



## *In silico* analysis and gene expression of heat stress responses genes in *Hordeum vulgare* L.



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### ARTICLE INFO

#### Keywords:

Heat-shock  
Barley  
Gene expression  
Gene ontology (GO)  
Bioinformatics analysis and nuclear factor Y subunit A2  
**Abbreviations:**  
GEO  
gene expression omnibus  
HSPs  
Heat shock proteins  
NF-Y  
NUCLEAR FACTOR-Y  
GO  
gene ontology  
DEGs  
differentially expressed genes; heat stress (HS)

### ABSTRACT

Barley has been selected or bred for specific adaptation to abiotic stresses in geographically distinct areas of the world. The purpose of this study is to identify differentially expressed genes and analyze biological processes related to heat stress in barley. The GEO dataset of heat-shock (HS) treated barley was statically analyzed using the integrated GEO2R tool in the package of Gene Expression Omnibus dataset. Cytoscape software and STRING website were used for Gene Ontology (GO) enrichment, protein-protein interaction analysis and network visualization. Our bioinformatics analysis displayed genes that were consistently differentially expressed in HS in barley plant. The results show that biological pathways and protein-protein interaction networks are associated with these genes. Based on the bioinformatics analysis results, NF-YA2 gene was selected as the hub gene responsible for HS. A qRT-PCR was designed to approve the *in silico* results and analyze the expression of NF-YA2. Barley plants were grown in the greenhouse, heat treated at 42 °C and then harvested. Identified genes were validated by means of qRT-PCR. According to the qRT-PCR results, NF-YA2 was up-regulated in sensitive plants and down-regulated in the tolerant plants. The results showed that NF-YA2 is a negative regulator in abiotic stress tolerance in barley.

### 1. Introduction

Barley is the second largest crop in Iran and the fourth most cultivated cereal in the world (Tuttolomondo et al., 2009). The area under cultivation of barley in the country was 1597000 ha in 2018 and approximately 50% of which have been cultivated with irrigation (Ebadzadeh et al., 2015a, b). Iran is located in arid and semiarid regions of the world and these climates cover more than 83% of the country (Modarres and da Silva, 2007). Due to the fact that barley has cultivated in almost all regions of Iran (Ahmadi et al., 2017), cultivation of this plant has encountered abiotic stresses.

Abiotic stresses lead to extensive loss of agricultural productivity worldwide (Lim et al., 2013; Shao et al., 2015; Tang et al., 2016). Heat-shock (HS) is known to have significant effects on cereal grain composition during grain development, such as disturbance of sucrose to starch conservation in barley (MacLeod and Duffus, 1988). Barley seedlings, subjected to HS, initially indicated a severe depression in leaf water potential, which was slowly recovered with time (Wahid and Shabbir, 2005). Understanding the physiological and molecular impact

of HS on plants as well as using the inherent potential of natural genetic diversity to tolerate extreme temperatures should be considered as essential components of scientific researches (Jedrowski and Brüggemann, 2015).

Heat shock proteins (HSPs) and reactive oxygen species (ROS)-scavenging enzymes, well known as target genes of HS-responsive TFs, are the major functional proteins that induced by HS (Ohama et al., 2017; Xu et al., 2016). Nuclear factor Y (NF-Y), a heterotrimeric transcription factor (TF) consisting of NF-YA, NF-YB and NF-YC subunits, is conserved across eukaryotic lineages (Swain et al., 2017). NF-Y, known as CCAAT-binding factor (CBF) or heme activator protein (HAP) is able to bind the *cis*-element. In most species, NF-Y is a trimeric transcription factor complex consisting of NF-YA (CBF-B/HAP2), NF-YB (CBF-A/HAP3) and NF-YC (CBF-C/HAP5) subunits (Mantovani, 1999). There are evidences that NF-Y subunits are important regulators of abiotic stress responses (Zhao et al., 2009; Ni et al., 2013; Han et al., 2013; Stephenson et al., 2007). The enhanced tolerance to several types of abiotic stress (flooding, N starvation, freezing and heat) was found to be the result of overexpression of At-NF-YA through modulating gene

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<https://doi.org/10.1016/j.bcab.2019.101061>

Received 30 November 2018; Received in revised form 2 February 2019; Accepted 23 February 2019

Available online 28 February 2019

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regulation (Leyva-González et al., 2012).

Many interesting questions in genetics may be addressed by microarray technology which reveals the pattern of genes expression and classifies samples (such as tumor samples) based on the patterns. In this article, the microarray data were analyzed by GEO2R, STRING, Cytoscape and PANTHER to identify the hub genes involved in heat response in barley.

## 2. Materials and methods

### 2.1. Data mining analysis

This study is an *in silico* analysis of the microarray data through various bioinformatics tools. Such studies need microarray dataset for a number of series and samples. Accordingly, the first step was microarray data mining. Normal microarray data about HS (1 platform, 1 series with 12 samples) on barley were generated from ncbi GEO (GEO accession number: GSE23896 <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23896>).

### 2.2. Gene expression data analysis

After grouping data into control and stress groups, microarray data was analyzed by GEO2R tool version R 3.2.3.

### 2.3. Gene response to heat stress

In the GEO2R analysis, contigs that showed a  $\geq 2.0$ -fold increase or a  $\leq 2$ -fold decrease in the LogFC signal intensities were identified as HS responding genes and saved. Sequences of contigs were downloaded from plant expression database (PLEXdb) ([www.plexdb.org](http://www.plexdb.org)). Accessions of genes around contigs were found by IPK barley blast server ([webblast.ipk-gatersleben.de/barley\\_ibsc/](http://webblast.ipk-gatersleben.de/barley_ibsc/)). Then barley unigenes were detected from NCBI UniGene database ([www.ncbi.nlm.nih.gov/unigene](http://www.ncbi.nlm.nih.gov/unigene)). In the process, primarily the Agilent probe IDs were converted to the corresponding NCBI IDs (UniGene) using plant expression database (PLEXdb), IPK barley blast server and ncbi UniGene.

### 2.4. Validation, characterization and networking

For the validation and characterization of data, *Arabidopsis thaliana* was used as the reference organism. Tair blast engine ([www.arabidopsis.org/Blast/index.jsp](http://www.arabidopsis.org/Blast/index.jsp)) and UniProt database ([www.uniprot.org](http://www.uniprot.org)) were applied to obtain the identified responding genes orthologues in *Arabidopsis thaliana*. Characterization was performed using Gene Ontology (GO). Functional categorization of the gene code names (Atg) of *Arabidopsis* orthologues are available at <https://string-db.org>. The results of *in silico* analysis were integrated with STRING database as a system for searching known or predicted interactions between proteins. Cytoscape software is an open source software platform (Shannon et al., 2003) for visualizing complex networks and integrating these with any type of attribute data, and was used for networking analysis. For Gene Ontology enrichment analysis, up and down regulated genes that were predicted to be target gene were analyzed using PANTHER (Protein Analysis Through Evolutionary Relationships) (<http://www.pantherdb.org/>). This database is a biological database of gene/protein families and their functionally related subfamilies.

### 2.5. NF-YA2 gene profiling

Profiling of NF-YA2 gene in response to HS was investigated using Affymetrix Barley Genome Array (GEO accession number: GSE23896). Profiling was performed by R software 3.5.0 and color heat map visualization was done by using associated software package graphics (<https://www.r-project.org>) for Bioconductor (<http://bioconductor.org>). Data were normalized according to logFC parameter and  $\geq 2.0$

&  $\leq 2$ -fold gene expression.

### 2.6. Plant material

In this study, two genotypes of barley were obtained from Seed and Plant Improvement Institute of Karaj (Karaj, Iran, <http://www.spii.ir/homepage.aspx?site=DouranPortal&tabid=1&lang=fa-IR>). For HS treatment, salt-sensitive (Fajr30) and salt-tolerant (Youssef) barley plants were grown in greenhouse for two weeks and then were kept in 42 °C for 3 h. The heat treated leaves were harvested, frozen in liquid nitrogen and stored at -70 °C.

### 2.7. Primer design

OligoCalc (Kibbe, 2007), Vector NTI software package (Invitrogen, Carlsbad, CA, USA) and OLIGOTECH software (OligoTech, Inc., Wilsonville, OR, USA) were used for primer design. The primers were designed to recognize 238 bp fragment of NFYA2 barley gene with 5'-TTCTCGCTCCGGCTCAC-3' (forward) and 5'-TGCAAGTCCTCCTCC TCA-3' (reverse) sequences. According to Janska et al. (2013), actin reference gene was amplified using the primer forward and reverse sets.

### 2.8. Quantitative RT-PCR analysis

Total RNA was extracted from barley plants by RNX-Plus (CinnaGen Co., Iran) and run on a 1% agarose gel (Fig. 4a). Then cDNA was synthesized by cDNA synthesis kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and fractionated on a 1% agarose gel (Fig. 4b). Fifty ng of RNA was used for cDNA synthesis as follows and the product was analyzed by qRT-PCR in presence of SYBR Green (Bio-Rad Co., LTD., America) in a Step One Real-Time PCR system: 15 s at 94 °C followed by 35 cycles consisting of 15 s at 94 °C, 40 s at 61 °C, and 40 s at 72 °C. A heat dissociation protocol (melting curves in the 60–95 °C range) was used for checking amplification specificity as the final step of the PCR.

### 2.9. Statistics analysis of qRT-PCR data

Analysis of difference in NF-YA2 gene expression between tolerant and sensitive plants was assessed by REST software version 2.0.13 (<http://rest.gene-quantification.info>) performed on 3 replication.

## 3. Results

### 3.1. Identification of genes responding to heat stress condition

Normal barley microarray data from HS including 1 platform (1 series with 12 samples) were downloaded from ncbi GEO and then analyzed using GEO2R tool. The microarray data mining, filtering and analysis have revealed 170 HS responding genes among 20000 genes with a  $\geq 2.0$ -fold increase or a  $\leq 2$ -fold decrease in the LogFC signal intensities.

### 3.2. Functional analysis

Protein interaction in both up and down regulated genes was preliminarily analyzed by STRING software (Fig. 1a and b). Network analysis results of *Arabidopsis* unigenes in STRING software were as follows (Fig. 1a and b):

As shown in Fig. 1a and b: STRING networking data supported a very weak interaction between down-regulated genes nevertheless; these results did not prove that up-regulated genes were more effective in response to HS. Further investigation was needed to better understanding the relationships between genes such as NF-YA2, NF-YB3, DPB3-1 and HSPs. Accordingly, GO analysis of the DEGs was performed using Cytoscape. The GO analysis was performed separately for up and

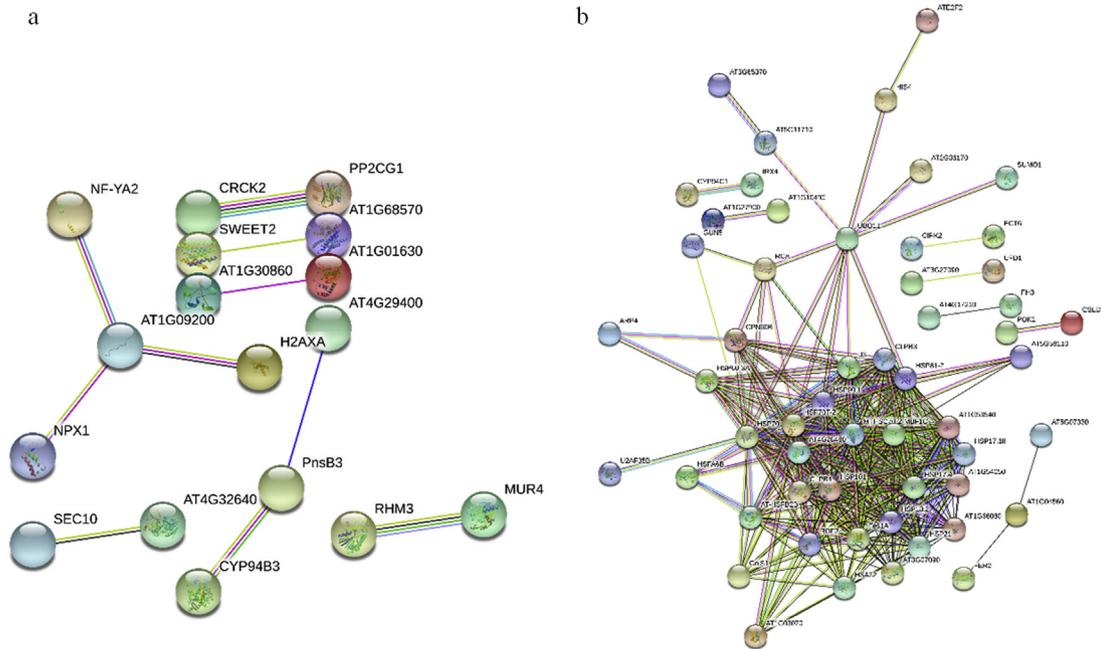


Fig. 1. Interaction between barley heat responded proteins, analyzed by STRING software. The list of identified proteins was subjected to String analysis to reveal their functional interactions. Each node represents a protein, and each edge represents an interaction. Fig. 1a and b shows the down-regulated and up-regulated genes, respectively.

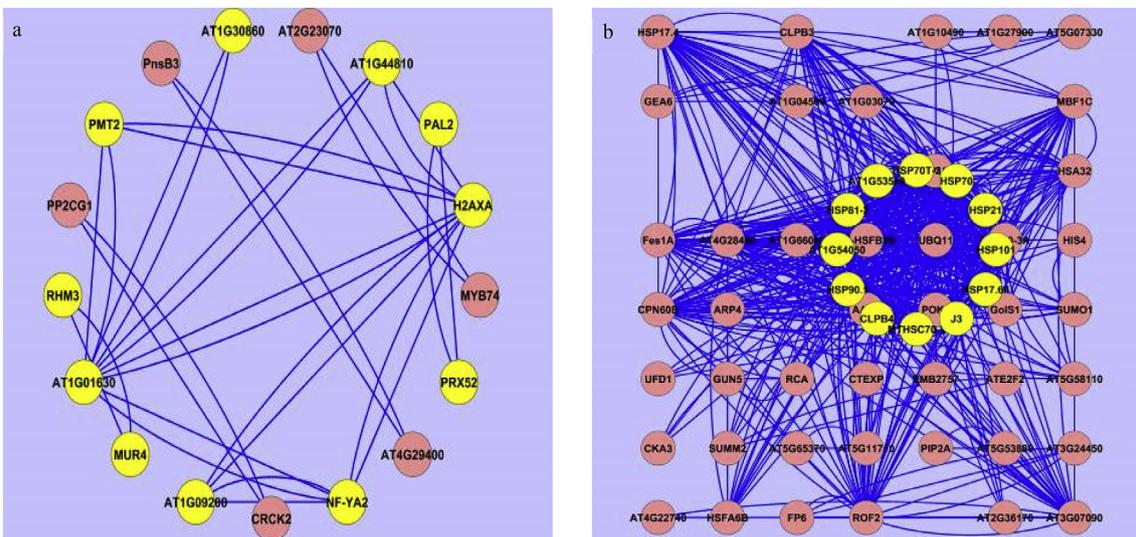


Fig. 2. Topological features of the interactive network in barley drawn up using Cytoscape. Yellow filled circles considered as the HS response hub genes. Red circles represent the genes with the least connectivity values and yellow ones represent the top genes with the highest connectivity values in response to HS in *H. vulgare* L. Blue edges describe the possible relationship between genes involved in HS condition. Fig. 2a and b shows the down-regulated and up-regulated genes of *H. vulgare* L. in response to HS, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

down-regulated genes of barley using the best genes based on *Arabidopsis* orthologs (Fig. 2a and b) to identify the biological processes associated with changes in gene expression in HS.

**Table 1**

The finalized data obtained from Cytoscape software on the HS responding genes in barley.

No.	Gene symbol	Methods	Unigene	Regulation	Putative Function
1	AT1G01630	Closeness.Degree. MNC	At.27921	Down regulation	Lipid binding protein
2	AT1G09200	Closeness.Degree. MNC	At.17610	Down regulation	Histone H3
3	AT1G30860	Closeness	At.40471	Down regulation	Ring/U-box protein
4	AT1G44810	Closeness.Degree. MNC	At.14883	Down regulation	Transcription factor
5	H2AXA	Closeness.Degree. MNC	At.24300	Down regulation	Histone H2
6	MUR4	Degree. MNC	At.20201	Down regulation	Transcription factor
7	NF-YA2	Closeness.Degree. MNC	At.19718	Down regulation	Transcription factor
8	PAL2	Closeness.Degree. MNC	At.21614	Down regulation	Transcription facto
9	PMT2	Closeness.Degree. MNC	At.11015	Down regulation	Transcription facto
10	PRX52	Closeness.Degree. MNC	At.28537	Down regulation	Peroxidase
11	RHM3	Closeness.Degree. MNC	At.27812	Down regulation	Rhamnose synthesis
12	AT1G53540	MNC	At.5366	Up regulation	Heat shock-like protein
13	AT1G54050	Closeness.Degree. MNC	At.11109	Up regulation	Heat shock protein
14	CLPB4	Closeness.Degree. MNC	At.28343	Up regulation	Casein lytic proteinase/heat shock protein
15	HSP101	Closeness.Degree. MNC	At.48370	Up regulation	Heat shock protein
16	HSP17.6II	Degree. MNC	At.20324	Up regulation	Heat shock protein
17	HSP21	Closeness.Degree. MNC	At.29484	Up regulation	Heat shock protein
18	HSP70	Closeness.Degree. MNC	At.23663	Up regulation	Heat shock protein
19	HSP70T-2	Closeness.Degree. MNC	At.13604	Up regulation	Heat shock protein
20	HSP81-2	Closeness.Degree. MNC	At.25243	Up regulation	Heat shock protein
21	HSP90.1	Closeness.Degree. MNC	At.25471	Up regulation	Heat shock protein
22	J3	Closeness	At.20860	Up regulation	Heat shock-like protein
23	MTHSC70-2	Closeness. Degree	At49002	Up regulation	Heat shock protein

Closeness, Degree and MNC were network-clustering algorithms, used by Cytoscape to find the top genes involved in the HS response. Regulation column shows the state of the gene expression under the impression of HS. Putative Function column represents the possible role of the genes in the cell.

### 3.2.1. Comparison of genes identified by cross-experiment and scholar mining

After characterization and networking of genes involved in HS, Scholar data mining was performed to find the heat responding genes. This literature mining approach is more efficient than the microarray analysis in identifying genes associated with HS, in the case of little expression change, which may be neglected in the microarray analysis. According to the literature review and methods steps listed above, NF-YA2 was chosen as hub gene involved in the HS response.

### 3.2.2. NF-YA2 gene profiling

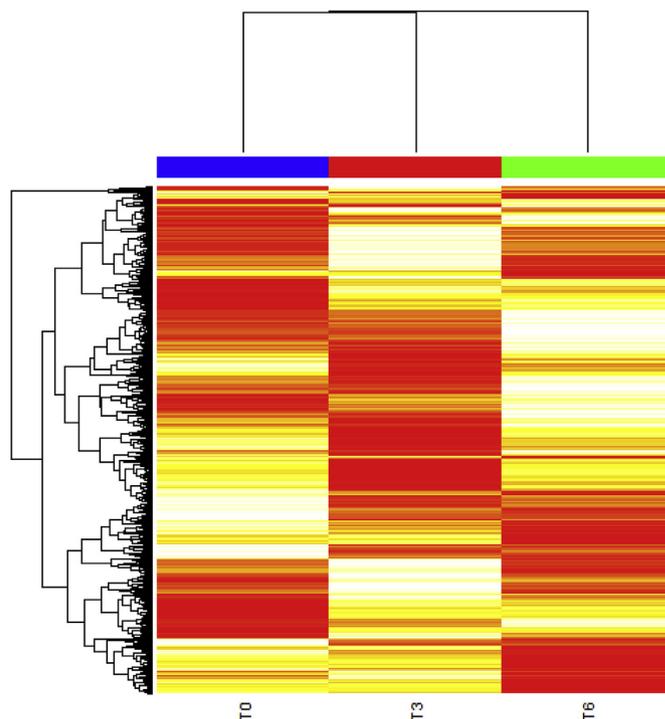
Heat map analysis results showed the profiles of genes from GO accession number GSE23896 which were affected under HS, were subject to at least 2-fold altered transcription levels. Heat map was generated by R software using default settings for the clustering algorithm.

### 3.2.3. Quantitative RT-PCR analysis

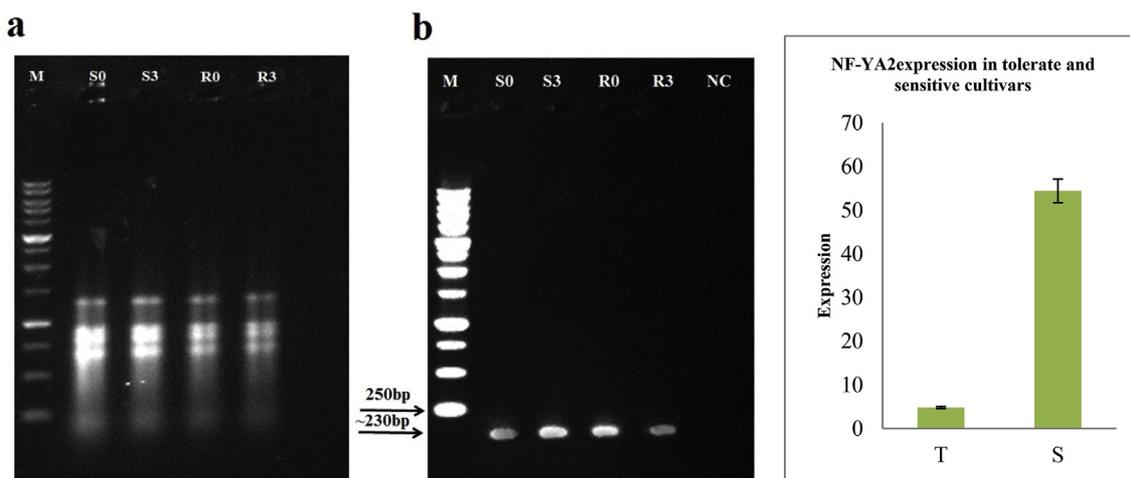
The analysis of difference in NF-YA2 gene expression was performed by the REST software. As showed in Fig. 4c, the vertical axis represents the level of expression and the vertical axis is related to the sensitive and tolerant plants. NF-YA2 expression under HS in the sensitive plants was much more than that in the tolerant ones. The results of qRT-PCR analysis indicate that NF-YA2 gene expressions in the tolerant and sensitive plants are 0.006 and 54.43, respectively (Fig. 4c).

## 4. Discussion

Interpreting the biological relevance of changes in expression (Lu et al., 2007) is a major challenge in microarray analysis. On the other hand, HS response of genes is one of the major worldwide concerns. Plants have evolved various physiological and molecular mechanisms to respond to HS (Qu et al., 2013). In order to determine the molecular mechanisms involved in the HS response of plants, it is needed to understand how plants respond and adapt to HS. Also, the results will be helpful in producing heat-tolerant crop. On the basis of the results of PANTHER analysis, most of the candidate genes were involved in cellular process and had catalytic activity. Gene ontology annotations of genes responding to HS are useful to reveal the respective roles of these



**Fig. 3.** Heat map illustration profiles from GO biological process category “developing barley caryopses” (GO accession: GSE23896) and detected to be expressed by any 12 array. Microarray data were obtained for different time points for heat stress, viz 0.5 h, 3 h and 6 h for developing barely caryopses (showed in blue, red and green, respectively) and analyzed with respect to the control. The genes affected under HS treatment with at least 2-fold altered transcription levels. Microarray data were normalized based on logFC parameter. Relative signal value are represented by color bar at the bottom of heat map, so white color represents no change in expression. Also, yellow and red colors show the down- and up-regulated genes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 4.** a: Screening of total RNA extraction from *H. vulgare* L. and validated based on size by 1 kb gene ladder. Lane M: 1 kb ladder; Lane S0: sensitive plant in control condition; Lane S3: sensitive plant in HS condition; Lane R0: tolerate plant in control condition; Lane R3: tolerate plant in HS condition. **Fig. 4b:** PCR product of NF-YA2 cDNA. Lane M: 1 kb ladder; Lane S0: sensitive plant in control condition; Lane S3: sensitive plant in HS condition; Lane R0: tolerate plant in control condition; Lane R3: tolerate plant in HS condition; Lane NC: none control. **Fig. 4c:** NF-YA2 expression level under HS condition in *H. vulgare* L. tolerant and sensitive cultivars. Lane S: sensitive plants, Lane T: tolerant plants.

genes in the cell. According to the heat map results, for the up-regulated genes in control condition, after 3 h heat treatment the expression decreased and the down-regulated genes changed into the corresponding up-regulated genes (Fig. 3). After 6 h heat treatment, the expression pattern was reversed again (Fig. 3). So a decreased or increased expression was observed for the down and up-regulated genes, respectively after 6 h heat treatment. It is noteworthy that the experimental data were in accord with the results of literature review and bioinformatics prediction results. Accordingly, we concluded that NF-YA2 is the most important heat responding gene. To our knowledge, it is the first report on the *in silico* analysis of NF-YA2 expression in response to HS in barley. Expression level of the NF-YA2 gene under HS in sensitive plants was far greater than that in tolerant plants. According to the results, it is not always necessary to increase the expression of the responsive genes to develop resistance against an abiotic stress. Also, the results suggest the involvement of simultaneous interaction of some important transcription factors such as NF-YA2.

NF-Y and all subunits required for DNA-binding (Maity&de Crombrughe, 1998; Mantovani, 1999) have been previously biochemically characterised. The NF-YB and NF-YC subunits form a tight dimer that offers a looser complex surface for NF-YA association (Franchini et al., 2005). NF-Y and the CCAAT box as its binding element, are among the first series of *trans*-acting factors and *cis*-elements identified in eukaryotes (Dolfini et al., 2012). On the basis of different studies, NF-YA core domain has been divided into two segments including a N-terminal domain responsible for NF-YC/NF-YB binding and a C-terminal one implicated in specific recognition of the CCAAT element (Olesen and Guarente, 1990; King et al., 1993; Mantovani et al., 1994). As was previously mentioned, overexpression of At-NF-YA2, 7 and 10 leads to the enhanced tolerance to several types of abiotic stress such as heat (Leyva-González et al., 2012). Based on our data (Fig. 1) NF-YA2 is linked to histone H3, which is one of the other hub genes responding to HS. The stress caused in acetylation of histone H3 but did not involve adjustments in DNA methylation (Lang-Mladek et al., 2010). Also, Histone H3 is linked by two lines, a straight one (passing through CRCK2) and a curve one, directly, with NF-YA2. Histone H3 is also linked with H2AXA (that is another Histone). On the other hand, Sato et al., (by interaction and expression pattern analyses) were reported that, the existence of a trimmer consisting NF-YA2, NF-YB3, and DPB3-1 that could synergistically activate a promoter of the heat stress-inducible gene with DREB2A in protoplasts associated with other influential factors like metabolism, transcription factors and stress related

genes (Sato et al., 2014). Hence, as mentioned previously (Liu et al., 2012), and based on our bioinformatics analysis HSPs were also up-regulated during HS.

The results of this study suggest that the *in silico* approach for analysis is a reliable strategy for discovering differentially expressed genes responding to HS. The expression of NF-YA2 gene was found up- and down-regulated in sensitive plants and tolerant ones, respectively. The increase in NF-YA2 levels could produce an interference in the formation of The existence of the trimmer consisting NF-YA2, NF-YB3, and DPB3-1, so that the complex is less effective to synergistically activate the promoters of the heat stress-inducible genes. Further studies are needed to support the knowledge on the role of these TFs.

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