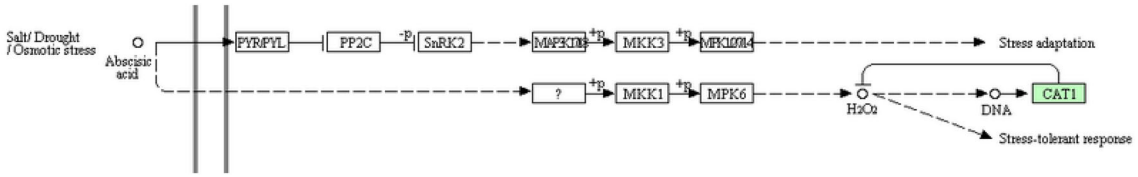


Fig. 6 Salt/Drought/Osmotic signaling pathway, adapted from the Kyoto Encyclopedia of Genes and Genomes (KEGG). Co-expressed genes identified in the present study highlighted by the green box

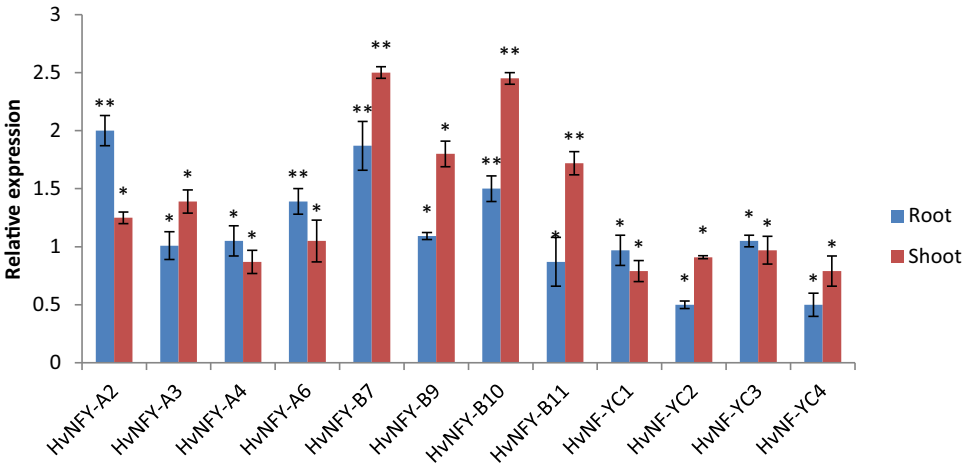


Fig. 7 Expression changes of *HvNF-Y*s in response to salt stress in roots and shoots. Their transcript levels were normalized to the 18SrRNA and then compared with control

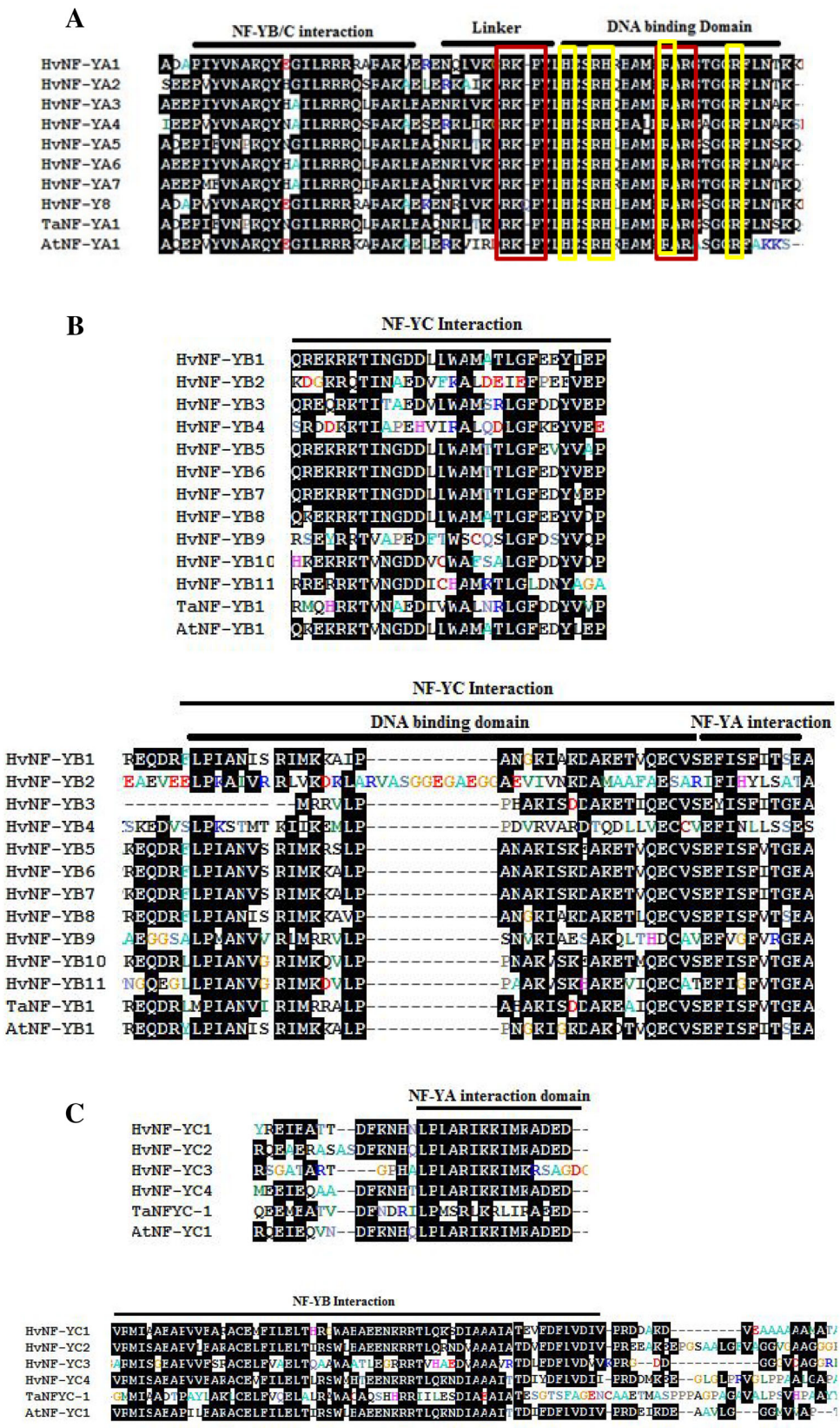


Fig. 8 Multiple sequence alignments of *HvNF-YA* (**a**) *HvNF-YB* (**b**) and *HvNF-YC* (**c**) as generated by the ClustalW software. In NF-YB/C interaction domain NF-YB and C create ahueteroduplex on NF-YA transcription factor. The red boxes highlighted the residues that is required for nuclear targeting

contained *HvNF-YC1*, *HvNF-YB9*, *HvNF-YC2* and *HvNF-YC3*.

Transcriptome analysis of *HvNF-Y* genes under different abiotic stresses were shown in the Fig. 2b. The results revealed that *HvNF-YA1* gene expressed in similar pattern under ABA and drought treatments condition and high expression of *HvNF-YA4* gene was observed under ABA, drought and cold stress conditions. As shown in the Fig. 2b, *HvNF-YA3*, *HvNF-YA2*, *HvNF-YA7* and *HvNF-YA8* expressed at similar pattern under drought stress condition and expression of *HvNF-YC3* gene was higher under ABA and drought stress conditions (Fig. 2b).

Co-expression networks and functional analysis

Further functional analysis of *HvNF-Y*s was done using construction of co-expression network (Fig. 3). Subsequently for functional annotation, co-expressed genes were mapped to KEGG database. Based on cut off of $P \leq 0.05$, photosynthesis, starch, sucrose metabolism and salt stress responding pathways were enriched (Figs. 4, 5, 6). In photosynthesis pathway, five components of photosystem II (PSII) were co-expressed with the *HvNF-Ys*. Other pathways correlated with the *HvNF-Y* expression were sucrose and starch pathways. The correspondence genes for sucrose and starch metabolism were sucrose synthase (EC 2.4.1.13), 4-alpha-D-glucan glucohydrolase (EC 3.2.1.3), beta-glucosidase (EC 3.2.1.21), starch synthase (EC 2.4.1.21), glucose-1-phosphate adenylyl transferase (EC 2.7.7.27) (Fig. 5).

Expression analysis of NF-Ys under salt stress

The expression response of 12 selected *HvNF-Y*s (8 *NF-YA* and 4 *NF-YC*) under salt stress condition (in both root and shoot parts) was validated using qRT-PCR. As shown in a Fig. 7, expression of the selected *HvNF-Ys* was changed in response to salt stress. *HvNF-YA1* and *HvNF-YA6* showed the highest differences in salt treated roots (Fig. 7).

Discussion

According to Gray et al. (2009), each protein was named with a two-letter code corresponding to *Hordeum vulgare* (*Hv*), followed by the *NF-Y* letter code and the family designation (*NF-YA*, *B*, or *C*). Finally, the identified genes were numbered based on their chromosomal positions (Cao et al. 2011).

In concordance with Siefers et al. (2009) and Cao et al. (2011), in the *HvNF-YA* phylogeny tree, paralogus genes were equidistant from each other. This is in contrast to the *HvNF-YB* and *HvNF-YC,* where clear blocks of the conserved regions was observed (Fig. 8b, c). NF-YA proteins consisted of two conserved parts; interaction and DNA-binding domains (Fig. 8a; Hackenberg et al. 2012) which are separated with a linker. The interaction domain generally has 20 amino acids and interacts with NF-YB/NF-YC heterodimer (Mantovani 1999). Presence of three clusters of basic residues (red boxs in Fig. 8a) which serve as a nuclear localization signal is a distinct characteristic of NF-YA proteins (Thon et al. 2010).

It has been suggested that evolutionarily constrains in NF-YA proteins is higher than NF-YB or NF-YC proteins (Cao et al. 2011). Structural studies revealed that NF-YA subunits are specifically responsible for all physical contacts with the CCAAT sequence (Romier et al. 2003). These residues are conserved in all eight *HvNF-YAs* (residues in yellow box in Fig. 8a) as well as in *NF-YAs* in other species (Thirumurugan et al. 2008)

Our results highlighted the regulatory potential of *HvNF-Ys* in photosynthesis process. As presented in Fig. 4, *HvNF-Ys* coordinately expressed with ATP synthase, ferredoxin, subunits of PSI, PSII and cytochrome b6f. In line with our results, functional CCAAT-boxes has been previously identified in the promoters of photosynthesis-related genes, but the role of *NF-Ys* in photosynthesis pathway is currently not well known. It has been also shown that NF-YB and NF-YA proteins regulate some of the photosynthesis-related genes such as chlorophyll a/b-binding protein (CAB) and the small subunit of Rubisco (RBCS) in stress conditions (Miyoshi et al. 2003; Warpeha et al. 2007).

The results of co-expression network analysis were consistent with the previous studies. Systematic analysis highlighted the *OsNF-YB1* roles in the grain filling (Xu et al. 2016). It has been reported that *OsNF-YB1* regulates the transcription of sucrose transporters through direct binding (Xu et al. 2016) and in *Vitis vinifera*, *NF-Ys* (*NF-YA1* and *NF-YB7*) are induced by sucrose, which hints the involvement of *NF-Ys* in the biosynthesis and/or transport of sucrose (Ren et al. 2016).

As shown in the Fig. 6, *Cat1* (Catalase) was co-expressed with *HvNF-Ys*. Catalase (EC 1.11.1.6) is considered as one of the main factors in plant tolerance to environmental stress (Navabpour et al. 2013; Shahriari Ahmadi et al. 2013; Panahi et al. 2013, 2014). It is one of the most important determinants of H_2O_2 levels during stress conditions. It has been proposed that one molecule of catalase eliminates about six million molecules of H_2O_2 per minute (Gill and Tuteja 2010). Recent evidences suggest the involvement of *HAP2E* and *NF-YC1* in the H_2O_2 signaling pathways (Chen et al. 2015; Thön et al. 2010). Feng et al. (2015) reported that H_2O_2 induces the *NF-YA1* and *NF-YB8* expression under stress conditions.

The previous studies demonstrated that NF-Y transcription factors play key roles in abiotic stresses (Han et al. 2013; Ni et al. 2013). Therefore, we attempted further validation of barley NF-Ys function during salt stress. Among *HvNF-YA* genes, *HvNF-YC2* and *HvNF-YC3* was strongly up-regulated under salt stress condition. Comparison of expression changes of *HvNF-Ys* which are defined as hub genes in the co-expression networks showed notable changes of *HvNF-Y3* gene in leaves and roots under salt stress condition. The results suggested that the most of *HvNF-Ys* might be the putative regulators in abiotic stress responses. This is also confirmed by the expression analysis of transcriptomic data under abiotic stress condition (Fig. 2b). In line with our results, it has been hypothesized that coordination of antioxidant defense systems such the production of CAT, thioredoxin reductases (TR) (EC 1.8.1.9) are regulated by NF-Ys (Ikbal et al. 2014). However, more research is needed to confirm of this hypothesis.

Conclusion

In conclusion, barley NF-Ys comprises relatively diverse gene family involved in different process such as starch metabolism, sucrose metabolism and photosynthesis. Functional analysis of *HvNF-Ys* by co-expression network and real time PCR revealed involvement of *HvNF-Ys* in adaptation to salt stress condition that can contribute to the maintenance of plant growth and productivity under stress conditions. The results of this study offer useful foundation for functional studies of *HvNF-Ys* and support the potential application of *HvNF-Ys* in the improvement of salt tolerance in cereals via biotechnological strategies.

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Authors' Contributions Conceived and designed the experiments: BP, SAM. Performed the experiments: BP, HAH,SAM, Analyzed the data: BP, SAM, HAH, MZM, KR. Wrote the paper: BP, SAM, KR, HAH, MZM.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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