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Data mining approaches highlighted transcription factors that play role in thermo-priming

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Abstract

Abiotic stresses including heat are major threats to crop plants especially considering the global warming facts. A pre-exposure to heat stress can prime plants and enable them to encounter a severe dose of stress which is lethal to unprimed plants. In this study, in order to identifying potential key elements involved in priming responses to heat stress we analyzed a microarray series in *Arabidopsis thaliana.* To this end we conducted differential expression analysis, clustering, annotation and network construction by using the publicly available tools. In agreement with experimentally validated results we noticed that different genes were differentially expressed between primed and unprimed plants from which genes encoding retro-elements and proteins involved in chromatin remodeling were noteworthy. The topology analysis of constructed network highlighted the role of TFs including zinc finger and DREB in heat acclimation after priming phase. A total of 33 transcription factors were differentially expressed in primed versus unprimed plants five of which were detected to be hub and bottleneck nodes in genes network that may play a role in heat stress priming and memory additionally as potential targets to discover new insights to improve stress resistance in crop plants.

Keywords: gene regulatory network, heat stress memory, priming, transcription factor.
Abbreviations: APX2_ascorbate peroxidase 2; AUPR_Area Under the PR curve; AUROC_Area Under the ROC curve; CCA1-_CIRCADIAN CLOCK-ASSOCIATED1; DE_differentially expressed; FDR_False Discovery Rate; GENIE3_GEne Network Inference with Ensemble of Trees; GLP4_germin-like protein 4; GOLS1_galactinol synthase1; GO_Gene Ontology; GRN_Gene Regulatory Network; GST_Glutathione S-transferase; HSPs_ heat shock proteins; HSFs_heat shock transcription factors; HS_heat stress; limma_Linear Models for Microarray Data; MBF1C_MULTIPROTEIN BRIDGING FACTOR 1C; NCBI_National Center for Biotechnology Information; qRT-PCR_Quantative Reverse Transcription–PCR; RMA_Robust Multiarray Averaging; ROC_Receiver Operating Characteristic; TF_transcription Factor.

Introduction

Plants are sessile organisms that should gauge and adapt to external stimuli such as extreme temperature, salinity and low humidity by different physiological responses (Mittler et al., 2012). These responses vary from an immediate to long lasting responses; Plants immediate responses to HS happens by physiological and genomics changes due to the many heat responsive genes (Thomashow, 1999) while long lasting responses can be achieved by genome modifications or when plants have been already exposed to a moderate stress that this exposure may act as a priming phase by imprinting in the plants memory (Stief et al., 2014). Therefore, plants will acquire a sort of thermo-tolerance if they expose to a moderate heat treatment (priming) before exposing to high temperature which is lethal to non-adapted plants. The acquired thermo-tolerance is maintained over several days due to the activation of several inner process that is genetically separable from inherited heat tolerance (Charng et al., 2006). Despite the relatively good understanding about immediate responses to acute HS, except for the biological basis, in molecular level it is only little known about the thermo-tolerance acquired by priming responses to severe HS. Nowadays data mining approaches are prominent strategies for extracting meaningful information from a growing wealth of biological data including microarrays and RNA-Seq that microarrays has been intensively used (Khosravi et al., 2015; Mantione et al., 2014) and elucidating high-fidelity gene associated networks from transcriptome data is one of the most important applications of computational systems biology. A GRN is a graph representation of biological units in which nodes represent genes and edges are the interactions between nodes. Generally, tools designed for recovering these interactions rely on similarity matrices indirectly measured by correlation matrices or mutual information (Feizi et al., 2013). However connectivity between nodes does not mean the causal relationships and similarity matrices usually includes many indirect links that should be identified and removed for increasing the reliability of GRN inference algorithms hence several sophisticated approaches attempted to remove indirect interactions and detect the causal relationships between gene pairs (Omranian et al., 2016; Huynh-Thu et al., 2010). On the other hand studying modularity in biomolecular networks is an efficient way to identify key components and complexes within transcriptional interactions. In graph theory in addition to hub nodes (proteins with high degrees), certain nodes with higher betweeness which are connected with the shortest paths are more likely to be key connectors and likewise critical points

controlling important dynamic components in biological networks (Faith et al., 2007) that removal of them causes biological systems fail to save their coherence thereby these nodes were named as bottleneck nodes. In this study, by exploiting the publicly available transcriptome data and analysis tools, we attempted to perform a transcriptome analysis of *Arabidopsis* thermo-tolerance after a moderate heat acclimation phase to the identification of potential key genes that are likely crucial for thermo-priming.

Results and Discussion

Transcriptional response to HS priming by identifying thermo-tolerance related genes

A total of 181 probsets were up-regulated in primed plants versus unprimed ones (supplementary Figure 2A and supplementary Table 1) including known genes in HS responses such as 16 HSPs; DNAj, HSP70b, HSP20-like chaperons, HSP70T-2, HSP17.4, HSP22.0, HSP23.6-MITO, HSP21, HSP17.6II, HSP17.6A, HSP18.2, HSP26.5-P, HSP17.6C-CI, HSP17.6-CII, HSP81-1, HSP23.5-M and HSA32. HSFs include HSFA2, HSFB2A, HSFA3, HSFA1E and ATHSF4, FKBP53 and FKBP65, APX2, MBF1C, a member of cytochrome P450 family, 3 members of dehydrogenase family, 7 genes of transporters family, 2 genes from Ca2+ signaling pathway, GOLS1, tetraspanin9 and GST family protein (ERD9) were another notable up-regulated genes. From the mentioned genes HSP17.6A, HSP22, HSA32, FKBP and APX2 are memory associated genes (Baurle, 2016) when FKBP1 mutant showed an impaired memory stress (Charng, et al., 2006). HSP22, HSP18.2, HSP21 and APX2 are examples of proteins that their expression sustain at least 3 days after exposing to HS (Stief et al., 2014). MBF1C acts in the up-stream of ethylene pathway that its expression is essential for basal thermo-tolerance and controls the expression of 36 different transcripts during heat stress, including the important transcriptional regulator such as DREB2A, HSFs and several of zinc finger proteins (Suzuki et al., 2008). In the up-regulation genes we noticed genes encoding transposable elements like Copia-type transposon which HSFA2 is required for their activation. There is couple of evidences that retrotransposons are activated after prolong HS and can induce neighbor genes by HS (Ito et al., 2011). Another interesting point about differential expression results was up-regulation of tetraspanin which recently was found to be related with stress memory process. Although there is no clear correlation between gene expression and DNA methylation in *Arabidopsis* (Colaneri et al., 2013), differential expression of several genes related to histone modification and DNA methylation including histone-lysine N-methyltransferase, ALFIN-LIKE 5 and histone acetyltransferase HAC1 in this study seem more interesting. In this regard H3K4me3 is a marker of gene activation and stress memory (Oh et al., 2008) that probably is activated with up and down regulation of ATXR2 and histone H2B respectively. ATXR2 is a histone N-lysine methyltransferase that activates a couple of genes in abscisic acid pathway in drought stress but there is no evidence on its role in *Arabidopsis* stress memory and has been shown to be involved in chromatin remodeling in plant response to water stress. 160 probsets were down-regulated (supplementary Figure 2B and supplementary Table 1) from which FKBP-like family protein, GSTU26, GSTU14 and 2 genes from Calcium-binding EF-hand family protein are noteworthy. Moreover GLP4 and pollen allergen 1 extensin were strongly down-regulated (FDR<0.01). Further inspection of gene expression was performed by grouping the genes into early (4 and 8 hours) and late (24 and 48) responses to HS (supplementary Table 2). In the early and late responses 97 and 5 genes were respectively up-regulated that the expression of several proteinases, NHX2, Copia-type reverse transcriptase-like protein, APX2, GolS1, LTP4 in early response and histone acetyltransferase of the CBP family 1 in late response are more interesting. Furthermore in early and late responsess 119 and 7 genes were down-regulated respectively. Down regulation of expansin A2 and pollen allergen 1 extensin in early responses is notably. Reportedly Yan et al., (2014) illustrated the role of expansins in osmotic stress tolerance in *Arabidopsis*. These proteins facilitate cell wall extension (Prasad et al., 2010). Down-regulation of extensins in this study might be biologically related to growth stalling at initial stage of HS (Sampedro and Cosgrove, 2005). Here to study the potential functions of the up and down regulated genes and classify them into four main categories namely biological process, molecular function, cellular localization and protein classes we used PANTHER database (supplementary Figure 3 and 4). We noticed that the most of DE genes were grouped in metabolic process (GO: 0006915), catalytic activity (GO: 0003824), cell part (GO: 0044464), nucleic acid binding (PC: 00176) and transferase (PC: 00220).

Differential responses of TFs to priming-associated thermo-tolerance

We used a list of 2576 *Arabidopsis* TF TAIR IDs compiled from Agris (Yilmaz et al., 2011), DAFT (Guo et al., 2005), PlantTFDB (Jin et al., 2014) and RARFT (Iida et al., 2005) for extracting the expression profiles of TFs in this study. We only focused on the highly expressed TFs which were expressed above the threshold (two-fold cut-off and with p-value < 0.01) whereby 33 TFs from different families such as HSF, NAC, WRKY, zinc finger, DREB and bZIP were passed the filter (Table 1). The hierarchical clustering of differential expressed TFs has been provided in supplementary Figure 5. Except for genes encoding AGL12, WRKY54, zing finger, bZIP, MYB and AP2/EREBP, the rest of TFs were up-regulated in early response phase and toward the late responses their expression were down-regulated. In *Arabidopsis thaliana* there are at least eight HSFs (Schramm et al., 2006) that in this study four of them including HSFB2A, HSFA3, HSFA1E and ATHSF4 were up-regulated. In *Arabidopsis* drought stress signaling pathway, HSFA3 is regulated by DREB2A that in turn will activate essential genes for sustaining protein homeostasis. In this list CCA1 is involved in phytohormone signaling pathway and a regulator in abscisic acid pathway. CCA1 is a Myb-related TF that activates the genes conferring the ability of tolerating a range of environmental temperatures (Rawat et al., 2011). As demonstrated in a study by Barah et al., (2016), among the identified TFs, At5g01380, At1g27730, At5g62020, At2g46830, At4g31800, At2g42150, At1g21000, At3g02990 and At5g22290 were shown to be responsive to cold, flagellin, salt and light stresses.

Co-expression network analysis

By using the 341 HS-regulated probsets between primed and unprimed plants we built a bipartite gene network using Pearson correlation coefficient (PCC threshold ≥0.70) and p-value ≤ 0.01 as a significant threshold for filtering out-ranged nodes. The constructed network is bipartite because they

Table 1. List of HS-responsive TFs between primed and unprimed plants.

Probset ID	TAIR ID	Description	logFC
	Up-regulated		
251114_at	At5g01380	Homeodomain	1.285
261086_at	At1g17460	TRFL3	1.105
264814_at	At2g17900	ASHR1	1.575
267140_at	At2g38250	Homeodomain	1.998
267026_at	At2g38340	DREB19	2.131
258133_at	At3g24500	ATMBF1C	1.779
259618_at	At1g48000	AtMYB112	1.495
256576_at	At3g28210	PMZ	3.530
261648_at	At1g27730	STZ	2.214
247509_at	At5g62020	AT-HSFB2A	2.098
248564_at	At5g49700	AHL17	1.175
247655_at	At5g59820	AtZAT12	2.258
266719_at	At2g46830	AtCCA1	2.165
253485_at	At4g31800	ATWRKY18	1.007
258157_at	At3g18100	AtMYB4R1	1.193
261610_at	At1g49560	Homeodomain	1.012
249139_at	At5g43170	AZF3	1.284
267631_at	At2g42150	bromodomain	1.723
262803_at	At1g21000	PLATZ	1.118
259800_at	At1g72175	DUF 1232	1.171
250910_at	At5g03720	AT-HSFA3	2.212
256356_s_at	At1g66500	Pre-mRNA cleavage	1.658
	At5g43620	complex II	
258603_at	At3g02990	ATHSFA1E	2.153
249944_at	At5g22290	FSQ6	1.2494
248611_at	At5g49520	ATWRKY48	0.823
246214_at	At4g36988	ATHSF4	1.490
	At4g36990		
	Downregulated		
251420_at	At3g60490	Integrase	-0.766
266516_at	At2g47880	Glutaredoxin	-1.44
263549_at	At2g21650	ATRL2	-1.258
261504_at	At1g71692	AGL12	-0.787
258734_at	At3g05860	MADS-box	-0.648
257382_at	At2g40750	ATWRKY54	-0.714
256446_at	At3g11110	RING/U-box	-1.094

logFC shows the expression ratios in primed plants in contrast to the controls.

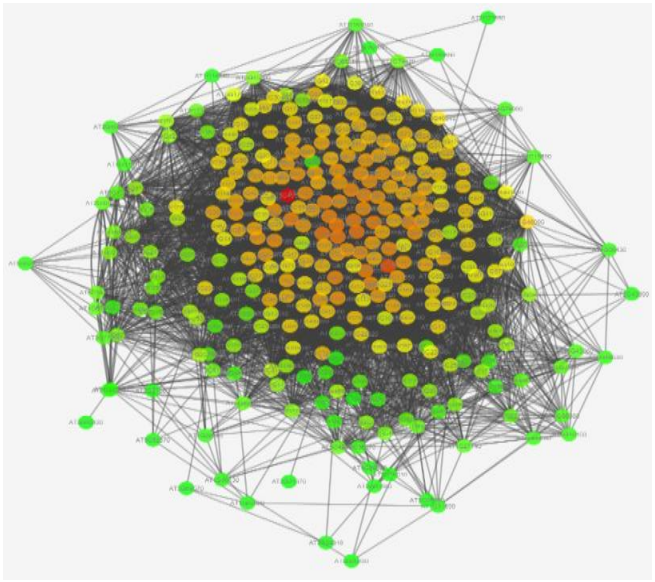

Fig 1. Co-expression network obtained by DE genes between primed and unprimed plants. We illustrated nodes with higher connectivity bigger and darker.

Table 2. The top 20 common hub and highest rank bottleneck genes derived from topology analysis of GRN constructed by GENIE3.

TAIR ID	Probset ID	Description	logFC
AT3G60490	251420_at	ERF/AP2 transcription factor family	-0.77
AT3G17030	257931_at	Nucleic acid-binding proteins superfamily	0.71
AT1G80130	262050_at	Tetratricopeptide repeat like superfamily protein	0.89
AT5G58070	247851_at	temperature-induced lipocalin	1.46
AT1G10370	264436_at	Glutathione S-transferase family protein	1.24
1AT3G07900	258648_at	O-fucosyltransferase family protein	-0.59
AT3G28210	256576_at	zinc finger	3.53
AT1G53940	263146_at	GDSL-motif lipase 2	-0.74
AT4G28520	253767_at	cruciferin 3	-1.67
AT4G35200	253179_at	DUF241	-0.89
AT2G19900	266690_at	NADP-malic enzyme1	1.76
AT5G17310	250074_at	UDP-glucose pyrophosphorylase 2	1.1
AT3G05860	258734_at	MADS-box transcription factor family protein	-0.65
AT5G64410	247284_at	oligopeptide transporter 4	-1.26
AT1G71692	261504_at	AGAMOUS-like 12	-0.8
AT5G15250	250162_at	FTSH protease 6	2.18
AT3G48720	252317_at	acyl-transferase family protein	-0.8
AT1G17190	262516_at	glutathione S-transferase tau 26	-0.8
AT4G25200	254059_at	ATHSP23.6-MITO	4.97
AT5G62020	247509_at	AT-HSFB2A	2.09

logFC shows the expression ratios in primed plants in contrast to the controls.

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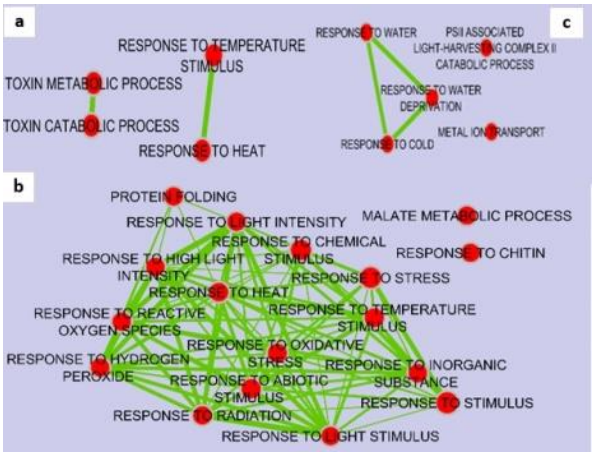

Fig 2a, **b**, **c**. After clustering of DE genes within co-expression network, each subnetwork was annotated to underlying GO categories.

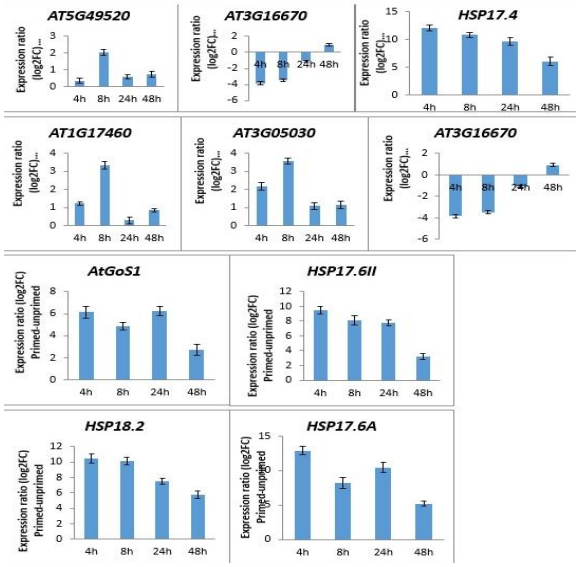

Fig 3. Quantative reverse transcription–PCR. Values were expressed in log2FC. Error bars indicate means±s.d. of three independent biological replicates each containing a pool of ~100 seedlings.

consist of two layers of nodes (supplementary Table 3). By utilizing the NetworkAnalyzer we set nodes with higher connections to bigger size and darker color (Figure 1). In co-expression network the WRKY (AT5G49520) and MYB-related (AT1G17460) TFs showed the highest connections (darker and bigger nodes). AT5G49520 encodes WRKY48 a stress induced TF and AT1G17460 encodes a TRF-like 3 TF a potential direct target of CCA1 that is expressed during expansion stages including petal differentiation and expanded cotyledon stage. Next to detect the potential clusters and densely interconnected nodes within co-expression network we performed cluster analysis and functional classification by BINGO Cytoscape plugin with Hypergeometric test and Benjamini and Hochberg FDR correction at significant level 0.05 (supplementary Table 3). As illustrated in the Figure 1 network was divided to three major sub-networks (Figure 2a, b and c); TRF-like 3 TF was grouped in subnetwork 2a. Genes within this sub-network were enriched for response to heat, temperature stimulus and toxin catabolic process. WRKY TF was placed in sub-network 2b. This sub-network was enriched for more process including response to reactive oxygen species, hydrogen peroxide, chitin, radiation and malate metabolic process that could imply on the role of some compounds like malate dehydrogenase in thermo-memory. Sub-network 2c included the least of genes enriched for response to metal ion transport and PSII associated light-harvesting complex II catabolic process. Proteases like FTSH6 is a product of this gene category. FTSH6 is a plastid metalloprotease which jointly with HSP21 regulate thermo-memory in *Arabidopsis* (Sedaghatmehr et al., 2016).

Topology analysis for detecting potential key genes

For conducting a precise topology analysis, inferring a more reliable GRN is essential. we therefore firstly build and then compared them by testing the first 10000 highly ranked edges over 4775 gene interactions in gold set list obtained from AGRIS database (supplementary Table 7). Evidently from supplementary Figure 7 computed AUROCs by R package minet showed the GENIE3 as the most powerful GRN inference algorithm to predict higher rates of true edges over the rest of compared GRN inference methods (Supplementary Figures 6, 7 and 8). While the aforementioned approaches reconstruct GRNs based on bilateral relationships, regression-based methods extract one-to-many interactions between nodes from measurement of gene expression (Linde et al., 2015). In this context GENIE3 infers GRNs by decomposing of network recovery procedure to p steps that p is the number of genes and each step is consisting of identifying genes that regulates a given target gene (Huynh-Thu et al., 2010). As a result we selected GENIE3 derived GRN for topology analysis and defined genes as hubs and bottlenecks if they are in the top 10% of degree distribution (genes that have the 10% highest number of neighbors) (Table 2). As we showed in Table 2, genes encoding proteases, transferases and transporter are shown as hubs and bottleneck. A total of five TFs among the hubs and bottlenecks were common between DE TFs (Table 1) including zinc finger (AT3G28210), two MADS-boxes (AT3G05860 and AT1G71692), ERF/AP2 (AT3G60490) and HSFB2A (AT5G62020). All members of the DREB2 family are involved in the regulation of heat-responsive genes. HSFB2A regulates the expression of HSP26.5, HSP25.3, HSP70b, APX2, and GolS1 that all of them were differentially regulated in this study. The expression of HSA32 was also related with expression of HSFB2A (Charng et al., 2006). Stress signals perception and transmission via calcium signaling and protein kinases following by the activation of stress responsive genes are the most important processes in plants response to different harsh environmental conditions (Padmalatha et al., 2012). Consistently, in this study several genes encoding proteins involved in calcium signaling and kinases including calmodulin like37, Calcium-binding EF-hand family protein, Calcium-dependent lipid-binding (CaLB domain) family protein, kinase superfamily protein, MAPK/ERK kinase 1 and calcium-dependent protein kinase 15 were up and Concanavalin A-like lectin protein kinase family protein and one of Calcium-binding EF-hand family proteins down-regulated. Hypothetically these signals in downstream will activate TFs especially hubs TFs detected in this assay such as zinc finger, MADS-box, DREB and HSF. Furthermore plant hormones signaling plays an important role in plant abiotic stress responses (Park et al., 2014). In agreement, genes encoding MBF1C involved in ethylene signaling pathway, CCA1 a key regulator of abscisic acid signaling and N-MYC downregulated like 1 a positive regulator of auxin signaling pathway were found to be up regulated while allene oxide cyclase1,2 involved in jasmonic pathway and Leucine-rich repeat protein kinase family protein involved in phytohormone signaling pathway down-regulated. Transcriptome changes due to the activation of aforementioned signaling elements and responsive TFs would impact on the expression of chaperons (HSPs) that there are tremendous of evidences in their role in response to HS (Bruce et al., 2007) and in this study sixteen of them were clearly up-regulated. Furthermore notable up-regulation of antiporter NHX2 involved in turgor regulation and stomatal function (Barragán et al., 2012), genes involved in detoxification (Thioredoxin/At5g06690 and Ascorbate/ At3g09640), stress responding genes (At2g24040, At3g53250, At4g11210, At5g15960 and At5g15970), redox exchanges (At5g06690 and At2g47880), energy production including DEAD/DEAH box helicase (At5g65900), ribonuclease T2 (At5g18040) and Adenine nucleotide alpha hydrolase (At1g68300) are examples of protecting strategies which considered to be achieved by priming responses to HS detected in this study.

Materials and Methods

Datasets used and pre-processing

We firstly downloaded GEO Series GSE72949 from NCBI (GEO, http://www.ncbi.nlm.nih.gov/geo/) database. In this experiment, *Arabidopsis thaliana* Col-0 4-d-old seedlings were subjected to heat regime of 4, 8, 24, and 48 hours as priming stimulus (that is, during the memory phase). For the HS phase, primed seedlings and control plants (unprimed) were subjected to a heat regime of 1.5 h, 37°C; 1.5 h recovery at 22°C; and 45 min, 44°C. After the priming HS treatment, seedlings were returned to normal growth condition for 3 - 4 days (recovery or memory phase). Supplementary Figure 1 is a schematic representation of experimental design used in this study. The gene expression supposed to be different between primed and unprimed plants due to the memory phase that primed plants have been exposed to a moderate heat. The subsequent bioinformatics analysis was done to identify the differentially regulated genes arise from memory phase.

Raw CEL files were normalized with RMA method embedded in affy R package (http://www.bioconductor.org). By using the limma R package, thermo-tolerance associated DE genes were identified between primed and unprimed

samples if expression level changes was above the defined threshold (absolute Log-fold change ≧ 1 and with p-value < 0.05). DE gene were functionally annotated by PANTHER server (http://www.pantherdb.org/geneListAnalysis.do) with default parameters.

Co-expression network analysis

In order to identifying potential signatures of transcriptomic response to thermo-priming we reconstructed co-expression network by setting p-value ≤ 0.01 and Pearson correlation coefficient ≥ 0.75. The network was visualized by Cytoscape 3.4.0. The genes within co-expression network were clustered to underlying sub-networks by Glay software embedded in clusterMaker Cytoscape plugin (http://apps.cytoscape.org/apps/clustermaker). Subsequently the modules were functionally classified by BiNGO Cytoscape plugin (Maere et al., 2005) to determine significantly over-represented GO terms. For network being more informative, we added publicly interaction databases including ATPID (http://www.megabionet.org/atpid/webfile/), AtPIN (atpin.bioinfoguy.net/) as well as Arabidopsis promoter information from AGRIS (http://agris.fao.org/agris-search/index.do).

Topology analysis of constructed GRN

For constructing GRNs, we utilized five algorithms considering their ability in recognition and removing of indirect links between genes including matlab implementations of Global Silencing by (Barzel and Barabási, 2013) and Network Deconvolution by (Feizi et al., 2014), Graphical Gaussian Models by (Schäfer and Strimmer, 2005) using GeneNet R package, R implementation of GENIE3 by Huynh-Thu et al., (2014) and CLR using spearman estimator embedded in minet R package (Faith et al., 2007). To assess the accuracy of algorithms in GRN discovery and choosing the better one for topology analysis, we drew the ROC curve that plots true positive rate versus the false positive rate by minet R package (https://www.bioconductor.org/). Centrality analysis of genes within the preferred GRN was performed by utilizing CytoNCA Cytoscape plugin (Tang et al., 2014).

qRT-PCR analysis

In order to testing reliability of our *in silico* analysis, we selected 10 genes from significantly HS-regulated genes for performing qRT-PCR analysis. Synthesis of cDNA and qRT-PCR by SYBR Green were done as described in (Balazadeh et al., 2010). qRT-PCR was done on an ABI PRISM 7900HT sequence detection system (Applied Biosystems Applera, Darmstadt, Germany). Expression levels were normalized against the expression level of ACTIN2. We designed the primers by Quantprime program (Arvidsson et al., 2008) (supplementary Table 6). The results presented are from three independent biological replicates from different plants (p-value < 0.05, student's t-test) (Figure 3 and supplementary Table 8).

Conclusion

In the present study we performed a comprehensive bioinformatics analysis to the identification of priming influences in transcriptome level. We used a reference network to assess the accuracy of constructed GRNs and reliability of identified hubs and bottleneck genes. Moreover we tested several of identified genes by qRT-PCR analysis. However by using more adequate number of samples the results would be more reliable. Furthermore gene expression network analysis at transcriptome level could be more intensified through merging studies with protein networks to draw more precise conclusions regarding predicted master regulators. Finally we used unweighted co-expression network and modularity analysis we then should be cautious about dynamic nature of living organisms via strictly analysis of statics networks.

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