

Transcriptome analysis of Arabidopsis to predict stress length-specific regulatory networks involved in drought response

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Abstract

Drought stress is one of the major environmental factors impairing crops productivity worldwide. Plants use various regulatory genes to reprogram genome activities to cope with such stresses. Among regulatory genes, transcription factors (TFs) function as terminal transducers and directly regulate the expression of wide spectrum of downstream genes. Multiplicity of the TF families and the complex interactions between TFs and cis-elements on the promoters of target genes as well as cross-talk between TFs in response to stress indicate the complexity of signaling networks involved in plant stress responses. This study aimed to use computational and statistical approaches to analyze a microarray dataset from Arabidopsis which covering different time periods of drought stress. After identifying and functional grouping of differentially expressed genes (DEGs), genes encoded TFs were determined and networked based on gene set enrichment analysis (GSE). Hierarchical regulatory network in each condition was assigned. After that, networks were used to conduct network topology analysis. Results indicated an obvious orientation in genome activity toward response to different cues; energy homeostasis and photosynthesis stability was occurred under drought stress. Also, 3787, 2931 and 5115 genes were differentially expressed under the early, moderate and prolonged drought stress, respectively, among them, 169, 140 and 261 TF were identified. Analysis of constructed regulatory networks of each drought condition revealed that plant recruits different but somewhat overlapping strategies to cope with stress in a long period of time. In each drought period, specific or common signaling pathways are activated using several numbers of transcription factors. It seems that among all identified TFs, *ARR5*, *ARR6*, *ABF3*, *MYB29*, *MYB76* and *SIGs* genes are good candidate to manipulate plant stress tolerance.

Keywords: Regulatory networks, transcriptomics, drought stress, singular enrichment.

Abbreviations: TFs_Transcription factors; DEGs_Differentially expressed genes; GSE_gene set enrichment; ARR_Type-A Arabidopsis Response Regulators; *ABF3*_ABF3 abscisic acid responsive elements-binding factor 3; MYB_myeloblastosis; SIG_Sigma factor; AP2/EREBP_APETALA2/ ethylene-responsive element binding proteins; NAC_NAM, ATAF, and CUC; b-ZIP_Basic Leucine Zipper Domain; Dof_DNA binding with One Finger; bHLH_basic helix loop helix; HD-Zip_Homeodomain-leucine zipper; HSF_Heat shock factor.

Introduction

Drought is a major factor impairing crops productivity worldwide (Farooq et al. 2012; Farooq et al. 2009). To cope with drought stress, plants use complex and multilayers mechanisms to reprogram their genome activity which results in change in genes expression and function (Ahuja et al. 2010; Ashraf 2010; Chen et al. 2014). Mainly, core regulatory networks underlying of such changes consist of several regulatory genes including transcription factors (TFs), protein kinases, protein phosphatases and miRNAs shaping the relevant transcriptome to show an adaptive response (Maruyama et al. 2012; Mehrotra et al. 2014; Mishra et al. 2006; Nakashima et al. 2009). Among all the mentioned components, TFs as one of the most important regulators, function as terminal transducers and directly regulate the expression of wide spectrum of downstream genes via binding to specific cis-elements in their promoter region (Yamaguchi-Shinozaki and Shinozaki 2005; Yamaguchi-

Shinozaki and Shinozaki 2006). In the recent decades, TFs gain the attention in researches that has been conducted to identify main tolerant genes (Gehan et al. 2015; Gollidack et al. 2011; Maruyama et al. 2012). Several TF families including AP2/EREBP, MYB, WRKY, NAC, bZIP, Dof, bHLH, HD-Zip, HSF, etc, were identified and their roles in plant stress tolerance have been investigated (Gehan et al. 2015; Gollidack et al. 2011; Yamaguchi-Shinozaki and Shinozaki 2006). However, it is not clear what TF(s) in such a huge gene families should be selected to manipulate stress tolerant. This issue rises from the fact that stress response in plants is an extremely complicated process (Pessaraki 2011). Multiplicity of the gene families and the complex interactions between TFs and cis-elements on the promoters of target genes as well as cross-talk between TFs in response to stress indicate the complexity of signaling networks involved in plant stress responses (Seki et al. 2003; Shamloo-

Dashtpagerdi et al. 2015; Tuberosa and Salvi 2006). Therefore, assigning the relations among TFs in response to drought stress will uncover the actual function of them facilitating improvement of drought tolerant crops. Microarray analysis is known as a powerful tool to discover and characterize genes involved in different stress responses in several plant species (Gul et al. 2016). Using this technology, it is possible to study of genome activity and identify regulation pattern of differentially-expressed TFs which may help us to study different pathways in drought stress condition. Although microarray analysis was used frequently to study plant response to drought stresses (Kathiresan et al. 2006; Mohanty et al. 2016; Rasheed et al. 2016) little is known about drought length specific regulatory networks. Characterization of TFs which function through a long drought period may introduce more reliable candidate gene(s) to use in crop stress tolerance manipulation. In this study, using computational approaches, a microarray dataset from Arabidopsis which covering different time periods of drought stress was analyzed. After assigning of differentially expressed genes (DEGs) followed by normalization and statistical analysis, ontology annotation was performed based on singular enrichment analysis. Furthermore, for each period, DEGs were determined. Several TFs were found among DEGs and their regulatory networks in each period were depicted. Results indicated that during drought stress, genome activity directed to response to internal and external cues and energy homeostasis, through regulatory complex and stress period specific regulatory networks are shaped during drought stress that had common and distinct TFs.

Results

Statistical analysis of microarray data to find differentially expressed genes

RMA algorithm normalized raw data successfully as the intensity value fitted well (Fig 1). ANOVA revealed that expression 6857 genes significantly changed during drought stress ($p\text{-value}\leq 0.05$) (Suppl Table 1). Paired wise comparison demonstrated that in contrast to normal condition, expression of 3787, 2931 and 5115 genes significantly differed in D1, D2 and D3 treatments, respectively ($p\text{-value}\leq 0.05$) that showed different magnitudes of expression changes (Suppl Table 2-4).

Ontology analysis of DEGs

Singular enrichment analysis was conducted for all DEGs identified by ANOVA to illustrate how the genome activities oriented in response to drought stress. From biological process aspect, DEGs were enriched in nine ontology groups (Fig 2-a). Results showed that during drought stress, genome activity was directed to metabolic process, translation and response to various internal and external cues such as "salt stress", "ABA", "cadmium ion", "cold" and "water deprivation" as well as photosynthesis. From point of view of molecular function ontology, a substantial of genome activities was related to regulatory and energy homeostasis mechanisms such as "protein binding", "transcription factor activity", "ATP binding", "catalytic activities", "Kinase activities" and so on (Fig 2-b), indicating that plant recruited

genome activity reprogramming as well as energy saving strategies to minimize adverse effects of drought stress.

Identification of differentially expressed TFs

From 3787 DEGs in D1, 169 genes were grouped in 42 distinct TF families (Table 1). NAC, AP2-EREB AND MYB families with 17, 14 and 14 members, respectively, comprised the largest groups. By contrast, 11 TF families contained only one gene (Suppl Table 5). 140 TFs belonged to 44 families were also identified among D2 DEGs (Table 1). Like as D1, NAC family had the most members (13 genes), while mTERF and AP2-EREB with 12 and 10 genes, respectively, were listed in next orders. Twenty one TF families had only one member in D2 (Suppl Table 6). A remarkable numbers of TFs was observed in D3 where, of 5115 DEGs, 261 TFs from 56 distinct families were assigned (Table 1). Unlike D1 and D2 conditions in which NAC family contained maximum number of genes, in D3, MYB family with 25 members had the most number of genes. NAC and AP2-EREB families with 19 and 18 members, respectively, were ranked as next families. Also, 13 families contained only one member in D3 (Suppl Table 7). Figure 3 represents the distribution of TF families among different drought periods. There was 35 common TF families among D1, D2 and D3. Furthermore, two families namely CSD and FHA were only observed in D1 (Fig 2). EIL, IWS1 and S1Fa-like families were only found in D2 (Fig 3). Nine TF families including ARID, BSD, Co-activator p15, CPP, DBP, FAR1, GeBP, PBF-2-Like and SRS specially existed in D3 (Fig 3).

Analysis of regulatory networks

Identified TFs in each drought period were networked and analyzed separately. So, three regulatory networks were obtained containing different number of nodes. In all obtained networks, higher betweenness centrality is shown with bigger nodes and the closeness centrality is illustrated using gradient coloring in which, red and green color represent highest and lowest closeness centrality, respectively. The magnitude of edge betweenness centrality also is represented with higher edges thickness. Betweenness obtain by counting the number of shortest paths going through a specific node which quantify the importance of node in a network (Boccaletti et al. 2006). The closeness of a node defined as the inverse of the average distance from all other nodes (Koschützki and Schreiber 2008). Also, edge betweenness considered as a standard measure of the impact of a node or a linkage in network and indicates to number of shortest paths between pairs of nodes that run through specific edge (Boccaletti et al. 2006; Koschützki and Schreiber 2008). Differentially expressed TFs between normal condition and D1 were located in a network with 52 nodes and 67 edges (Fig 4-a). Topology analysis of network highlighted ABA, methyl jasmonate and H₂O₂ pathways among different signaling pathways indicating their important roles in early drought responses. In addition, Abscisic acid responsive elements-Binding Factor 3 (ABF3) and Type-A response regulator 6 (ARR6) transcription factors had the most centrality indices, respectively, implying their pivotal roles in predicted regulatory network. Expression of ABF3 significantly increased during all drought stress time points while ARR6 exhibited a decreased expression compared to normal condition (Suppl Table 1).

Table 1. A summary of pairwise expression profiling and transcription factor found using Arabidopsis microarray data in drought stress. N, D1, D2 and D3 represent non stress, early drought, moderate drought and prolonged drought respectively. DEGs: Differentially expressed genes.

	D1			D2			D3		
	DEGs	TFs	TF families	DEGs	TFs	TF families	DEGs	TFs	TF families
N	3787	169	42	2931	140	44	5115	261	56

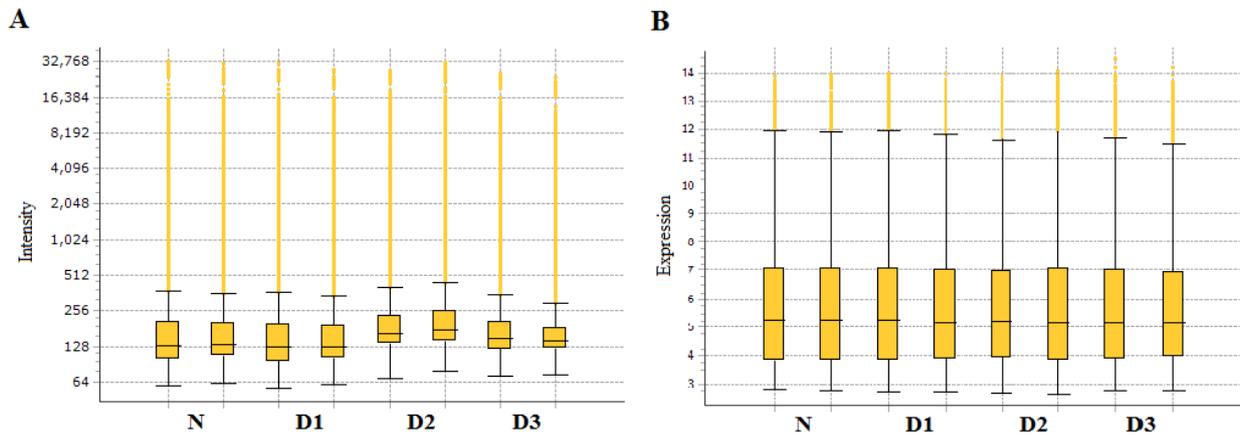


Fig 1. Normalization of microarray data of drought stress in Arabidopsis. A: Intensity values before normalization. B: Expression values after normalization. N, D1, D2 and D3 represent non stress, early drought, moderate drought and prolonged drought respectively.

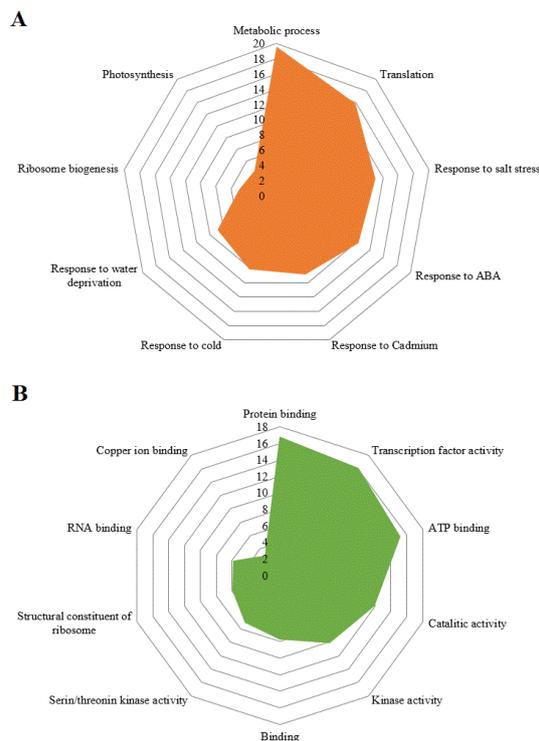


Fig 2. The percentages of functional modules of DEGs according to A: biological process and B: molecular functions.

Figure 3

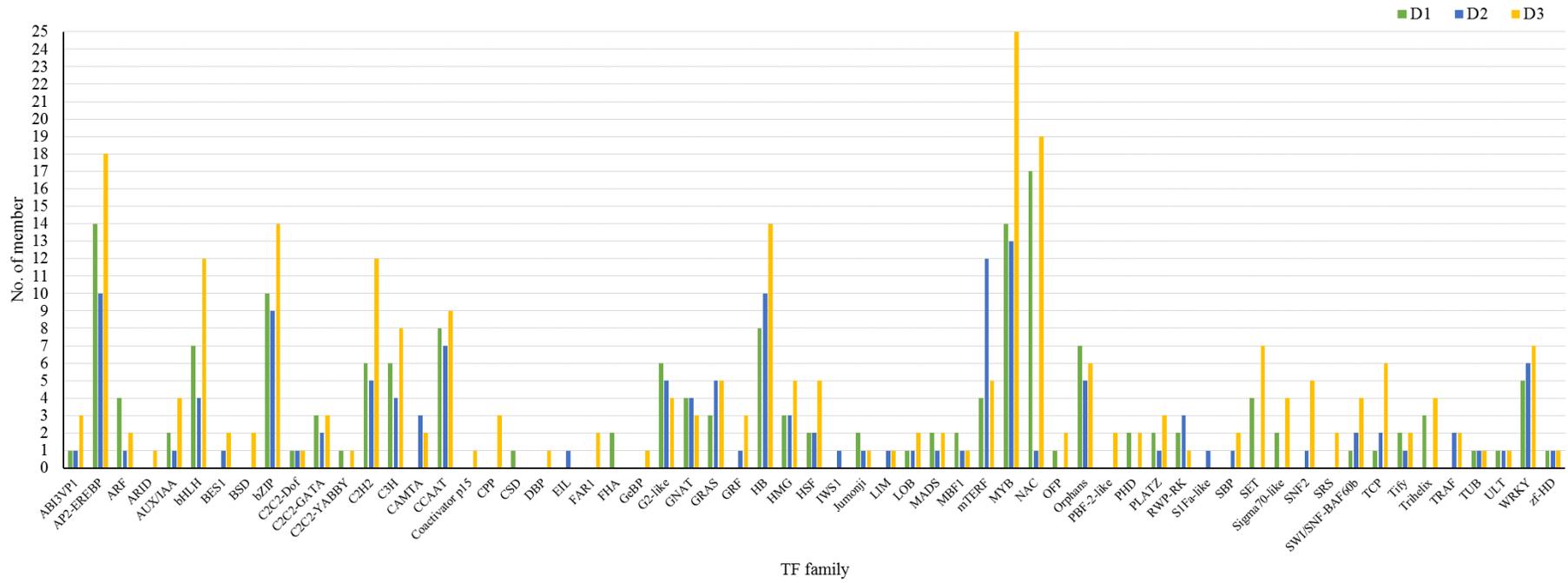


Fig. 3. Distribution of transcription families in three drought conditions. D1, D2 and D3 represent early drought, moderate drought and prolonged drought, respectively.

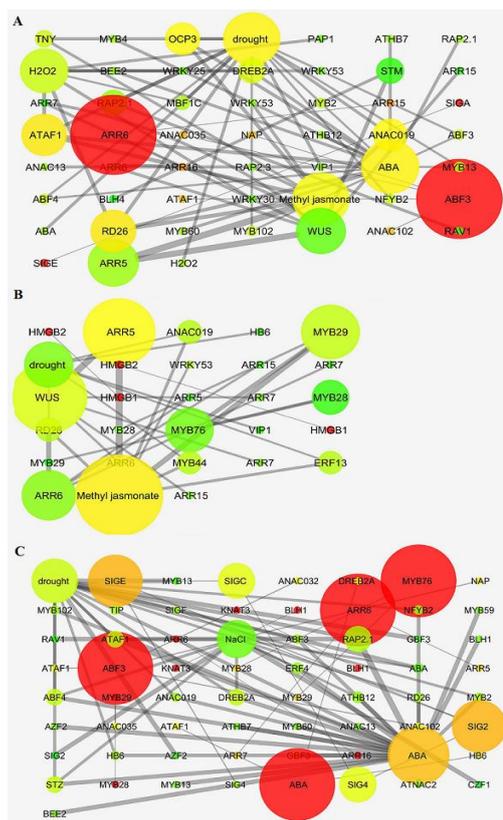


Fig 4. Topology analysis of predicted networks based on differentially expressed transcription factors. A: Network of early drought (D1). B: Network of moderate drought (D2) and C: Network of prolonged drought. In all three networks, nodes with bigger size indicate higher betweenness centrality. Red color represents highest and green color indicates the lowest closeness centrality. Edges with higher thickness reveal the high value of edge betweenness centrality.

Predicted regulatory network in D2 was consisted of 28 nodes and 28 edges (Fig 4-b). In regulatory network of D2, only methyl jasmonate signaling pathway was bolded (Fig 4-b), considering the regulatory network in D1, this emphasizes significant impact of methyl jasmonate in response to average drought periods. Although *ARR6* had a notable centrality indices, however, *ARR5* and *MYB29* genes were borne more centrality values in average in D2 network. A network with 65 nodes and 74 edges was constructed in long-term drought stress (Fig 4-c). Interestingly, like as D1, ABA signaling was activated again in response to drought stress. Also, salinity stress signaling as NaCl term was highlighted in network which suggests that in response to prolonged drought condition, high osmotic and ionic stress signaling pathways are also activated. As shown in figure 3-c, three TFs namely *ABF3*, *ARR6* and *MYB76* gained most centrality values. Moreover, four members of plastid sigma factors (SIG) including *SIG2*, *SIG3*, *SIG4*, and *SIG5* with relatively high centrality values were highlighted only in D3 networks. Statistical analysis of genes expression showed that all identified SIGs downregulated under drought stress.

Discussion

Centralization of genome activity under drought stress

Results of singular enrichment analysis obviously demonstrated that under drought stress, plant genome reprogramed to regulate mechanisms that enable plant to

sense stress, transduce its signals and make changes in own physiology and biochemistry properties. The roles of TFs and protein kinases, as two major regulatory components (Duque et al. 2013), were emphasized along with the energy homeostasis and photosynthesis. The results were consistent with the fact that plant stress responses cause reduction in cellular energy and photosynthesis thereby plants need to constantly adjust their energy and photosynthesis-associated functions (Alqurashi et al. 2016; Yamori 2016). More details about undying molecular mechanisms of such alterations in genome activity are discussed below.

Different signaling pathways in different drought periods

In early drought treatment (D1), signaling pathways of ABA, methyl jasmonate and H₂O₂ were highlighted. The ABA signaling pathway was also bolded in prolonged drought condition. Previous studies revealed that up to 25% of the Arabidopsis genes are affected by ABA and over 60% of the ABA-regulated genes are responsive to various abiotic stresses (Fujita et al. 2011; Zeller et al. 2009). Our results showed that NAC, AP2-EREB and MYB TF families were more represented among DEGs. Most of the members of these TF families are under the control of ABA (Golldack et al. 2011). *ABF3* which highlighted as main regulatory network gene in D1 and D3 conditions, is a b-ZIP protein that bind to the G-box-type ABA Responsive Element (ABRE). *ABF3* known as one of the core ABA signaling components which is highly

inducible by high salinity and osmotic stresses (Yoshida et al. 2010). Overexpression of Arabidopsis *ABF3* in transgenic rice increased tolerance to abiotic stresses (Oh et al. 2005). It has been revealed that one of the most important positive regulator of ABA signaling pathway namely SnRK2D/SnRK2.2 protein kinase interact with *ABF3* to active target genes in ABA signaling (Yoshida et al. 2010). From our results, it seems that during early drought condition, accumulation of ABA and thereby activation of stress avoidance mechanisms such as stomatal closure, synthesis of osmolytes and etc. via ABA responsive genes mainly such as *ABF3*, shape the dominant strategy to cope with drought. Highlighted H_2O_2 component reinforced our suggestion, because ROS act as intermediates in many ABA-regulated events at low concentrations and they act as signal molecules that triggers tolerance to stresses (Acharya and Assmann 2009; Quan et al. 2008). H_2O_2 can be induced by ABA and accumulates in guard cells (Zhang et al. 2001) It has been shown that MAP kinases *MPK3* and *MPK6* induced by H_2O_2 (Jammes et al. 2009; Xing et al. 2008). Under osmotic stress *MPK6* leads to stomatal closure and positive regulation of Heat shock factors (*HSFs*) (Liu 2012; Pérez-Salamó et al. 2014).

We found some evidences indicating that there is a crosstalk between Cytokinin (CK) and ABA signaling pathways in response to drought stress. Two numbers of type-A ARR including *ARR5* and *ARR6* which known as main CK signaling components (Kunikowska et al. 2013), were delineated as one of the important networks components. Both genes exhibited decreased expression in drought stress. In a study, it has been demonstrated that individual *arr5* and *arr6* mutants in Arabidopsis had enhanced cold stress tolerance (Ha et al. 2012). Moreover, expression of *ABI5*, an important ABA signaling gene (Raghavendra et al. 2010), was negatively controlled by *ARR4*, *ARR5* and *ARR6* (Wang et al. 2011). So, these authors suggested that these four proteins may form a protein complex to control the interplay between CK and ABA signaling in response to stresses (Wang et al. 2011). Altogether, it can be concluded that during drought stress, CK signaling suppressed and consequently, ABA signaling intensified in overall through down-regulation of *ARR6* and *ARR5* genes, triggering abiotic stress responses.

Topology analysis illustrated that besides the ABA and CK signaling pathways, methyl jasmonate pathway play important roles in plant adaptation to adverse conditions, particularly in D1 and D2 drought. Some previous reports suggested an involvement of methyl jasmonate with stress responses. For example, methyl jasmonate was reported to be involved in the adaptation of barley to salt stress (Walia et al. 2007). Also, it was associated with cold stress responses in the *Thellungiella halophylla*, a tolerant model plant (Wong et al. 2005). It seems that methyl jasmonate and ABA work synergistically or antagonistically in response to biotic and abiotic stresses. Under drought stress, methyl jasmonate modulates membrane lipid peroxidation and antioxidant activities to improve stress tolerance (Anjum et al. 2011). Furthermore, it has been demonstrated that H_2O_2 acts as secondary messenger in methyl jasmonate-induced genes transcription (Orozco-Cárdenas et al. 2001), which is in accordance with our findings that indicated H_2O_2 and methyl jasmonate are the significance components of drought regulatory networks of D1 and D2 conditions. Interestingly, two significant identified MYB transcription

factors namely *MYB29* and *MYB76* in D2 and D3 conditions, are involved in methyl jasmonate-mediated genes regulation (Gigolashvili et al. 2008). Soybean *MYB76* conferred abiotic stresses tolerance in transgenic Arabidopsis through regulation of several stress-responsive genes (Liao et al. 2008). This gene was highlighted in both D2 and D3 conditions implying that it contributes in response to drought stress in both average and intense stress.

Exclusive highlighting of four members of plastid sigma factors namely *SIG2*, *SIG3*, *SIG4* and *SIG5* in D3 suggests a stress length specific function of these genes. All of the sigma factors from higher plants are nuclear encoded and regulate plastid gene transcription (Chi et al. 2015). Multiple sigma factors may have specialized or overlapping redundant functions based on the promoters they recognize (Chi et al. 2015). Based on expression and network topology analysis, prolonged drought stress may result in hampered plastid transcription which in turn, decreases plant photosynthetic capacity. These results are in accordance with results of singular enrichment analysis in which, photosynthesis associated genes were over-represented to compensate stress damages. Reduction in photosynthesis and consequently diminished final plant productivity due to abiotic stress, specially drought and heat stresses, are well documented (Xu et al. 2015). So, SIGs genes are one of the good candidates to manipulate photosynthetic pathway toward drought tolerance improvement.

Materials and methods

Plant materials such as species cultivar etc. that the data retrieved from databases. I also need authors to add at what stress condition of plant the data was generated (such as drought condition).

Source of microarray data sets

Raw microarray data of drought stress experiment (AC: E-GEOD-24177) was downloaded from EBI (<http://www.ebi.ac.uk/arrayexpress/>). In brief, a controlled and sub-lethal moderate drought treatment system was used in *Arabidopsis thaliana* ecotype Columbia (Harb et al. 2010). Three drought treatment including 1 and 10 days after moderate drought as well as prolonged drought were considered (Harb et al. 2010).

Expression profiling and ontology analysis

Expression data was normalized using robust multi-array averaging algorithm (RMA) (Irizarry et al. 2003) via Expression Console 1.2 software (Affymetrix). Normal data was used in FlexArray 1.6 software (Blazejczyk et al. 2007). Analysis of variance (ANOVA) procedure was applied using same software to find DEGs through drought experiment. Furthermore, Bayesian t-test (Fox and Dimmic 2006) was used to paired wise comparison between experimental conditions (P -value ≤ 0.05). Gene ontology annotation was performed using GeneCodis (<http://genecodis.cnb.csic.es/>) (Tabas-Madrid et al. 2012), based on singular enrichment analysis (Nogales-Cadenas et al. 2009) via hypergeometric test followed by FDR correction (Benjamini and Hochberg 1995). Radar charts were used to represent the percentage of functional modules.

Identification of TFs

The DEGs were annotated and classified as transcription factors using PlantTFact (<http://plantgrn.noble.org/PlantTFcat/>) (Dai et al. 2013). All identified differentially expressed TFs were subjected to next analysis step.

Construction and topology analysis of regulatory network

Gene Set Enrichment (GSE) analysis based on Fisher exact test was applied to assign significant sub-networks among TF genes in each drought period at p -values ≤ 0.05 using Pathway Studio software 7 (Nikitin et al. 2003; Subramanian et al. 2005). Hierarchical regulatory networks were constructed through connecting all obtained significant sub-networks using same software. Topology analysis of constructed networks was carried out by Cytoscape software 3.1.1 (Scardoni et al. 2009; Shannon et al. 2003). Betweenness and closeness centrality as well as edge betweenness parameters were considered as major centrality parameters to find TF that has largest influence on the connection of nodes through the networks (Boccaletti et al. 2006; Girvan and Newman 2002).

Conclusion

Study of regulatory networks during different period of drought stress clearly demonstrated that plant recruits different but somewhat overlapping strategies to cope with stress in a long period of time. In each drought period, specific or common signaling pathways are activated using several numbers of transcription factors. Transcription factors related to auxin signaling namely *ARR5* and *ARR6* were highlighted in all drought periods indicating they are interesting candidate genes to further studies. In addition, *ABF3* gene which represented in D1 and D2 networks as main node, because of its broad spectrum of target genes, has great potential to researchers for manipulate plant drought tolerance. Finally, *SIGs* transcription regulators may enable researchers to improve photosynthesis and yield stability under drought stress, which is the ultimate goal of plant breeders.

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