

# Transcription Factors and microRNA Genes Regulatory Network Construction Under Drought Stress in Sesame (*Sesamum indicum* L.)

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## Research article

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# Abstract

**Background:** Drought is one of the most common environmental stresses affecting crops yield and quality. Sesame is an important oilseed crop that most likely faces drought during its growth due to growing in semi-arid and arid areas. Plants responses to drought controlled by regulatory mechanisms. Despite this importance, there is little information about Sesame regulatory mechanisms against drought stress. **Results:** 458 drought-related genes were identified using comprehensive RNA-seq data analysis of two susceptible and tolerant sesame genotypes under drought stress. These drought-responsive genes were included secondary metabolites biosynthesis-related Like F3H, sucrose biosynthesis-related like SUS2, transporters like SUC2, and protectives like LEA and HSP families. Interactions between identified genes and regulators including TFs and miRNAs were predicted using bioinformatics tools and related regulatory gene networks were constructed. Key regulators and relations of Sesame under drought stress were detected by network analysis. TFs belonged to DREB (DREB2D), MYB (MYB63), ZFP (TFIIIA), bZIP (bZIP16), bHLH (PIF1), WRKY (WRKY30) and NAC (NAC29) families were found among key regulators. mRNAs like miR399, miR169, miR156, miR5685, miR529, miR395, miR396, and miR172 also found as key drought regulators. Furthermore, a total of 117 TFs and 133 miRNAs that might be involved in drought stress were identified with this approach. **Conclusions:** Most of the identified TFs and almost all of the miRNAs are introduced for the first time as potential regulators of drought response in Sesame. These regulators accompany with identified drought-related genes could be valuable candidates for future studies and breeding programs on Sesame under drought stress. **Keywords:** *Sesamum indicum*, Drought stress, Regulatory networks, miRNA, Transcription Factors.

## Background

Sesame (*Sesamum indicum* L.) is an ancient oil crop that was cultivated around the world for many years. It is now identified as an important crop due to its numerous applications, including in foods, pharmaceutical and industrial [1]. Sesame seeds are an excellent source of protein, oil, and antioxidants. The protein and oil content of seed was reported to be about 25% and 50% respectively [2]. The sesame seed oil contains fatty acids such as linoleic acid (38–49%), oleic acid (36–54%), palmitic acid (8–12%), stearic acid (3.5–7%) and small amounts of others [2]. Moreover, it is rich in natural antioxidants, including sesamin, sesamol, and tocopherols, which provide oil stability against oxidation [2]. Some reports suggested possible protective effects of Sesame oil consumption against heart disease, diabetes, arthritis, and cancers [3, 4, 5]. These nutritional, medical and industrial benefits have increased the importance of Sesame and led to an ever-growing demand for it.

Sesame traditionally cultivated in arid and semi-arid regions of the world and drought is one of the most common abiotic stresses in these areas. So, it is very likely for Sesame to be faced with moderate to severe drought stress during at least one or two stages of its growth. Although, Sesame significantly has been adapted to dry environments and could regain moisture from the lower layers of the soil by developing its tap root system under drought conditions [6]. However, drought stress mostly limits the

potential of Sesame production and adversely affects its growth, development, metabolism, and yield [7]. Therefore, improving drought-tolerant could be one of the most important Sesame breeding goals.

To improve plants against environmental stresses including drought, their response patterns to desired stress should be identified and studied. The plant response to drought is controlled by several regulatory mechanisms. Transcriptional regulation by transcription factors is one of these mechanisms that plants use to deal with stresses. Transcription factors (TFs) are proteins that bind to cis-regulatory elements located in the promoter of their target gene and regulate its expression. In recent years, several families of plant TFs including MYC, MYB, bZIP, NAC, WRKY, and AP2 / ERF have been identified and proved that they play key roles in drought tolerance [8, 9]. In Arabidopsis, it has been shown that the expression of maize ZmMYB3R improves drought and salinity tolerance [10]. The expression of Soybean GmNAC085 transcription factor in Arabidopsis significantly led to improved drought tolerance of transgenic plants by positively regulating growth rate and reducing transpiration and cell membrane damage [11]. It was reported in rice that overexpression of OsNAC5, 6, 7, and 9 genes result in enlarged root and enhanced drought tolerance [12]. The SIWRKY58 and SIWRKY72 genes have been shown to be associated with improved drought tolerance in tomatoes [13]. Dossa et al. [14] introduced AP2si16 as a potential candidate gene in breeding programs for drought tolerance, in a study on the AP2/ERF genes of Sesame.

The plant regulatory mechanisms are not limited to the transcriptional level. Post-transcriptional regulation is another prominent plant control process in the face of drought stress. At this level we can refer to microRNAs (miRNAs), non-coding single-stranded RNAs that regulate gene expression using sequence-specific cleavage or translation inhibition of the target transcripts [15, 16]. Plant miRNAs derive from their precursors that are transcripts of exon and intron of coding regions or intergenic regions [17, 18, 19]. After multiple processing steps, the mature miRNA forms from the miRNA gene. Mature miRNA then loaded onto the argonaute protein and along with some other proteins forms the RISC complex [17]. After all, miRNA by base pairing with mRNA would guide RISC to cleave the target or repress its translation [15, 16]. In recent years, studies on miRNA-mediated gene regulation of plants under abiotic stresses, including drought have been growing, and their results revealed the crucial role of miRNAs in the plant response to stress [20, 21]. A study on maize showed that miRNAs such as miR474, miR168, miR528, and miR167 might be involved in regulating tolerance mechanisms against drought stress [22]. In barley, miR169b and miR1432 have been reported to control the expression of drought-responsive genes including NFY-A and Calmodulin-related proteins and thus regulate stress response [23].

The development of high-throughput technologies in recent years and their combination with bioinformatics analysis enabled the researchers to have a clearer vision of drought response mechanisms in plants and helped them to identify the drought tolerance candidate genes more efficiently for use in the breeding program. Recently, studies on the role of transcription factors in Sesame responses to drought stress were increased and have been growing [14, 24, 25, 26]. However, our knowledge about many of Sesame TFs and their roles under drought stress is limited and insufficient. Meanwhile, most of these researches considered TFs response separately, and collective and network-based studies that focused on the interactions between TFs and stress-related genes have less seen. On

the other hand, studies about Sesame miRNAs are rare and their regulatory roles in response to drought stress are unclear. The present study aimed to identify Sesame drought-related genes by performing a comparative RNA-seq-based analysis and then construct gene regulatory networks (GRNs) using the predicted relations of identified genes with Sesame TFs and miRNAs. Finally, hub genes were identified by network analysis as potentially key regulators in drought stress to be verified and used in future breeding programs.

## Results

### Differentially Expression Analysis

To identify the general mechanism of drought response in Sesame, 290 differentially expressed genes (DEGs) that were shared between all genotypes and samples were selected as drought-responsive core (DRC) gene-set (Fig. 1 and Additional file 1: Table S1). Protein coding genes like 'LEA protein Dc3' (105157537), 'dehydrin DHN1-like' (105179059), 'heat shock protein' (105164479), 'alcohol dehydrogenase 1' (105159420), 'oleosin S1-2' (105175017) and some ncRNA genes (110012740, 105162944) were up-regulated, from this set. On the contrary, expressions of protein-coding genes such as 'auxin-binding protein 19a' (105157802), 'pectate lyase 5' (105168345), 'cytokinin dehydrogenase 1-like' (105162564) and ncRNAs (105173052, 105173721) were recorded as down-regulated. Most of the DRC genes had similar expression levels in both genotypes under low drought stress, but respond to higher drought intensities was different between tolerant and susceptible samples. Notably 'SPX domain-containing protein 3-like' (105173318), 'monogalactosyldiacylglycerol synthase 2' (105166432), 'pathogenesis-related protein 1-like' (105162859), 'expansin-A10' (105159984) and some uncharacterized ncRNA and protein-coding genes (110012421, 105163110, 105162944, 105168585) showed higher expression compared to sensitive samples.

On the other hand, another 168 DEGs which only differentially expressed in tolerant samples at high drought levels were selected as drought tolerance-related (DTR) gene-set to understand the specific mechanism of Sesame drought tolerance (Fig. 2 and Additional file 1: Table S2). Up-regulated genes including 'flavonoid 3'-monooxygenase-like' (105175177), 'casparian strip membrane protein' (105177354), '2-oxoglutarate-dependent dioxygenase AOP1' (105157562), 'extracellular ribonuclease LE-like' (105156533) and 'sucrose transport protein SUC2-like' (105176204) were found in this set. Genes like 'NIM1-interacting 2-like' (105156738), 'glutamate receptor 2.9-like' (105155884), 'receptor-like protein 12' (105163973) and 'pathogenesis-related protein 5-like' (105166178) were also down-regulated.

### Enrichment Analysis

To determine the functional properties of each set, genes were annotated by Gene Ontology (GO) terms and Kyoto Encyclopedia Genes and Genomes (KEGG) pathways. Cellular parts such as nuclear chromatin, apoplast, nuclear chromosome part, cell wall, and some others were significantly enriched for DRC genes (Fig. 3-a). They were also significantly enriched for the response to light intensity, hydrogen peroxide, heat, toxic substance, and some others from the biological process category (Fig. 3-b).

Molecular functions such as DNA-dependent ATPase and galactosyltransferase activities were significantly enriched for the DRC set (Fig. 3-c). KEGG pathways including 'protein processing', 'DNA replication', 'pentose and glucuronate interconversions', and 'galactose metabolism' were significantly annotated for them (Fig. 5-a). On DTR genes biological processes including inorganic anion, anion, and nitrate transport, and response to nitrogen compound were significantly enriched (Fig. 4-a). From molecular function aspect, various transmembrane transporter (inorganic anion, anion, and secondary active), and symporter (carbohydrate:proton, carbohydrate:proton and solute:proton) activities were significant, on DTR set (Fig. 4-b). Moreover, 'Phenylpropanoid biosynthesis', 'starch and sucrose', 'pyrimidine metabolism', and 'cyanoamino acid metabolism' pathways were also significantly enriched based on the KEGG database (Fig. 5-b).

## Gene Regulatory Networks

Three gene regulatory networks (GRNs) were constructed for each set using predicted relations between studied genes, TFs, and miRNAs of Sesame (Additional file 1: Table S3-8). The degree of nodes in all networks followed a power-law distribution which indicated that they were scale-free ( $0.7 < R^2 < 0.87$ ). General properties of networks are shown in Table 1.

**Table 1** General properties of gene regulatory networks.

Network	Nodes	Edges	DRGs	TFs	miRs
TF-DRC	289	1121	228	61	-
miR-DRC	375	947	247	-	128
TF-DTR	116	235	76	40	-
miR-DTR	276	580	147	-	129
miR-TF-DRC	489	2659	281	80	128
miR-TF-DTR	349	1280	156	64	129

DRG: Drought-Related Gene, TF: Transcription Factor Gene, miR: miRNA Gene.

To identify key regulators, the degree and betweenness centrality were calculated for each network. Degree centrality is the simplest measure of gene connectivity in the GRNs that calculated by the number of direct connections of that gene with other genes. Regulator genes with the highest degrees control a large number of drought-responsive genes and therefore could play an important role in regulating plant response to stress and inducing tolerance. Betweenness was measured by the number of shortest paths that pass through each node. Genes with high betweenness are crucial regulators in GRNs that act as bridges between other genes and connect them [27]. Thus, nodes with high degree and betweenness centrality considered as hub genes (Additional file 1: S9-12).

TFs including ‘transcription factor IIIA’ (105155794), ‘MYB-related protein 306’ (105157040), ‘dehydration-responsive element-binding protein 2D-like’ (105166097), ‘zinc finger protein ZAT5’ (105161312), and some others were hub genes in TF-DRC network as shown in Table 2. Notably, most of the top hub TFs in this network were the members of MYB family. In the DTR-TF network, ‘transcription factor PIF1’ (105165703), ‘homeobox protein knotted-1-like 1’ (105168958), ‘light-inducible protein CPRF2’ (105172269) and ‘basic leucine zipper 43’ (105177566) were among top hub TFs (Table 3). TFs including, ‘light-inducible protein CPRF2’ (105172269), ‘transcription factor PIF3’ (105169008) and ‘transcription factor PIF1’ (105165703) were common between the two networks. However, they found as hub genes only in TF-DTR network. Commonly miR9773, miR5658 and miR156f were top hub miRNAs in both miR networks (Table 4). The majority of miRNAs were shared between two miR networks but there were some different hub miRNAs between two networks, like miR396b, miR172d and miR8140 in miR-DRC and miR169i and miR169a and miR395 in miR-DTR.

**Table 2** Top 10 hub TFs of TF-DRC network based centrality measures.

Gene ID	Description	Betweenness		Degree		Family
		Value	Rank	Value	Rank	
105155794	transcription factor IIIA	27389.361	1	95	1	zf-C2H2
105157040	myb-related protein 306	6199.0103	2	46	2	MYB
105166097	dehydration-responsive element-binding protein 2D-like	5865.903	3	39	4	AP2
105161312	zinc finger protein ZAT5	5856.9683	4	25	13	zf-C2H2_6
105171783	myb-related protein Myb4	5674.037	5	44	3	MYB
105168111	transcription factor MYB63	4036.9114	7	38	5	MYB
105155728	myb-related protein 306	3645.386	8	33	6	MYB
105162220	myb-related protein Myb4-like	3306.7559	9	30	10	MYB
105165703	transcription factor PIF1	2107.3755	19	30	9	HLH
105166028	transcription factor E2FC	2938.6282	11	9	95	E2F_TDP

MCODE algorithm was applied to the integrated miR-TF-DRG network of each set to detect key regulatory relations of Sesame under drought stress (Additional file 1: Table S13-14). Seven modules (c1, c2, c3, c4, c5, c6, and c7) were identified in the DRC network and five (d1, d2, d3, d4, and d5) in DTR network (Fig. 6). Module c1 with 25 nodes and 38 edges was the largest detected cluster in the DRC network. Notably, genes such as DHN1 (105165756), ABP19a (105173675) (module a1), pectate lyase 18 (105157315) (module a2), DHN1-like (105179059) (module a3), pectate lyase 8 (105171845) and sucrose synthase 2 (105163347) (module a7), TFs like MYB63 (105168111), DREB 2D-like (105166097) (module c1), MYB-related 306 (105157040), PIF1 (105165703) (module c2), bZIP16 (105159270), CPRF2 (105172269)

(module c3) and transcription factor IIIA (105155794) (module c4), and miRNAs like miR169a, miR399g (module c1), miR156b (module c2) were among detected modules in DRC network. Module d1 was also the largest cluster in the DTR network with 27 nodes and 41 edges. Genes like SUC2-like (105176204), CYP82D47-like (105178083) (module d1), cytokinin dehydrogenase 7 (105180056) (module d2) and TFs like MYB108 (105178858), WRKY30 (105160383), NAC29 (105175179) (module d1), and miRNAs including miR171b, miR529d (module d1), miR169a, miR395c (module d2), were found in modules of DTR network.

**Table 3** Top 10 hub TFs of TF-DTR network based centrality measures.

Gene ID	Description	Betweenness		Degree		Family
		Value	Rank	Value	Rank	
105165703	transcription factor PIF1	2581.837	1	11	7	HLH
105168958	homeobox protein knotted-1-like 1	2401.3303	2	5	36	KNOX1,2
105172269	light-inducible protein CPRF2	1746.6625	5	11	8	bZIP_C
105177566	basic leucine zipper 43	1578.3323	6	8	13	bZIP_1
105178858	transcription factor MYB108	1504.9016	7	5	39	MYB
105178907	calmodulin-binding transcription activator 5	1357.1499	8	8	14	CG-1
105174207	dof zinc finger protein DOF3.5	1300.5356	9	8	12	zf-Dof
105169008	transcription factor PIF3	984.0747	16	9	10	HLH
105179013	probable WRKY transcription factor 50	1070.4827	14	6	31	WRKY
105178586	protein FAR-RED ELONGATED HYPOCOTYL 3	989.2037	15	8	15	FAR1

**Table 4** Top 10 hub miRNAs of miRNA networks based centrality measures.

miR-DRC					miR-DTR				
Gene	Betweenness		Degree		Gene	Betweenness		Degree	
	Value	Rank	Value	Rank		Value	Rank	Value	Rank
miR9773	21457.668	1	41	1	miR9773	13463.416	1	28	1
miR156f	14251.105	2	32	2	miR5658	4960.732	3	13	6
miR156e	13407.189	3	31	3	miR156f	4769.096	4	13	5
miR5658	10172.956	4	24	5	miR156b	4678.1597	5	13	4
miR156b	8392.074	5	27	4	miR169i	3451.3743	8	7	43
miR529d	6910.4907	7	18	9	miR156a	2916.2463	17	12	8
miR396a	4287.992	20	19	7	miR169a	3415.93	9	9	17
miR172d	6028.376	10	19	8	miR395k	3137.5925	12	9	16
miR396b	3994.1416	24	17	10	miR529d	2701.2659	18	8	33
miR156a	5670.0586	11	22	6	miR828b	2529.5598	21	9	19

## Discussion

The present study conducted to explore regulatory networks that control Sesame respond to drought stress. To this end, the drought-responsive genes firstly were identified using a comparative analysis of transcriptome data of two susceptible and tolerant Sesame genotypes. To investigate the general and specific tolerance response of Sesame in the facing with drought stress, two DRC and DTR gene sets were selected based on similarity and difference between susceptible and tolerant genotypes DEGs, respectively. As shown in results, genes from families like LEA, Dehydrin, and HSP were found up-regulated in the DRC set. Many studies confirmed the positive role of these gene families against oxidative stresses including drought [28, 29, 30]. Other genes like alcohol dehydrogenase 1 (ADH1) and oleosin S1-2 were also detected in the DRC set. Shi et al. [31] in a study on function of ADH1 in *Arabidopsis* under abiotic stress showed that overexpression of ADH1 enhanced tolerance to abiotic stress including drought by conferring ABA sensitivity, increasing expression of stress-related genes and accumulations of more protective osmolyte (such as sugars and sucrose) [31]. Little is known about the role of oleosin during drought stress. Oleosins provide stability to oil bodies and prevent their fusion to an enlarged abnormal oil-structure. The irregularly enlarged oil bodies caused the deformation of the cell nucleus and disrupt its function [32, 33]. Oleosin conferred freezing tolerance to *Arabidopsis* seeds through this mechanism [32]. Oleosins may likely stabilize lipid bodies during the dehydration under drought conditions with a similar function. Genes such as pectate lyase 5 and auxin-binding protein 19a (ABP19a) were found down-regulated among DRC genes. Pectate lyase was down-regulated in *Arabidopsis* and Tomato during drought stress, similar to our results [34, 35]. Pectate lyase is involved in cell wall modification and formation of root structures [34, 36]. Auxin-binding proteins have also been found down-regulated in maize and potato during drought stress [37, 38]. Similarly, ABP was reported to have a role in cell wall modification and cell expansion [39]. It seems the genes related to cell wall modification and degradation were suppressed by drought stress in both Sesame genotypes. Cytokinin dehydrogenases (CKXs) are flavoenzymes that catalyze the oxidation of cytokinins. Overexpression of CKX resulted in high drought tolerance by slowed growth, enhanced root system, and reduced shoot structures. However, these features such as slow growth and decreased leaves and shoots were present even under normal condition which are undesirable [40, 41]. CKX1-like was down-regulated in all samples (presented in DRC gene set) but CKX7 has been found up-regulated in the DTR gene set. After all, cytokinin seems to play an important role in response to drought conditions.

In DTR set up-regulated genes like flavonoid 3'-monooxygenase-like and 2-oxoglutarate-dependent dioxygenase AOP1 were present. Flavonoid 3'-monooxygenase also known as flavonoid 3'-hydroxylase (F3H), is a member of 2-oxoglutarate-dependent dioxygenase family that has a key role in flavonoids biosynthesis. Flavonoids are plant secondary metabolites which involved in various biological functions including response to abiotic stresses. Many reports in various plants have been showed that F3H improved drought tolerance [42, 43]. The AOPs are a subfamily of 2-oxoglutarate-dependent dioxygenases which involved in glucosinolates (secondary metabolites) biosynthesis. It was suggested that glucosinolate accumulation has a role in drought tolerance through controlling stomatal closure and preventing water loses [44]. Secondary metabolites and oxoglutarate-dependent dioxygenase family seem to play important roles in drought tolerance, as both AOP1 and F3H are members of them. The

casparian strip membrane protein (CASP) and extracellular ribonuclease LE-like (RNase LE-like) also found among top up-regulated genes in the DTR set. CASPs are transmembrane proteins that mediate casparian strips formation by localizing lignin deposition sites [45]. It has been suggested that lignification and casparian strip formation might lead to drought tolerance by preventing toxic compounds uptake and water loss [46, 47, 48]. RNase LE is an extracellular class I RNases [49]. The class I RNases has been reported that induced by Pi deficiency, senescence, wounding and osmotic stresses including drought [50]. RNase LE has a phosphate-scavenging function in response to P<sub>i</sub> deficiency by hydrolyzing extracellular RNAs [51, 52]. One of the consequences of drought stress in plants is the decrease in concentrations of nutrients especially P which caused by the decrease in P uptake of roots [53]. Thus, RNase LE up-regulation might contribute to the drought stress tolerance using this function. The sucrose transport protein (SUC2-like) was also up-regulated in the DTR set. It was shown in Arabidopsis that SUC2 up-regulated during water deficit and increased C export to the roots by sucrose phloem loading [54]. Genes such as NIM1-interacting 2-like, glutamate receptor 2.9-like, receptor-like protein, and pathogenesis-related protein 5-like were found down-regulated in the DTR set. Although there have been reports that overexpression some of these genes have a positive effect on tolerance to abiotic stresses [55, 56, 57], but better-known function of all of them is resistance to disease and biotic stresses [58, 59, 60, 61, 62, 63]. Based on the results, it seems likely that tolerant genotype at high drought levels down-regulate the expression of some of the disease-related genes (not all) and thus spend their stored energy on producing other vital compounds to survive under severe drought stress. Overall, previous studies support most of our selected genes as drought-related.

Interactions between identified drought-related genes and gene regulators including TFs and miRNAs were predicted. Based on results, the transcription factor IIIA (TFIIIA) and ZAT5 from the Cys2/His2 zinc finger family were hub genes in the DRC-TF network. Overall, C2H2 zinc finger proteins improve plant tolerance during drought stress by increasing level of ABA, proline, soluble sugars and ROS scavenging enzymes like superoxide dismutase (SOD) and peroxidase (POD) through ABA-dependent and ABA-independent pathways, as described by Wang et al. [64]. MYB-related protein 306 and some other MYB TFs found in the DRC-TF network (Table 2) as hub genes belonging to the R2R3-MYB subfamily. The R2R3-MYB TFs regulating important plant biological processes such as primary and secondary metabolism, cell fate and identity, developmental processes, and responses to biotic and abiotic stresses [65]. Tang et al. [66] showed that overexpression of OsMYB6, an R2R3-MYB TF have enhanced drought tolerance and it was probably raised from increased proline content, decreased MDA content, higher CAT and SOD activities and induced expression of abiotic stress-response genes [66]. Another identified TF with high centrality measures in the DRC-TF network was dehydration-responsive element-binding 2D-like (DREB2D-like), a member of the DREB family of AP2/EREBF superfamily. There is not much information about DREB2D and its functions against abiotic stresses in plants. Ansari et al. [67] in a study on muskmelon genotypes (*Cucumis melo* L.) showed that high up-regulation of DREB2D improved tolerance of tolerant genotype under severe drought stress. Moreover, it was reported that DREB2-type genes involved in drought stress response and tolerance [68, 69, 70].

On the other hand, the PIF1 and PIF3 were among DTR-TF network hub genes. Phytochrome-interacting factors (PIFs) are members of basic helix-loop-helix (bHLH) transcription factors family. ZmPIF1 was shown that enhances drought tolerance and prevents water loss by reducing the stomatal opening in transgenic rice plants [71]. ZmPIF3 could also improve drought tolerance of transgenic rice plants by positive regulation of relative water content, chlorophyll content, fluorescence, and drought-responsive genes expression [72]. Knotted1-like-homeobox 1 (KNOX1) was another hub TF that found in the DTR-TF network. It was suggested that KNOX1 could be used to improve crops drought resistance [73]. CPRF2 and basic leucine zipper 43 (bZIP43), members of plant bZIP TF family also were seen among DTR-TF network hub genes. Many members of the bZIP family were reported that positively regulate tolerance to abiotic stresses including drought [74, 75]. Yang et al. [76] showed that overexpression of OsbZIP62 improved drought tolerance by regulating the expression of stress-related genes [76].

As previously shown, in both miR-DRG networks miR9773, miR5658 and miR156f had the highest centrality measures. Studies on miR9773 and miR5658 and their regulatory function during abiotic stresses including drought have just begun. The expression level of ta-miR9773 was significantly increased in Einkorn wheat under drought stress [77]. It was suggested that sly-miR5658 regulates drought stress response by controlling hormone signal transduction pathway genes in both sensitive and tolerant genotypes of tomato [78]. Studies on miR156 also demonstrated its regulatory roles in response to drought and salinity stresses in alfalfa (*Medicago sativa*) [79, 80]. The miR396b and miR172d were hub genes in the DRC-miR network. miR396 identified as a key regulator for drought tolerance in many plants including rice [81]. The gma-miR396 family was found as positive (leaves) and negative (roots) regulator under low water availability stress in Arabidopsis plants [82]. miR172 family has been reported that involved in drought stress response in potato (*Solanum tuberosum*) and *Echinacea purpurea* L. [83, 84]. In the DTR-miR network, miR169 and miR395 were identified as hub miRNAs. Several studies demonstrated that miR169 plays a significant role in drought stress responses in various plants [85, 86, 87]. miR169 family has been shown down-regulated in leaves but upregulated in roots of wheat under drought stress [88]. miR395 also has an important role in response to drought stress and was found up-regulated in several plants under drought stress [88, 89, 90].

Important regulatory interactions between DRGs and regulators (TFs and miRNAs) in the miR-TF-Gene networks were identified using the MCODE clustering algorithm. Genes like DHNs, ABP19a and pectate lyases presented in DRC modules were previously shown as the drought-responsive gene. Sucrose synthase (SUS2) was also found in DRC modules which identified as drought-responsive genes [91, 92]. MYB63, TF found in DRC modules is a transcriptional activator of lignin biosynthetic genes [93]. Lignin has a positive role in drought tolerance [94] and due to this fact, MYB63 is possibly involved in plant drought responses. DREB 2D-like, MYB-related 306, PIF1 and CPRF are also drought-related TFs as previously discussed. The bZIP16 another transcription factor found in DRC modules was reported to regulate drought resistance [95]. As shown earlier, miR169 and miR156 have a role in regulating drought responses. miR399 was also shown that regulates plant responses to salt, ABA, and drought [96, 97]. On the other hand, SUC2-like and CKX7 genes were presented in DTR modules which their relations with drought stress previously have been described. CYP82D47-like which is a member of the CYP82 family

also has been found in DTR modules. The CYP82 of Soybean has been reported that enhances tolerance to salinity and drought stresses [98]. Among TFs presented in DTR modules, members of MYB, WRKY and NAC family can be seen. MYB108 has been found to regulate responses to biotic and abiotic stresses including drought [99, 100, 101]. WRKY30 has been shown that positively regulate drought tolerance [102, 103]. NAC29 also enhanced salt and drought tolerance in transgenic Arabidopsis [104]. miRNAs like miR171 and miR529 were found in DTR modules. miR171 family was identified as drought-responsive microRNAs which regulate plant response during the stress [105, 106, 107]. miR529 also has been shown that involved in drought stress responses [105, 107, 108]. The relation of miR169 and miR395 with drought responses have been previously discussed. Given that these genes are related to drought stress, identified relations and other genes of modules might be involved in Sesame drought stress responses.

## Conclusion

In this study, we identified a total of 458 drought-related genes (290 DRC and 168 DTR) using RNA-seq data analysis of two susceptible and tolerant sesame genotypes under drought stress. Drought-related genes like LEA protein Dc3, DHN1-like, ADH1, HSP, CKX7, F3H, SUC2, SUS2 were identified. Then, their relation with gene regulators (TFs and miRNAs) were predicted using bioinformatics tools. A total of 117 TFs and 133 miRNAs that might be related to drought stress were identified. Using predicted relations between DRGs and TFs or miRNAs the regulatory gene networks were constructed. Possible key regulators of Sesame drought response were identified using centrality measures. The TF and miRNA regulatory networks were merged and miR-TF-DRG networks were constructed. Key regulatory relations between miRNAs, TFs and DRGs were identified using modules detecting. The TFs like TFIIIA, MYB-related protein 306, DREB2D-like, PIF1, PIF3, KNOX1, and CPRF2 found as hub TFs. miRNAs like miR9773, miR5658, miR156, miR396, miR395, miR172, and miRNA169 were among hub genes. Moreover, TFs including MYB63, MYB108, bZIP16, WRKY30, and NAC29 were detected in modules of DRG-TF-miR networks. miR171, miR399, miR171, and miR529 also were identified in modules. Most of the identified TFs and almost all miRNAs of this study have not yet been reported for Sesame drought responses. These findings could be used in the next studies on Sesame to validate their function in drought stress. Finally, the validated genes and regulators could be used in Sesame future breeding programs to improve drought tolerance.

## Methods

### Data Collection

Raw RNA sequencing data of 30 Sesame leaf samples collected from two drought-tolerant and drought-sensitive genotypes each one at five time-points (before stress and when soil moisture reached to 16%, 13%, 10% and 8% named T0, T1, T2, T3, and T4 for tolerant samples and S0, S1, S2, S3, and S4 for sensitive samples respectively) with 3 replicates, were obtained from NCBI BioProject (<https://www.ncbi.nlm.nih.gov/bioproject>) database under accession number PRJNA478474 [109].

## Data Processing

The quality of the raw data was checked using FASTQC [110]. Then, excluding low-quality nucleotides and reads and also adaptor removing were done using Trimmomatic [111]. After filtering data clean reads were mapped to the reference genome sequences of Sesame available at NCBI Genome database (<https://www.ncbi.nlm.nih.gov/genome>) under genome ID 11560 using HISAT2 [112]. Count matrix was calculated based on matched reads using HTSeq [113]. Count data were normalized using the TMM method included in the edgeR package [114] under the R platform [115]. Differentially expressed genes (DEGs) analysis was performed on normalized data using the edgeR package [114]. DEGs with  $FDR < 0.01$  and  $|\log FC| > 3$  were retained for each sample. Genes that differently expressed in all samples were selected as drought-responsive core (DRC) gene set. On the other hand, genes that exclusively differently expressed in tolerant samples under severe drought (T4 and T4T3) compared to sensitive samples at the same condition, as well as other tolerant samples were also selected as drought tolerance-related (DTR) gene set. The analysis was then performed separately on these two sets of genes.

## Enrichment Analysis

Each of the sets was functionally annotated using Gene Ontology (GO) main categories including cellular components (CC), molecular functions (MF) and biological processes (BP). GO terms for each gene obtained from their Arabidopsis homologues that were identified by blastp of BLAST+ software [116]. Target genes were also assigned to biological pathways using the Kyoto Encyclopedia Genes and Genomes (KEGG) pathway database [117]. Enrichment analysis was done using clusterProfiler under the R platform [118].

## TF-Gene Network Construction

Sesame TFs that targeted gene sets were identified using TF Enrichment tool of PlantRegMap database [119]. TFs that possess a significantly over-presented number of targets in gene sets based on Fisher's exact tests were selected (adjusted p-value cut-off= 0.05). TF-Gene networks were constructed using regulatory relations between selected TFs and genes of each set.

## miR-Gene Network Construction

A list of mature Sesame miRNA sequences was obtained from the Sinbase database [120] and previous studies [121, 122]. miRNA that targeted gene sets were predicted by psRNATarget web-based tool

(expectation-value cut-off= 5) [123, 124]. Using predicted miRNA-target gene interactions miR-Gene regulatory network was constructed for each set.

### **miR-TF-Gene Network Construction**

The bidirectional interactions of identified miRNAs and TFs with each other were predicted using the Regulation Prediction tool of PlantRegMap database (TFs target miRNAs) and psRNATarget website (miRNAs target TFs). The integrated miR-TF-Gene GRNs were constructed by merging TF-Gene, miR-Gene and miR-TF networks of each set.

### **Network visualization and Analysis**

Networks were visualized by Cytoscape software [125, 126]. Centrality measures including degree and betweenness centrality were calculated for GRNs using CytoNCA, a Cytoscape plug-in [127]. The Molecular Complex Detection (MCODE) algorithm [128] was applied to find network modules using clusterMaker, Cytoscape clustering plug-in [129].

## **Abbreviations**

ABP, Auxin-Binding Protein; ADH, Alcohol Dehydrogenase; bHLH, basic Helix-Loop-Helix; BP, Biological Process; bZIP, Basic Leucine Zipper; CASP, Casparian Strip Membrane Protein; CC, Cellular Component; CKX, Cytokinin dehydrogenase; DEG, Differentially Expressed Gene; DRC, Drought Responsive Core; DREB, Dehydration-Responsive Element-Binding; DRG, Drought-Related Gene; DTR, Drought Tolerant-Related; F3H, flavonoid 3'-hydroxylase; FC, Fold Change; FDR, False Discovery Rate; GO, Gene Ontology; GRN, Gene Regulatory Network; HSP, Heat Shock Protein; KEGG, Kyoto Encyclopedia Genes, and Genomes; KNOX, Knotted1-like-Homeobox; LEA, Late Embryogenesis Abundant; MF, Molecular Function; miR, miRNA, microRNA; PIF, Phytochrome-Interacting Factor; SUC, Sucrose transport; SUS, Sucrose Synthase; TF, Transcription Factor.

## **Declarations**

### **Ethics approval and consent to participate**

Not applicable.

### **Consent for publication**

Not applicable.

### **Availability of data and materials**

All RNA sequencing data used and analysed during the current study are available in the NCBI BioProject under accession numbers PRJNA478474 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA478474>).

### **Competing interests**

The authors declare that they have no competing interests.

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### **Authors' contributions**

MAB, SKK, MMN, and AMN designed the research. MAB performed the research, analyzed the data and wrote the manuscript. SKK and AMN supervised the research. SKK, MMN, AD, PM, MMN, and AMN reviewed and edited the manuscript. All authors read and approved the final manuscript.

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# Additional File Legends

## Supplementary Tables:

**Table S1** Relative expression of top 15 Up and Down-regulated genes of DRC set under drought stress in all samples (selected based T4 samples). Samples collected at 16%, 13%, 10%, and 8% soil moisture named T1, T2, T3, and T4 for tolerant genotype and S1, S2, S3, and S4 for sensitive genotype, respectively.

**Table S2** Relative expression of top 10 Up and Down-regulated genes of DTR set under drought stress in T4 samples.

**Table S3** Source vs. Target Table of the TF-DRC network. Source includes TF IDs and Target includes DRG IDs.

**Table S4** Source vs. Target Table of the TF-DTR network. Source includes TF IDs and Target includes DRG IDs.

**Table S5** Source vs. Target Table of the miR-DRC network. Source includes miR IDs and Target includes DRG IDs.

**Table S6** Source vs. Target Table of miR -DTR network. Source includes miR IDs and Target includes DRG IDs.

**Table S7** Source vs. Target Table of the miR-TF-DRC network. Source includes miR and TF IDs and Target includes miR, TF, and DRG IDs.

**Table S8** Source vs. Target Table of the miR-TF-DTR network. Source includes miR and TF IDs and Target includes miR, TF, and DRG IDs.

**Table S9** Centrality measures of TF-DRC network nodes ranked by Betweenness.

**Table S10** Centrality measures of TF-DTR network nodes ranked by Betweenness.

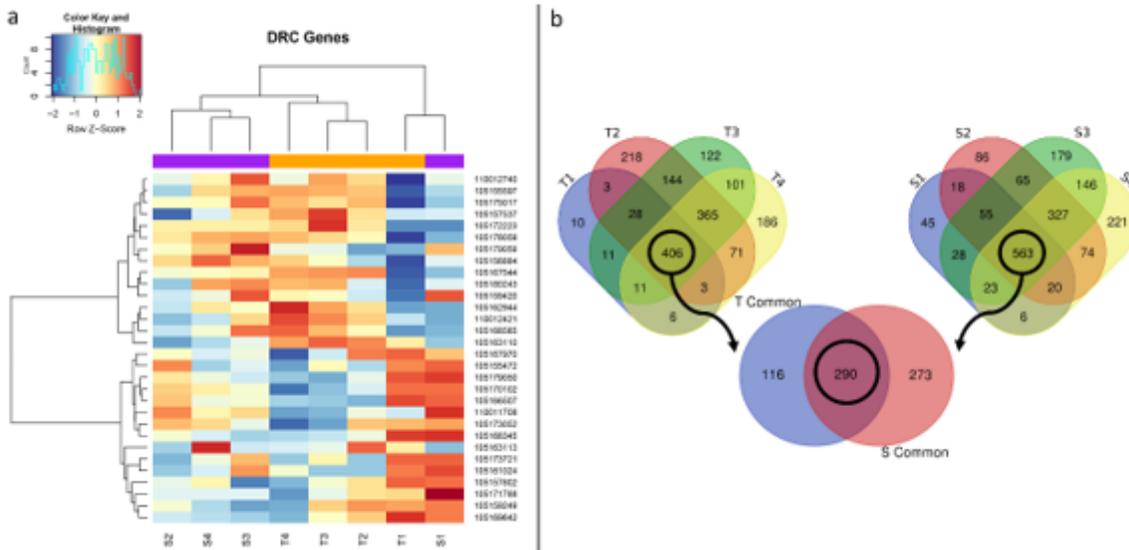
**Table S11** Centrality measures of miR-DRC network nodes ranked by Betweenness.

**Table S12** Centrality measures of miR-DTR network nodes ranked by Betweenness.

**Table S13** General details of detected modules in the miR-TF-DRC network.

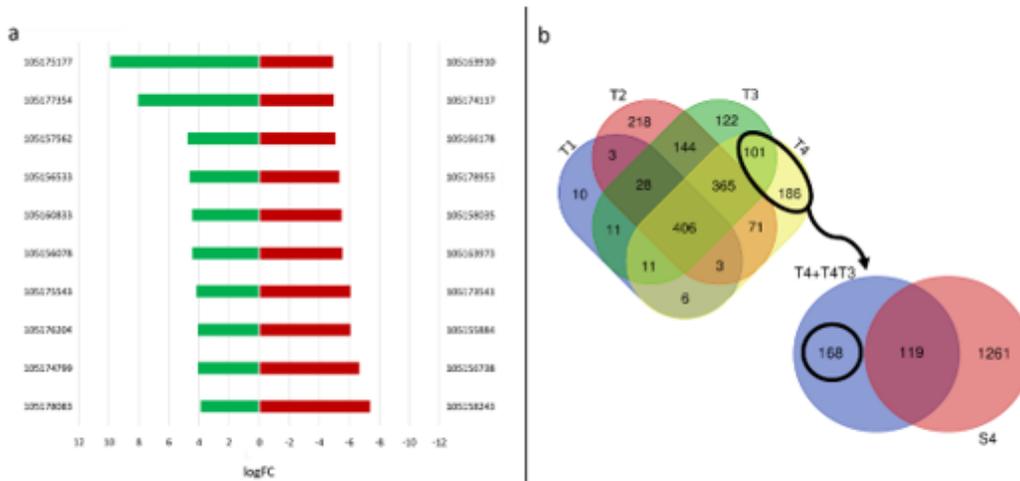
**Table S14** General details of detected modules in the miR-TF-DTR network.

## Figures



**Figure 1**

a) Relative expression (logFC) pattern of top 15 Up and Down-regulated genes of DRC set under drought stress (selected based T4 samples). b) Venn diagram of common differentially expressed genes shared between all samples named as DRC genes (290 DEGs). Samples collected at 16%, 13%, 10%, and 8% soil moisture named T1, T2, T3, and T4 for tolerant genotype and S1, S2, S3, and S4 for sensitive genotype, respectively.



**Figure 2**

a) Relative expression of top 10 Up and Down-regulated genes of DTR set under drought stress on T4 samples. b) Venn diagram of genes that only differentially expressed under severe drought level and tolerant samples named as DTR genes (168 DEGs). Samples collected at 16%, 13%, 10%, and 8% soil moisture named T1, T2, T3, and T4 for tolerant genotype and S1, S2, S3, and S4 for sensitive genotype, respectively.

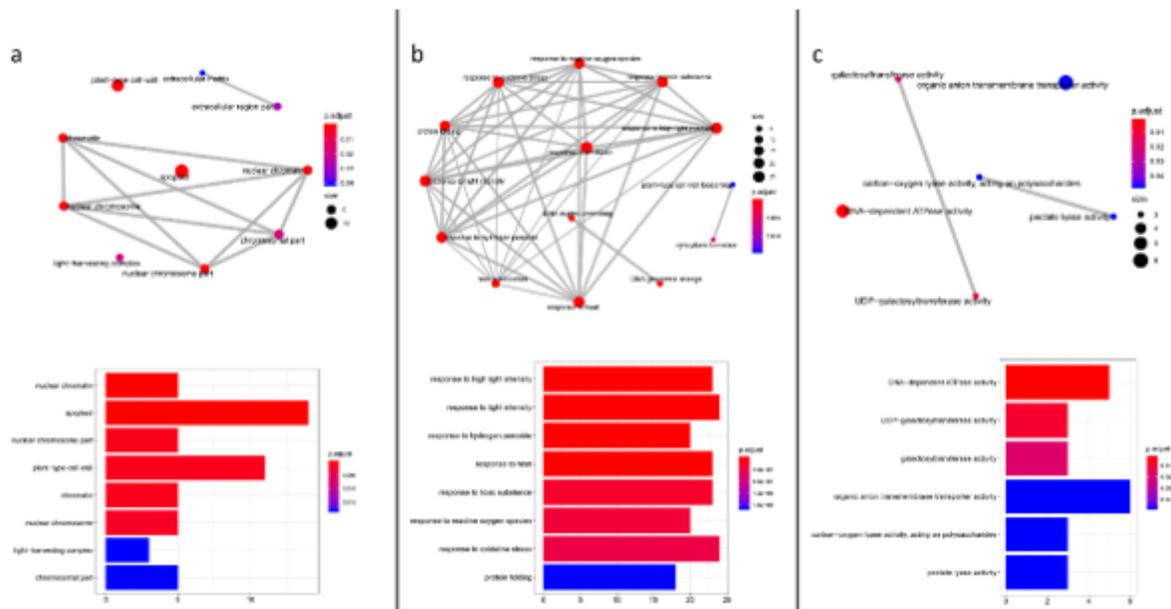


Figure 3

Significant enriched GO terms categories including a) Cellular Components b) Biological processes c) Molecular Functions in DRC set (adjusted p-value cut off= 0.05).

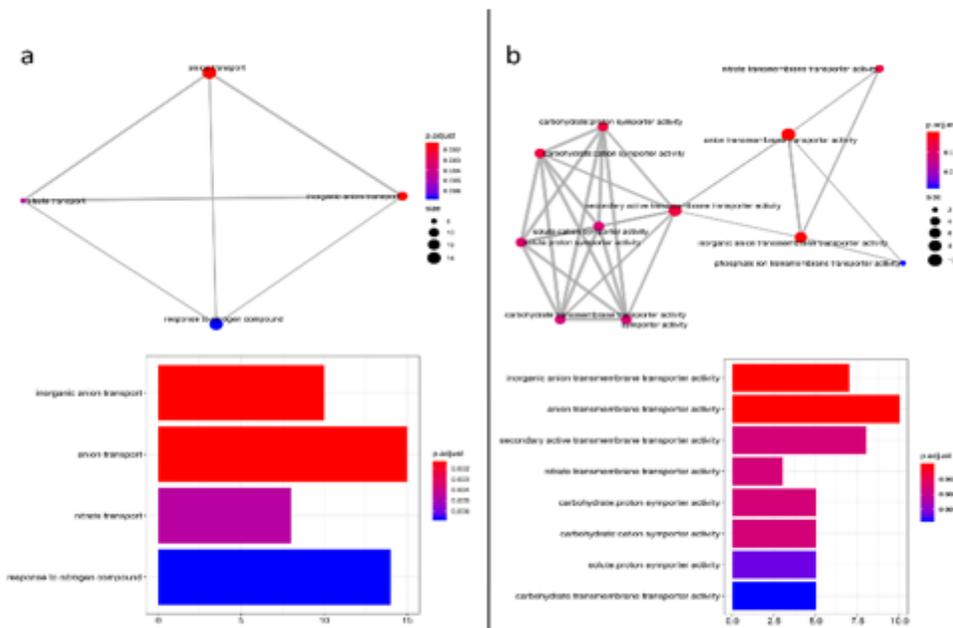
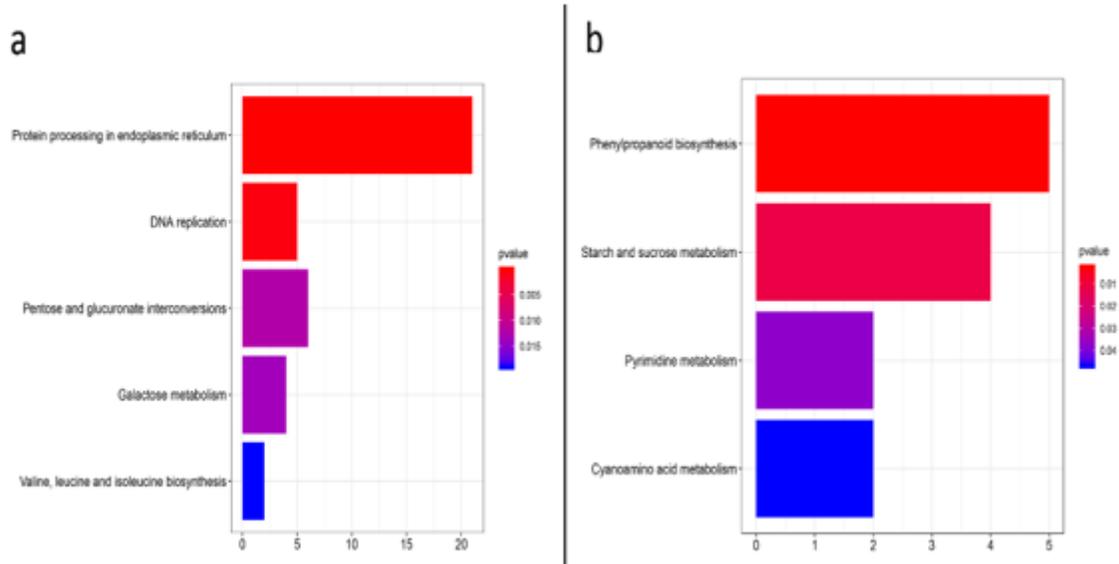


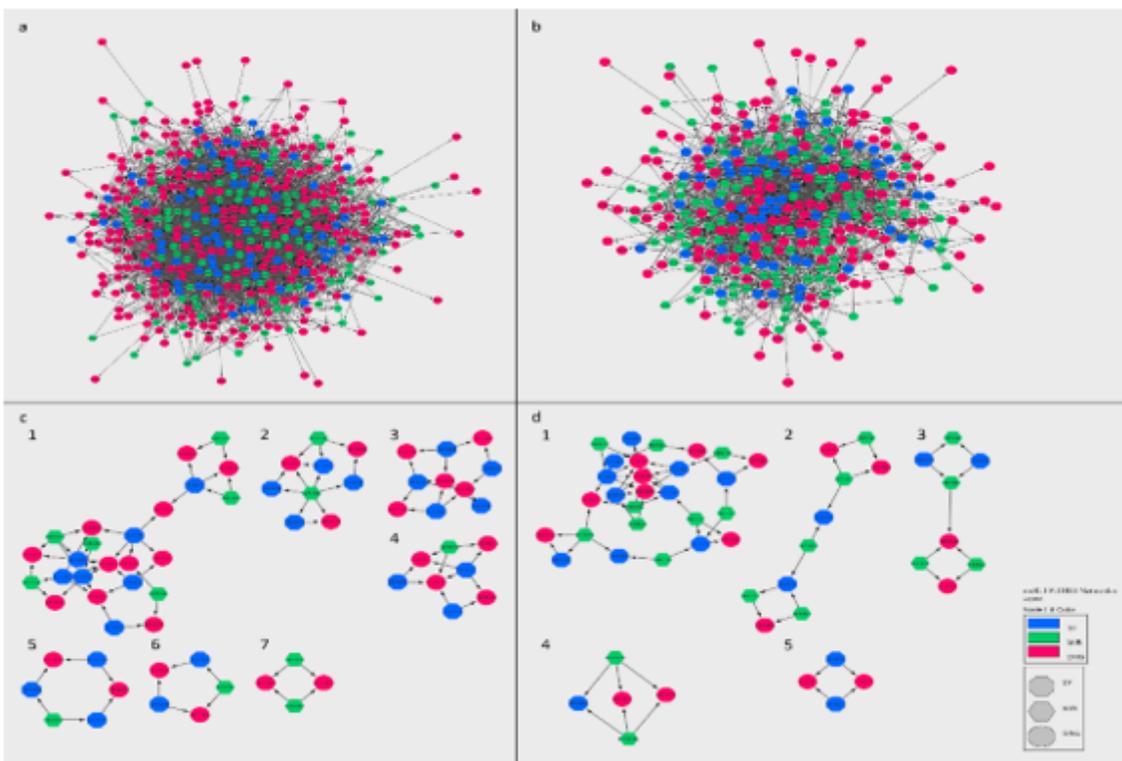
Figure 4

Significant enriched GO terms categories including a) Biological processes b) Molecular Functions in DRC set (adjusted p-value cut off= 0.05).



**Figure 5**

Significant enriched KEGG pathways in a) DRC and b) DTR sets (p-value cut off= 0.05).



**Figure 6**

miR-TF-DRG networks of a) DRC and b) DTR sets and their detected modules (c and d respectively).

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile1.xlsx](#)