Genomics 112 (2020) 412–422

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

Abiotic stress induced miRNA-TF-gene regulatory network: A structural perspective

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ARTICLE INFO

Keywords:
miRNA
Transcription factors
Abiotic stress
Regulatory network
Target-mimics
Arabidopsis

ABSTRACT

MicroRNAs (miRNAs) and transcription factors (TFs) are the largest families of trans-acting gene regulatory species, which are pivotal players in a complex regulatory network. Recently, extensive research on miRNAs and TFs in agriculture has identified these trans-acting regulatory species, as an effective tool for engineering new crop cultivars to increase yield and quality as well tolerance to environmental stresses but our knowledge of regulatory network is still not sufficient to decipher the exact mechanism. In the current work, stress-specific TF-miRNA-gene network was built for Arabidopsis under drought, cold, salt and waterlogging stress using data from reliable publically available databases; and transcriptome and degradome sequence data analysis by meta-analysis approach. Further network analysis elucidated significantly dense, scale-free, small world and hierarchical backbone of interactions. The various centrality measures highlighted several genes/TF/miRNAs as potential targets for tolerant variety cultivation. This comprehensive regulatory information will accelerate the advancement of current understanding on stress specific transcriptional and post-transcriptional regulatory mechanism and has promising utilizations for experimental biologist who are intended to improve plant crop performance under multiple Abiotic stress environments.

1. Introduction

Plants are exposed to a wide array of environmental fluctuations that lead to various physiological and metabolic changes, which in turn adversely affect the growth and productivity. Abiotic stresses are the principal cause of decrement in crop production globally and are responsible for lowering the average yield of major crops by > 50% [1,2].

To combat the changes in the environment, plants undergo morphological and physiological adaptations which require complex rearrangements of gene expression networks controlled at transcriptional and post-transcriptional level. Transcription factors (TFs) and microRNAs (miRNAs) are the key players in plant regulatory network. TFs activate or repress the transcription of their targets transcripts by binding to their promoter regions whereas miRNAs control the expression of their targets transcripts by cleavage and translational repression [3,4]. The TFs and the miRNAs control gene expression on transcriptional and post-transcriptional levels respectively and also they interact with each other to further fine-tune gene expression [3]. They play essential roles in attenuation of plant growth and development under stress condition [5]. For example, miR156 play a critical role in plant development and phase change by targeting SPL transcription factors [6]. A recent study in Arabidopsis found that miR156-mediated down-regulation of SPL increased plant response to environmental stresses, including heat stress and heat stress memory [7]. Mir160-mediated regulation of ARF10, ARF16, and ARF17 are involved in root development and stress response [8]. According to a study, auxin-induced miR164 helps in maintaining a homeostatic balance within the auxin signaling pathway by inhibiting NAC1 transcription factor and thus, down-regulating auxin signaling [9]. At the same time, the biogenesis and the activities of miRNA themselves were under tight surveillance transcriptionally or post-transcriptionally [10].

A distinct mechanism was discovered by Franco-Zorrilla et al. [11] known as “target mimicry” which involves regulation of miRNA activities in plants. This mechanism includes another class of RNAs described as endogenous target mimics or eTMs [11]. An eTM is generally a long non-coding RNA (lncRNA), but can also be part of a protein-coding transcript [12,13]. In the literature, eTMs are also often described as miRNA decoys in plants [14] and miRNA sponges or competing endogenous RNA (ceRNA) in animals [15], although these terminologies are now being used interchangeably in plant and animal

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https://doi.org/10.1016/j.ygeno.2019.03.004
Received 31 July 2018; Received in revised form 17 December 2018; Accepted 8 March 2019
Available online 12 March 2019
0888-7543/

systems. Thus eTMs add a new class to the expanding repertoire of riboregulators and represent an essential component of competing endogenous RNA regulatory network (ceRNET) [16,17].

Based on computational programs and deep sequencing, an immense population of miRNA, TFs and mRNAs were uncovered in different plant species under stress condition; however, there is no study reported that have aimed to understand a dynamic relationship among them. To partially address this issue, a semi-experimentally validated TF-miRNA-gene regulatory network was constructed for differentially expressed miRNA and genes under abiotic stress condition (drought, waterlogging, salt and cold). This network contains five types of regulatory information: miRNA regulating genes, TF regulating genes, TF regulating TF, TF regulating miRNA genes and target-mimics regulating miRNAs. To build this network meta-analysis of publically available abiotic stress transcriptome data was performed for getting differentially expressed mRNA and miRNAs under drought, waterlogging, salt and cold stress. Then, these miRNAs were subjected to Argonaute 1 (AGO1) enrichment analysis. In addition, those enriched in AGO1 were considered to have a potential of performing target cleavages and were included in target prediction and degradome sequencing based validation. In order to provide another layer for modulating miRNA and mRNA activities, experimentally validated transcription factors regulating miRNA and mRNA, target mimics for AGO1 enriched miRNAs were also searched from the database. Then, we applied concepts from the theory of complex networks to study the level of connectivity among the differentially expressed molecular species under abiotic stress, namely – genes, transcription factors, and miRNAs.

Under stress, all these molecular species are interacting with each other and hence, there is a continuous signaling occurring among them within a cellular boundary. This scenario very naturally leads to a signal-boundary paradigm, a hallmark of various complex adaptive systems [18]. Such interaction among molecular species through signaling results into a network thereby permitting a structural perspective of the cellular dynamics.

The term network is an informal description for a set of elements with interaction between them and in a formal way it is a universal presentation of complex system that is modeled as graph. Formally a network N is a four tuple $(V_\lambda, E_\lambda, \psi_\lambda, \wedge)$ along with an algorithm A such that for $\wedge \neq \emptyset$, $i \in \wedge$, V_λ is a set of vertices V_i, E_λ is a set of edges E_i, ψ_λ is incidence function $\psi_i : E \rightarrow [V]^2$ where $[V]^2$ is the set of not necessarily distinct unordered pairs of vertices such that (V_i, E_i, ψ_i) is a graph given by the algorithm A(i). The incidence function ψ provides structure to a graph by associating to each edge an unordered pair of vertices in the graph as $\psi(e) = \{V_i, V_j\} : V_i, V_j \in V, \forall e \in E \subseteq [V]^2$. Here i is the temporal component by virtue of which a network can evolve as per the given algorithm A [19].

A network is thus an empirical object, the underlying graphs for which can be either deterministic as completely determined by an algorithm or stochastic as obtained by modeling real world data. However, a graph is an algebraic object such that an unlabelled graph represents an isomorphism class of otherwise labeled graphs. We call a network as static network if the temporal component $\wedge$ consist of single element i, otherwise the network is a dynamic network [19]. In present study, we define a miRNA-TF-gene regulatory network N in which V_λ is the set of molecular species (structural genes, TF and miRNA) differentially expressed under particular abiotic stress, and E_λ is the set of flows of signal between two different molecular species under the influence of specific abiotic stress.

The gene regulation by miRNA and TF in Arabidopsis thus forms a dynamic regulatory network $N = (V_\lambda, E_\lambda, \psi_\lambda, \wedge)$ with $\wedge$ consisting of four different abiotic stresses namely, salt, cold, drought and waterlogging stress. Here the vertices of underlying graphs represent molecular species (structural gene, TF, miRNA) that are connected by edges if there is experimentally validated evidence on regulatory interaction.

2. Results

2.1. Differential expression analysis

Plants are constantly challenged by a complex array of environmental stresses that require their fast and proper response in order to adapt and survive. Drought, salinity, cold and waterlogging are four important abiotic stresses that adversely affect the productivity of plants. Morphological and physiological adaptations to stress condition plants undergo several re-arrangements at transcriptional and post-transcriptional level. In order to canvass these re-arrangements, mRNA and miRNA differential expression analysis using meta-analysis approach was performed to find genes and miRNA dysregulated in response to these stresses.

2.2. Dysregulated genes under abiotic stress

Arabidopsis thaliana (Arabidopsis) microarray expression data consisting of 22,810 probe sets (genes), 149, 241, 64 and 225 samples related to cold, drought, waterlogging and salt stress respectively. After normalization and sample outlier detection 43, 34, 7 and 16 samples from cold, drought, waterlogging and salt stress respectively were discarded and meta-analysis was performed on remaining datasets. In total 7790 genes were found differentially expressed under at least one stress with foldchange expression > 2 (Supplementary Table S3). According to results, the maximum numbers of genes were differentially expressed under cold stress and minimum under waterlogging stress. When dysregulated genes were compared across the four stresses it was found that only 12 genes were commonly expressed across the four stresses, interestingly, all these genes showed up-regulation under drought stress but down-regulation under salt and waterlogging stress. Whereas, under cold, drought and salt stress much higher number of (245 genes) were commonly dysregulated; among them, only 12 genes had the same kind of regulation. Under cold and drought stress, the maximum number of common genes (157) showed conserved expression pattern whereas under drought and salt stress the maximum number of genes showed reversed expression pattern. The results showed that 74% (5748) of total differentially expressed genes were stress specific (Supplementary Table S3 and Fig. 2). The pathway analysis revealed that metabolic pathway is commonly affected under all four abiotic stresses where as cold, drought and salt stress shares several other pathways like- photosynthesis, plant hormone signal transduction pathways, MAPK pathway, Circadian rhythmetc. The list of genes and associated biological pathways information is given in Supplementary Table S3.

2.3. Dysregulated miRNA under abiotic stress

In the present study, Arabidopsis miRNA expression responses to drought, salinity, cold and waterlogging stresses were surveyed taking advantage of the public sRNA HTS data of Arabidopsis. Following the workflow described in Fig. 1 the miRNAs were identified for control and each stress samples (Supplementary Table S4) and then raw counts of identified miRNAs were used to find differentially expressed miRNAs in response to abiotic stresses. Overall, 110 miRNA's were differentially expressed in response to at least one abiotic stress under study. The results showed that under salt stress the highest numbers of miRNAs were expressed (74: 12 up-regulated, 62 down-regulated) followed by cold stress (63: 49 up-regulated, 14 down-regulated) but under salt stress > 80% of miRNAs were down-regulated whereas in response to cold stress ~ 78% of miRNAs were up-regulated (Fig. 3&Supplementary Tables S4& S5). On comparing the differential expression analysis results of individual stresses, it was found that there were no miRNA common among all four stresses although under cold&drought&salt; drought&salt&waterlogging and cold&salt&waterlogging combination 20, 1 and 1miRNAs were commonly expressed respectively (Fig. 3) and

certain miRNAs were also found which showed expression specific to stress.

2.4. Argonaute 1 (AGO1) enrichment analysis

In plants, miRNAs, exert their regulatory activities through target cleavages. The miRNA incorporate into specific complexes (RISCs) and guide the complexes to the target transcripts to perform cleavages. It has been reported that AGO1 is an RNA slicer selectively recruiting miRNAs and short interfering RNAs in plants [38]. In this consideration, AGO1-enriched miRNAs were identified. As a result, 92 AGO1-enriched miRNAs were extracted from differentially expressed miRNAs in response to abiotic stresses (Supplementary Table S6).

2.5. Identification of the targets of the AGO1-enriched miRNAs

Aforementioned, the miRNA allied with the AGO1 silencing complexes are more likely to alter gene expression through cleavages. In this analysis, targets were predicted only for the AGO1-enriched miRNAs. psRNAtarget, a web-based online search tool with default parameters was used to predict the targeted transcripts. As a result, 646 target binding sites were predicted for 92 AGO1-enriched miRNAs expressed under abiotic stresses (Supplementary Table S7).

Degradome sequencing data is a valuable resource for large-scale validation of the miRNA-target duplex interactions [39]. Previous studies established that the 5′ ends of miRNA-cleaved mRNA fragments would correspond to the nucleotide that is complementary to the 10th nucleotide of the miRNAs. Therefore, the cleaved RNA targets should have distinct peaks in the degradome sequence tags at the predicted cleavage site relative to other regions of the transcript [28,39]. In the present study, 14 degradome sequencing datasets of Arabidopsis were assigned to do a comprehensive validation of the predicted targets using CleaveLand4 version 4.4 pipeline. Abundance of the sequenced tags was plotted on each transcript and the cleaved target transcripts have been grouped into five categories based on the relative abundance of the degradome tags mapping at the miRNA target site through the height of the degradome peak at each occupied transcript position (Categories 0, 1, 2, 3 and 4) (Supplementary Table S8).

In total, 123 psRNAtarget predicted (AGO1-enriched) miRNA-target interaction was validated by degradome sequencing data analysis from which 61 were classified as category 0, 2 as category 1, 38 as category 2, 3 as category 3 and 19 as category 4. Category 0: > 1 raw tags at the

Fig. 1. Workflow for sRNA transcriptome data analysis and their target validation.

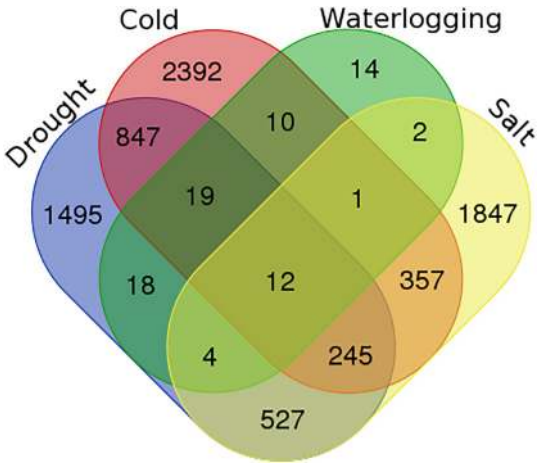

Fig. 2. Venn diagram for differentially expressed genes under drought, cold, waterlogging and salt stress in Arabidopsis.

position, abundance at the position was equal to the maximum on the transcript, and there is only one maximum on the transcript; category 1: > 1 raw tags at the position, abundance at the position was equal to the maximum on the transcript; and there was more than one maximum position on the transcript; category 2: > 1 raw tags at the position, abundance at the position was less than maximum but higher than the median for the transcript; category 3: > 1 raw tags at the position, abundance at the position was equal to or less than median for transcript; category 4: only 1 raw tag at the position. The validated interactions involved 85 genes and 60 miRNAs highly abundant under the influence of abiotic stresses (Supplementary Table S9).

The validated miRNA target genes were found to be involved in Ubiquitin mediated proteolysis, Plant hormone signal transduction, ABC transporters and metabolic pathways (Supplementary Table S9).

2.6. Abiotic stress specific TF-miRNA-gene network realization

miRNA-TF induced regulatory networks were constructed for all four abiotic stresses under study. Each stress specific network is a combination of five types of regulatory information: miRNA regulating genes, TF regulating genes, TF regulating TF, TF regulating miRNA genes and target-mimics regulate miRNAs. The experimentally validated TF regulation for differentially expressed (DE) genes and miRNAs and target-mimics for DE miRNA were fetched from publically available databases (Fig. 4). And miRNA-target interactions were validated by degradome sequence data analysis. The final network of miRNAs, gene, and TF proteins are the large directed graphs consisting of (2058, 3720), (1971, 3807), (1865, 3787) and (251, 401) nodes-edges pair in cold, drought, salt and waterlogging stress specific regulatory networks

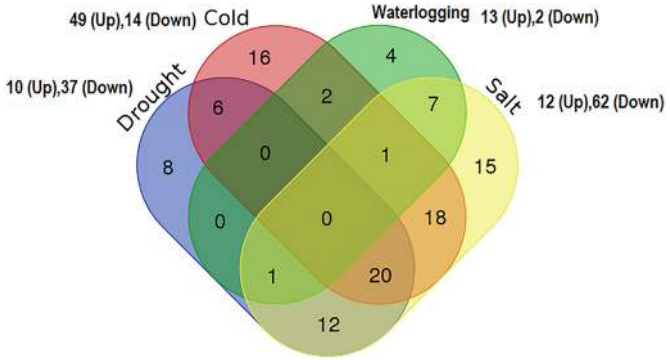

Fig. 3. Venn diagram for differentially expressed miRNA under drought, cold, waterlogging and salt stress.

respectively. To examine the constructed networks are random or complex or regular type of network, their degree distribution was studied in the context of the power law.

Maximum likelihood estimation was used to estimate the value of exponent α and Kolmogorov-Smirnov method was used to find y_{min} for all four networks in-degree and out-degree distribution. The values of $y > y_{min}$ and corresponding degree sequence of the underlying graphs for the networks was plotted separately on a double logarithmic scale (Fig. 5). All four networks in-degree and out-degree distribution plot follow a straight line that shows scale-free nature of the networks. Hubs in a scale-free network are the nodes with the highest degree. We measured the degree share of hubs as the ratio of the degree of a node to the total number of nodes in a network and found that the nodes with the highest degree have > 40% of degree share in all the four networks.

The assortativity for all four networks was calculated and found negative values of assortative mixing for in-in, out-out, in-out and out-in degree pairs across the networks which demonstrated the presence of interaction between nodes having the higher degree with nodes having lower degree (Table 1). It showed disassortative nature of networks which implies in case of failure of high degree nodes (hubs) the network is likely to become disconnected. We computed transitivity (TG) and average shortest path length (ASLG) for four TF-miRNA induced stress-specific networks under study and compared their values with transitivity TER and average shortest path length ASLER of Erdos-Renyi random network of same order and sizes. Then calculated small-worldness (S) for cold, drought, salt and waterlogging stress-specific regulatory network based on the formula given in eq. 1. It was found, all four networks have value > 1which implies that all four networks under study possess the small-world property. The computed values are summarized in Table 2.

A scale-free network having high clustering and, if the average clustering coefficient C(N) follows inverse proportional trend line with the degree of nodes then the network is also said to have hierarchical organization and hence modularity [40]. For all four stress-specific TF-miRNA-gene regulatory networks we found aforesaid scaling and the value of linear correlation coefficient between clustering coefficient and degree were 0.875, 0.875, 0.880, 0.836 and 0.697 for cold, drought, salt and waterlogging stress-specific regulatory network respectively, thus revealed a linear correlation between degree and clustering coefficient. Indeed, most of the transcription factors with a few edges (small N) regulate one gene (TF or non-TF) or miRNA. Each such transcription factor 't' has a clustering coefficient equal to one, as all transcription factors 't' have interaction to are part of the same regulatory pathway, and are therefore connected to each other. The high degree nodes are transcription factor which regulates many genes or miRNA, which are the part of different regulatory pathways and hence, their neighbors are not necessarily connected to each other, consequently, they have smaller clustering coefficient.

We calculated centralities for the network and determined the most central molecular species (miRNA/TF/gene). The list of top ten molecular species based on network centralities for cold, drought, salt and waterlogging stress specific regulatory network were given in Supplementary Table S10.

A graph has a strongly connected component (SCC) if there is a path in each direction between each pair of nodes [41]. It can be easily understood that only miRNAs and TFs could be present in the SCC. Non-TF genes cannot be included as they don't regulate any other molecule. It was found that there were three SCC in drought- and salt-, two SCC in cold- and one SCC in waterlogging- stress specific regulatory network. It was noted that miRNA159a and miRNA172c are member of SCC in cold, drought and salt- stress specific regulatory network. A list of the TFs and miRNAs present in the SCC can be found in Supplementary Table S11. Functional annotation and literature survey indicated the role of these miRNA and TFs in hormone activated signaling pathways, flower development, cell fate specification and their expression improves tolerance to several abiotic stresses.

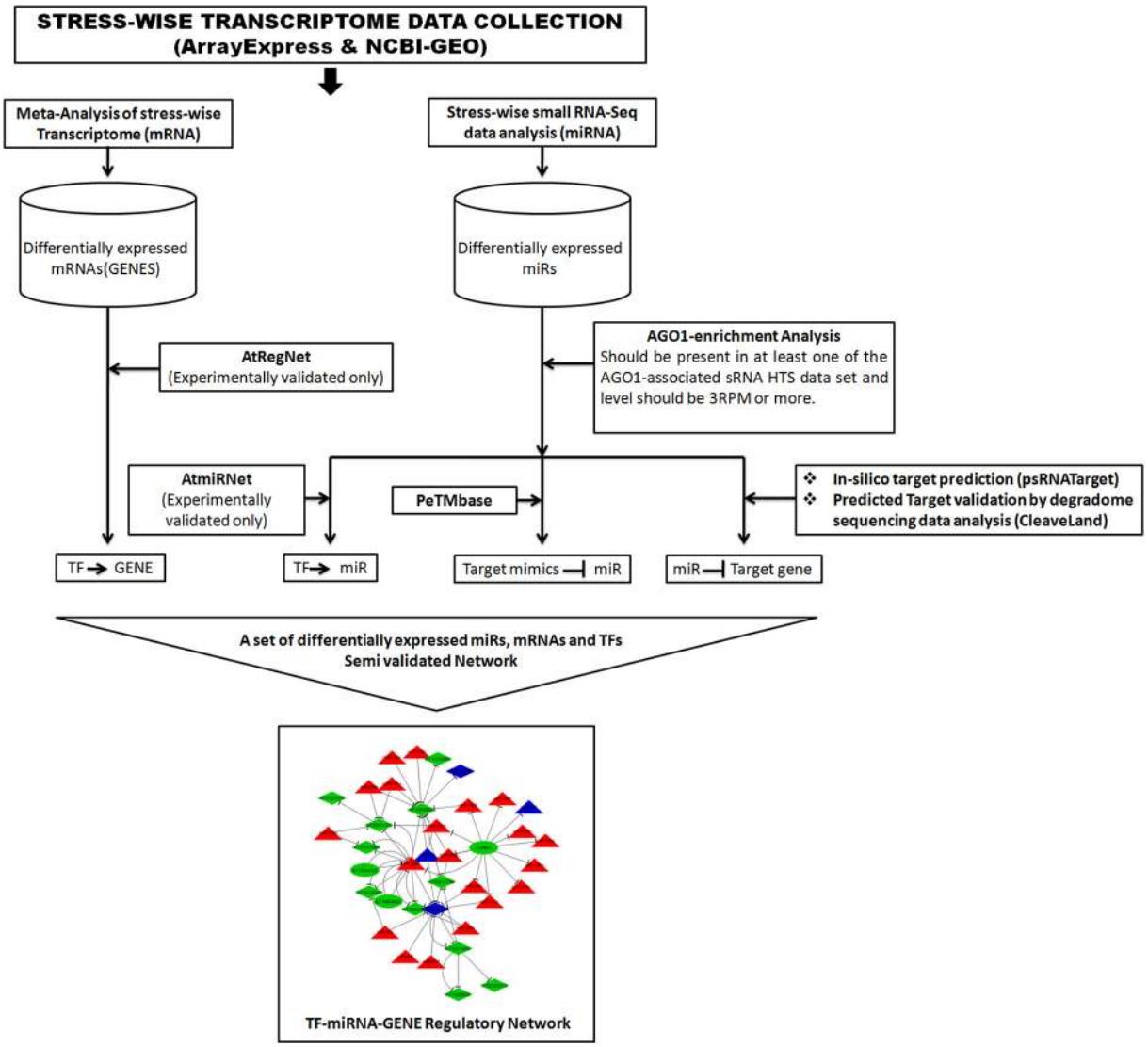

Fig. 4. Workflow describing data preprocessing, integration and induction for TF-miRNA-gene regulatory network generation for Arabidopsis under abiotic stress.

3. Discussion

The objective of this work was to reconstruct abiotic stress-specific miRNA-TF regulatory interaction network of Arabidopsis in response to four of the most important environmental stresses that affect agricultural productivity, namely; cold, drought, salt and waterlogging stress using meta-analysis approach. A meta-analysis is a statistical approach for combining the results of multiple studies. It has been used to determine the genes differentially expressed under abiotic stress condition in recent studies [42–44]. We took advantage of the public abiotic stress-related mRNA microarray and sRNA HTS data of Arabidopsis to find dysregulated genes and miRNA under cold, drought, salt and waterlogging stress. It was observed that a large number of genes (245) and miRNAs (20) were commonly differentially expressed under cold, drought and salt stress as compared to only 12 genes and no miRNAs were commonly expressed with waterlogging stress. This indicates sharing of same stress signaling pathways under cold, drought and salt stress which are different from pathways affected by waterlogging stress, this is further validated by presence of CYCD3 (AT4G34160), AUX1(AT5G01240), AUX/IAA (AT5G43700), CRE1 (AT5G35750), ARR-1 (AT2G41310) and PYR/PYL (AT5G53160) plant hormonal signal transduction pathways related genes and group of miRNAs (miR163, miR165, miR166, miR169, miR172, miR395 and miR399) among commonly expressed genes and miRNA under cold, drought and salt stress. CYCD3 encodes a cyclin D-type protein involved in cell division [45], AUX1 is an auxin influx carrier and AUX/IAA is an auxin-responsive protein which together participates in cell enlargement and plant growth [46,47], CRE1 encodes histidine kinase- a membrane-bound cytokinin sensor and ARR1 encodes an A- type response Regulator under abiotic stress condition, they form two compartment system that transduces extracellular signals to cytoplasm through phosphotransfer between two components [48]. And PYR/PYL is an abscisic acid receptor which interacts with clade A protein phosphatase 2Cs (PP2Cs) and inhibits its activity. Inhibition of the PP2Cs then leads to the activation of the SnRK2 family protein kinases that phosphorylate and activate downstream effectors involved in cold, drought and salt stress adaptation, like - guard cell slow anion channel named, SLAC1, required for stomatal closure under stress conditions [49]. Recent studies suggested that families of miRNAs which we found commonly expressed in at least two stress conditions under study are highly conserved and commonly expressed under various abiotic stresses in plants but their differential expression dependents on the specific stress condition [50]. For example, we found that miR169 inhibited by drought but induced by salt stress. The target of miR169, nuclear factor Y (NF-Y) subunit A 5 (NFYA5 (AT1G54160)) was found to be significantly induced under drought but repressed under salt

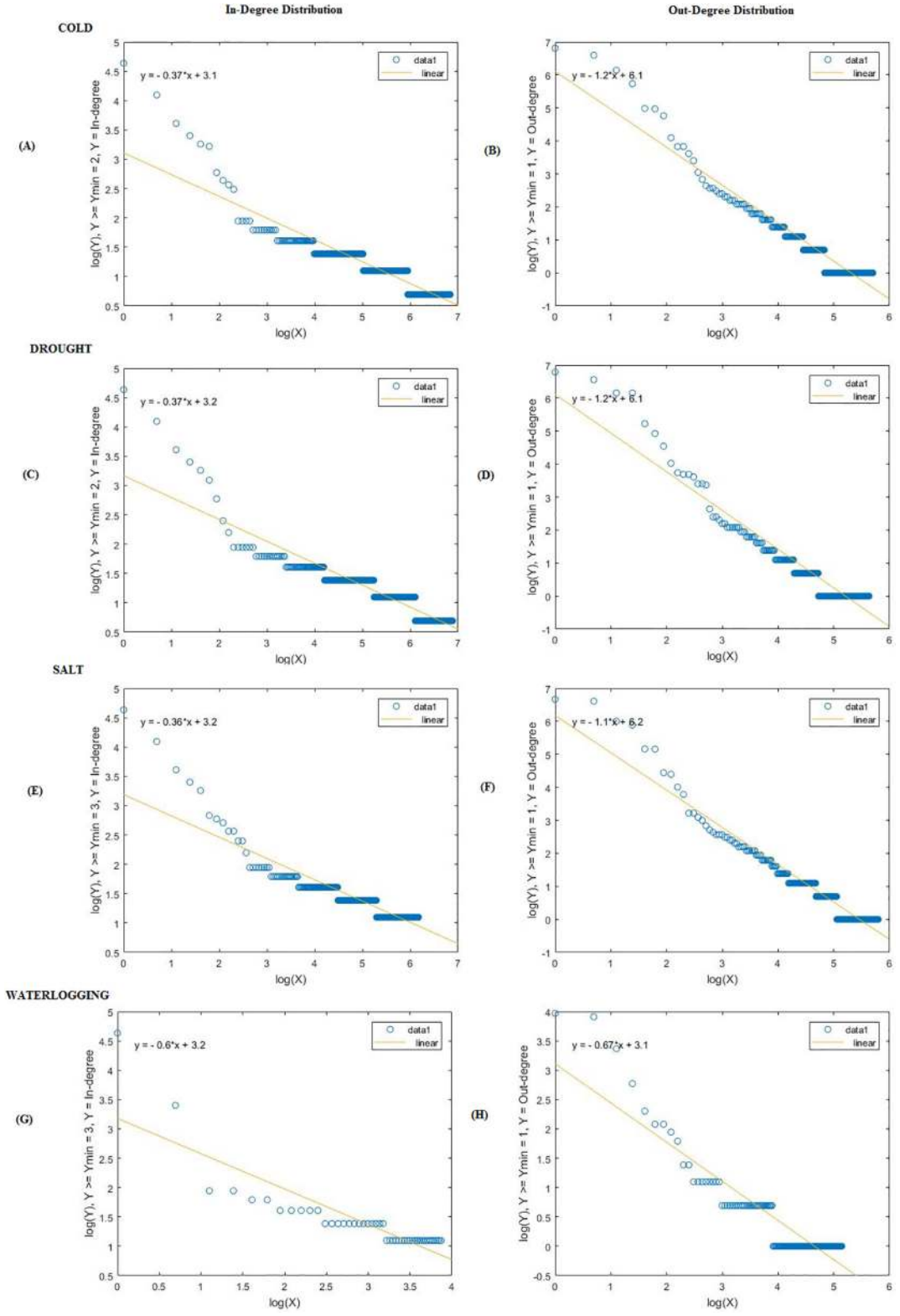

Fig. 5. Plot of degree sequence on a double logarithmic scale is found to be a straight line for each stress in-degree and out-degree showing Scale-free behavior of networks.

stress. Arabidopsis plants with the NFYA5 knockout or over-expressing miR169 showed sensitivity to drought stress and enhanced water loss whereas, over-expressing NFYA5 transgenic Arabidopsis plants showed resistance to drought stress [51] and it was reported that under salt stress miR169 was induced and its target was repressed [52].

To gain more insight about the key players and regulatory mechanism functional under the influence of abiotic stresses, we built the abiotic stress specific experimentally validated miRNA-TF-gene regulatory network for aforementioned differentially expressed genes and miRNA under abiotic stresses where interaction among these three molecular species was assembled using degradome sequencing data and several databases. The network structural measures study revealed that

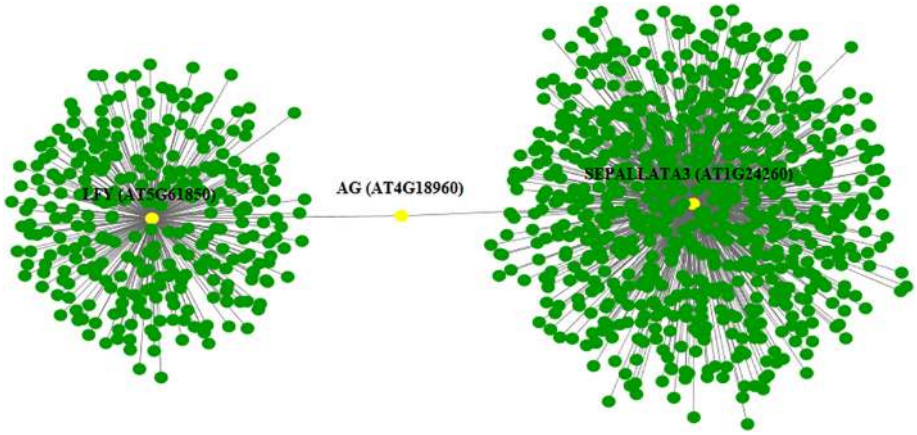

Fig. 6. Interaction of transcription factor AG with two other hub transcription factors LFY and SEPALLATA3.

Table 1
Topological attributes of the TF-miRNA-gene network of Arabidopsis for cold,drought, salt and waterlogging stress.

Topological attributes	Cold	Drought	Salt	Waterlogging
No. of edges	3720	3807	3787	401
No.of nodes	2058	1971	1865	251
Largest SCC size	17	23	23	8
Graph diameter	9	9	10	6
Characteristic path length	3.22	3.586	3.526	2.635
Average number of neighbors	3.594	3.84	4.038	3.1
Assortativity (In-In)	−0.0354	−0.52194	−0.46251	−0.211976256
Assortativity(In-Out)	−0.00168	−0.00069	−0.0015	−0.014040488
Assortativity(Out-In)	−0.4787	−0.50506	−0.44583	−0.399855057
Assortativity(Out-Out)	−0.46335	−0.02343	−0.03429	−0.168572516

all four regulatory networks are scale-free and have small world effect. This reflects several important features of the networks like- stability, invariant to changes of scale and an efficient, quick flow of signals within the network, which showed that the networks are highly robust and can cope with a relatively high amount of perturbations in single genes. That mean, if the failure occurs at random then it rarely causes a dramatic change in properties such as - average short path length and clustering coefficient. This follows from the fact that shortest paths between nodes flow through hubs, and if a non-hub node is deleted it is unlikely to interfere with the path between other non-hub nodes. As the fraction of non-hub nodes in a small world network is much higher than the fraction of hubs, the probability of deleting an important node is very low and if hub failure occurs then also network will generally not lose its connectedness because of the other hubs [53].

We computed various centrality measures that highlighted important transcription factors and miRNAs playing a crucial role in maintaining homeostasis and efficient flow of signal when plant experiences environmental stress. For instance, the TFs and miRNAs which best ranked for degree centrality are actually the hubs in the network. A comparative study showed that most of these hubs are conserved across all four stresses and literature survey illustrated their participation in calcium- and hormonal- signaling pathways, vegetative phase change regulation, transition to flowering, xylem formation, circadian clockwork etc.

It was observed that the TFs which are not hubs but are top-ranked according to betweenness centrality and closeness centrality are important because they lie on communication paths and can control information flow. They act as a relay point for passage of signal between hubs. This is especially true for all four stress-specific regulatory networks under investigation for the reason that the hubs in these networks tend not to be linked as indicated by negative values of assortativity. For example, a transcription factor AG (AT4G18960), it interacts with two hub transcription factors namely, LFY (AT5G61850) with 470 neighbors and SEPALLATA3 (AT1G24260) with 911 neighbors (Fig. 6). LFY and SEPALLATA3 transcription factors are known for their role in flower development pathway [57]; [54]; [55]. LFY was also found regulating expression of several other transcription factors and miRNAs which play role in multiple abiotic stresses like- RAP2.2, WRKY15, DREB19, DREB26, DREB2A, miR156, miR160, miR169 etc. [58–60]. It activates the expression of AG transcription factor that further regulates the expression SEALLATA3 transcription factor which has a vital role in floral organ identity [57]. Thus, AG is acting as a relay point between two hubs that coordinating timely flowering process under the influence of several environmental stresses.

We also explored strongly connected components (SCC) in cold, drought, salt and waterlogging stress-specific regulatory networks. The largest SCCs were of size 23 in drought and cold, 17 in salt and 8 in waterlogging stress specific network. It was found that miR159 and miR172c were commonly present in SCC of cold, drought and salt stress-specific regulatory networks which reflect their important role in multiple abiotic stresses. Both were also top-ranked according to betweenness centrality in aforementioned networks that indicated their vital place in the networks to facilitate the process of propagation or spread of signal across the network. miR159 is known to post-transcriptionally regulate MYB gene and function in leaf, flower and seed maturation. According to a study on transgenic rice plants, over-expressing miR159 were sensitive to heat stress which indicate under-expression of this miRNA may contribute to heat stress tolerance [61]. miR172 regulate mRNA of a floral homeotic gene AP2 that involve in floral development [6]. It regulates its target by translational inhibition.

Table 2
Calculations of small-world-ness of the four miRNA-TF-gene regulatory networks.

Network	Transitivity_network	Transitivity_random	ASD_network	ASD_random	Swness
Drought	0.011473526	0.002489971	2.808114616	5.601384543	9.191432
Salt	0.012588448	0.002334176	2.822705358	5.280555524	10.0891
Water	0.075114793	0.009844135	3.089243028	4.273848606	10.55638
Cold	0.010326638	0.001355524	2.832328681	5.676242634	15.26754

According to a study by Nova-Franco et al. Up-regulation of miR172c enhanced root growth, improved rhizobial infection, improved nodulation and hence nitrogen uptake [62]. Several studies reported the significant expression of miR159 and miR172c under multiple abiotic stress and their proven role in plant development showed their importance and need of further in-depth study for revealing their function in improving tolerance to various abiotic stress [50,60].

4. Material and methods

4.1. Data sources

High-throughput sequencing datasets of Arabidopsis sRNAs and microarray-based (GPL198) mRNA expression were retrieved from GEO (Gene Expression Omnibus; http://www.ncbi.nlm.nih.gov/geo/) and ArrayExpress Archive (https://www.ebi.ac.uk/arrayexpress/) pertaining to waterlogging, cold, drought and salt stress conditions (Supplementary Table S1).

The AGO1-associated sRNA HTS datasets was downloaded from GEO. GSM707682, GSM707683, GSM707684, GSM707685 and GSM253622 samples were prepared from the Arabidopsis AGO1protein which belongs to GSE28591 and GSE10036 series.

The Arabidopsis degradome sequencing datasets - SRP033352, SRP022925, SRP078966 and SRP055491 (Supplementary Table S2) were downloaded from EMBL European Nucleotide Archive (http://www.ebi.ac.uk/ena).

The miRNA sequences were obtained from miRBase (release 21; http://www.mirbase.org/) [20]. The transcripts of Arabidopsis genes and the gene annotations were retrieved from The Arabidopsis Information Resource (TAIR), release 10 https://www.arabidopsis.org/ [21].

4.2. Accumulation of dysregulated genes and miRNAs related to abiotic stress

4.2.1. Differentially expressed genes by meta-analysis of abiotic stress mRNA expression data

The mRNA stress-wise expression datasets were normalized using GCRMA R package [22] and outlier samples were detected by ArrayQualityMetrics R package [23]. Then, differentially expressed genes were determined by the function RPadvance in the Bioconductor package [24] and pathway analysis using KEGG database [25].

4.2.2. Differentially expressed miRNAs by analysis of miRNA-Seq data

The raw reads from HT sequencing were first subjected to the preprocessing analysis, which includes adaptor/barcode trimming, getting rid of the low-quality tags and sequence quality check. After getting the clean reads, the length distribution of the clean tags and common and specific sequences between samples were summarized. rRNA, scRNA, snoRNA, snRNA, tRNA, exon, intron and repeat sequence tags were removed based on GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) and Rfam (12.2) database (http://rfam.sanger.ac.uk/). The pre-processed reads were aligned to reference genome (TAIR10) and coordinates were extracted in internal BED format. The coordinates were then compared to Arabidopsis miRNA GFF file and different measures of the expression level are generated like the read count (total number of reads assigned to the reference RNA), adjusted read count (read count normalized by the number of times that the read maps to the library or the genome) and normalized RPM (reads per million). The mapping and read count calculation were done by miRanalyzer [26]. EdgeR and DeSeq were used for differential expression analysis of miRNA pertaining to waterlogging, drought, cold and salt stress conditions (Fig. 1).

4.3. Identification of AGO1-enriched differentially expressed miRNA

The differentially expressed miRNAs from various abiotic stresses were subjected to AGO1-enrichment analysis by applying following rules: 1) the miRNA should be detectable in at least one of the AGO1-associated sRNA HTS data sets 2) its normalized accumulation levels should be 3 RPM or higher.

4.4. Building the TF-miRNA induced regulatory network

4.4.1. miRNA-gene data

The AGO1-enriched miRNAs were subjected to target prediction by the psRNATarget tool [27] using default parameters. The degradome sequencing data were utilized to validate the predicted miRNA-target duplexes. The degradome sequencing data were analyzed by CleaveLand v4.4.4 [28]. This tool removes structural RNAs from degradome data and then aligns it to reference transcriptome using Bowtie. Alignment parameters allow zero or one mismatch and are only allowed to the forward strand of the transcriptome then the alignments are parsed to quantify the density of observed 5′ ends at each nucleotide of the transcriptome and create degradome density files. For each exact match to the sense strand of an mRNA transcript, a 26-nt long query mRNA subsequence is generated by extracting 13-nt long sequences upstream and downstream of the location of the 5′-end of the matching degradome sequence. The query sequences are aligned to small RNA sequences using GSTAr.pl (generic small RNA transcript Aligner) which is based on RNA-RNA thermodynamic prediction. All alignments having the 5′-end of the degradome sequence coincident with the 10th nucleotide of the complementarity to the small RNA are retained. To differentiate spurious results from real targets, the pipeline re-runs using randomly shuffled small RNA sequences to estimate signal-to-noise ratios.

In order to provide another layer of miRNA activities modulation, target mimics were fetched from PeTMbase (Plant endogenous target mimics database) [29].

4.4.2. TF-gene data

TFs are the most important regulators function as terminal transducers and directly regulate expression of several downstream genes. They also act as inter-mediators of the secondary gene regulation by miRNAs and other TFs. Their activities determine how cell function and respond to the environment. We extracted experimentally validated transcription factors regulating all the differentially expressed genes found in the meta-analysis of abiotic stress related mRNA expression datasets from AtRegNet database [30]. We also extracted the TF-TF relationship from the same database.

4.4.3. TF-miRNA data

The experimentally validated transcription factors regulating all the differentially expressed AGO1-enriched miRNAs from AtmiRNET database [31].

4.5. Computation of network measures

4.5.1. Scale-free behavior identification

The scale-free behavior of the networks were studied by degree distribution plot for in-degree and out-degree separately and all the required calculation were done as pervious work [19].

4.5.2. Small-world behavior identification

A network behaves as a small-world if any node in the network can be reached by any other node in the network by traversing a path consisting of only a small number of vertices. It is observed that average shortest distance (ASD) is similar for a complex network and Erdos-Renyi random network of same order and size but clustering coefficient (CG) and transitivity (TG) of the complex network is higher than that

for the Erdos-Renyi random network (CER) and (TER) [32,33].

The small-world-ness (S) can be defined as

$$S = \frac{T_G \times ASL_{ER}}{ASL_G \times T_{ER}} \quad (1)$$

Where ASLG and ASLER are average shortest path length for the given network G and Erdos-Renyi random network (ER) of same order and size. If the value of small-world-ness (S) is greater than one then network is said to have small world behavior.

Clustering coefficient for the directed network can be defined as [34]:

$$\boldsymbol{C(i)} = \frac{\mathbf{1}}{\boldsymbol{n}} \sum_{i \in N} \frac{\boldsymbol{t_i}}{(\boldsymbol{k_i^{out}} + \boldsymbol{k_i^{in}})(\boldsymbol{k_i^{out}} + \boldsymbol{k_i^{in}} - \mathbf{1}) - \mathbf{2}\sum_{j=N} \boldsymbol{a_{ij} a_{ji}}} \quad (2)$$

And transitivity for directed network can be defined as:

$$\boldsymbol{T} = \frac{\sum_{i \in N} \boldsymbol{t_i}}{\sum_{i \in N} \left[[\![(\boldsymbol{k})]\!]_i^{\boldsymbol{out}} + \boldsymbol{k_i^{in}})(\boldsymbol{k_i^{out}} + \boldsymbol{k_i^{in}} - \mathbf{1}) - \boldsymbol{v2} \sum_{j \in N} \boldsymbol{a_{ij} a_{ji}} \right]} \quad (3)$$

Where,

t_i is the number of directed triangles around 'i'

$$\boldsymbol{t_i} = \frac{\mathbf{1}}{\mathbf{2}} \sum_{j,h \in N} (\boldsymbol{a_{ij}} + \boldsymbol{a_{ji}})(\boldsymbol{a_{ih}} + \boldsymbol{a_{hi}})(\boldsymbol{a_{jh}} + \boldsymbol{a_{hj}})$$

N is total number of nodes in a network.

K_{iout} is the out-degree of 'i' node.

K_{iin} is the in-degree of 'i' node.

axy and axy is the connection status between 'x' and 'y' in both direction.

4.5.3. Assortative mixing

Assortativity is a graph metric which represents to what extent nodes in a network associated with other nodes in the network, being of similar sort or being of opposing sort. Generally, the assortativity of a network is determined for the degree of the nodes in the network. According to which, in a network if nodes of higher degree on average connected to nodes of higher degree and lower degree nodes on average connected to lower degree nodes then the network show assortative mixing and if higher degree nodes on average connected to low degree nodes then the network is called disassortative. This concept was given by [35].

Assortative mixing is calculated as

$$\mathbf{r} = \frac{\left\{ \mathbf{M^{-1}} \sum_i \mathbf{j_i}\, \mathbf{k_i} - \left[\mathbf{M^{-1}} \sum_i \frac{\mathbf{1}}{\mathbf{2}} (\mathbf{j_i} + \mathbf{k_i}) \right]^2 \right\}}{\mathbf{M^{-1}} \sum_i \frac{\mathbf{1}}{\mathbf{2}} (\mathbf{j_i^2} + \mathbf{k_i^2}) - [\mathbf{M^{-1}} \sum_i \mathbf{1/2}(\mathbf{j_i} + \mathbf{k_i})]^2} \quad (4)$$

Where, j_i and k_i are the degrees of the node at the end of the ith edge where $i = 1$……..M.

Assortativity is expressed as a scalar value, r, in the range $-1 \leq r \leq 1$.

4.5.4. Network centrality calculation

In a network, some nodes are more important than others. Network centrality measures give a way to quantify the different ways that a node can be important. To find important nodes (representing – gene, miRNA or transcription factor): degree centrality, betweenness centrality, and closeness centrality were computed for all four regulatory networks.

The degree is a centrality measure that counts how many neighbors a node has. In case of a directed network, it is of two types: In-degree (number of incoming links) and out-degree (number of outgoing links). The degree centrality can be computed as marginal of adjacency matrix B. If we consider directed links (i, j) are ordered from i to j then,

In-degree centrality,

$$C_{D_{IN}}(i) = \sum_{j \in N} B_{ji} X_j$$

Out-degree centrality,

$$C_{D_{OUT}}(i) = \sum_{j \in N} B_{ij} X_j \quad (5)$$

Where N is the total number of nodes present in a network and X is a vector with each entry as 1.

Betweenness centrality is based on communication flow [36]. It measures the number of times the information passes through a node laying in the shortest paths between two nodes. Hence, the betweenness (δUV(N)) of a node (N) is calculated considering couples of nodes (U, V) and counting the number of the shortest paths linking those two nodes, which pass through node N.

$$\delta_{UV}(N) = \sigma_{UV}(N)/\sigma_{UV}$$

Where σ_{UV} is the number of all shortest path between U and V. The betweenness centrality of a node is given as:

$$C_B(N) = \sum_{U \neq V \neq N} \delta_{UV}(N) \quad (6)$$

Closeness centrality is a measure which estimates how fast the flow of information would be through a given node to other nodes. It considers a node more central if the total sum of the distance from the given node to all other nodes is minimum.

$$C_C(i) = \sum_{j \in J} 1/\mathrm{dist}(i,j) \quad (7)$$

Where dist (N, i) is the distance between node i and j [37].

4.5.5. Strongly connected component identification

A strongly connected component of a directed graph G is a maximal set of vertices $C \subseteq V$ such that for every pair of nodes i and j, there is a directed path from i to j and a directed path from j to i.

All the network measures were calculated in MatLab release 2017a.

5. Conclusion

Gene expression regulation is accomplished by a complex network of various regulatory elements, like, chromatin modification, transcription, post-transcriptional modification, translation, regulation by other non-coding RNAs (lncRNA) etc. Gene expression variation responding to environmental stresses helps the plant to adapt and survive. However, the underlying gene regulatory networks controlling gene expression is largely unexplored. In the present work, we reconstructed abiotic stress-specific miRNA-TF-gene regulatory network. Nevertheless, the various network structural measures study of the four different stress-specific miRNA-TF-gene regulatory networks appears to enlighten some significant findings. All four networks were found to be scale-free, represent small-world and have a hierarchical organization which reveals the stable nature of the networks where the efficient and quick flow of information occur by multilevel propagation of deregulatory signals. It also gave hints about important genes, TFs, and miRNAs shared by multiple stresses acting as a hub or relay point for signal transmission. This opens a new scope to further study this genes/TF/miRNA to prove their potential role as a target for cultivating multiple stress tolerant variety.

The links in all four networks are locally dense due to high clustering noticed because of small-world nature of networks. We consequently inferred that efficient and quick flow of signals occurs within the network. It was also observed that for all four networks the clustering coefficient varies as the inverse of degree for a node which is the indication of hierarchical organization. This further implies that there is a multilevel propagation of regulatory signal under stress. A small number of differentially expressed molecules (TFs or miRNAs) have a cascading effect leading to further regulation of a large number of other molecules (stress-responsive genes) in response to stress.

To summarize, in present work we have provided some structural insights into the cellular dynamics in the context of multiple abiotic

stresses, which emphasize the role of miRNAs and TFs as mainstream regulators of gene expression.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

RS, SU, SB and AS conceived and designed the study; RS and SU performed the analysis and wrote the manuscript and SB, AS, BB and GS critically revised the manuscript. All of the authors read and approved the manuscript.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2019.03.004.

Acknowledgments

AS acknowledges support from Shiv Nadar University, India for infrastructure facility and CSIR for providing financial support as fellowship to RS.

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