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RESEARCH ARTICLE

Screening of salt stress in the overexpressed type of *Arabidopsis thaliana* (L.) Heynh. for the identification of significant hub genes using a systems biology approach

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Abstract

Worldwide, it is known that abiotic and biotic stresses can affect the production of crops by a declining trend. To control the situation, SnRK2 (a subfamily 2 of SNF1-related protein kinase) overexpression levels can induce salt tolerance. This study used a dataset for 2 types of *Arabidopsis thaliana* including the wild and PtSnRK2.7 overexpressed in mock and salt conditions to compare and identify the salt stress-responsive genes. A computational systems biology approach was employed to identify the differentially expressed genes and determine their mechanisms in terms of molecular functionalities, cellular components, KEGG enrichment pathways and plant ontology analyses. The results indicate that the 15 genes identified for PtSnRK2.7 overexpressed type in mock against salt conditions were upregulated (AT1G19180 and AT2G23150 were downregulated) and related to various environmental stresses. Furthermore, 8 out of 15 identified genes were downregulated for the wild type exposed to salt stress and the rest were upregulated. And, the only upregulated gene found differentially expressed between wild and overexpressed types in salt stress conditions was AT4G15110. In contrast, the other two AT1G15010 and AT4G19430 were downregulated and involved in transient stress and inactivation of chloroplast, respectively. Taken together, it has been shown that *A. thaliana* PtSnRK2.7 overexpressed type can resist salt stress. Finally, more experimental studies and computational systems biology methodologies are needed to reveal and confirm the responsive gene for salt stress in *A. thaliana*.

Keywords

Systems biology, *Arabidopsis thaliana*, differentially expressed genes, function, plant ontology, gene ontology

Introduction

Globally, the agricultural products suffer from stressful environmental conditions such as high temperature, high soil salinity and drought, which hamper a lot of accounting for large cultivable areas in many countries like India, the US and China (1, 2). Among various types of abiotic stressors limiting the productivity of the crop, salt stress is considered plant toxicity that interferes significantly with cellular and physiological processes such as photosynthesis, chlorophyll contents, chlorosis and necrosis due to ion toxicity (3-5). So, the management of modern agriculture in the presence of salt stress, which increases annually due to the prolonged shortages in the wa-

ter supply, is essential. Hence, several biotechnological techniques (e.g., *Arbuscular mycorrhizal* fungi (AMF)) are needed to alleviate the adverse effects of salinity (6). The plants are structured as a nonlinear model such that any input can impose a complicated output. This property of plants' nature makes them be reprogrammed in physiological, molecular, and developmental processes to optimize salt stress response (7). One of the well-known plant model systems is *Arabidopsis thaliana* from the Brassicaceae family, with 120 Mbp of DNA organized into five chromosomes. That has specifically become the focus of molecular genome-based analysis research for over forty years to provide the most basic information for all eukaryotes (8, 9). Several studies were performed on inducing various stressors in *A. thaliana* to investigate the changes in the microarray gene expression levels (10-12).

SnRKs (sucrose non-fermenting related protein kinase) with 3 subfamilies of SnRK1, SnRK2 and SnRK3 are involved in associating abiotic stresses and their corresponding metabolic responses (13). Among these, members of SnRK2 (i.e., subfamily 2 of SNF1-related protein kinase) family, plant-specific serine/threonine kinases, have taken special interests of researchers while studying the plant responses to various stressors (e.g., abiotic stresses and abscisic acid-dependent signaling pathway) (13). Several genome-wide analysis studies were performed on the SnRK2 family using some plant models such as *Gossypium hirsutum* and *Populus trichocarpa* to appraise the overexpression levels of SnRK2 members *Arabidopsis thaliana* dealing with environmental stresses (14, 15).

Moreover, the rationale of the current systems biology research to study the overexpressed *Arabidopsis thaliana* with PtSnRK2.7 overexpression is to investigate the differentially expressed genes (DEGs) in the presence and absence of the overexpression in salt stress situations. However, the control of the position will bring the policy-makers many advantages in terms of commercial or economic purposes.

Materials and Methods

The overall flowchart of the current study, starting from the dataset selection, pre-processing steps, and post-processing to analysis procedures, were depicted in Fig. 1.

Data Source

The GSE79997 dataset was downloaded from the NCBI GEO database (i.e., https://www.ncbi.nlm.nih.gov/geo/) with twelve samples consisting of 4 groups of wild and SnRK2.7 overexpressed types in mock and salt treatment status, which was the only available GEO dataset. The platform of this microarray dataset was GPL198 [ATH1-121501] Affymetrix *Arabidopsis* ATH1 genome array which includes twelve CEL files (GSM2109727, GSM2109728, GSM2109729, GSM2109730, GSM2109731, GSM2109732, GSM2109733, GSM2109734, GSM2109735, GSM2109736, GSM2109737, GSM2109738). The four groups were denoted by wild-*mock*-*salt*, wild-over-*mock*, wild-over-*salt*, over-*mock*-*salt*. Considering the group naming, it should be noted that the italic format of the font represented the mock and salt stress conditions and the wild and SnRK2.7 overexpressed types used the normal font.

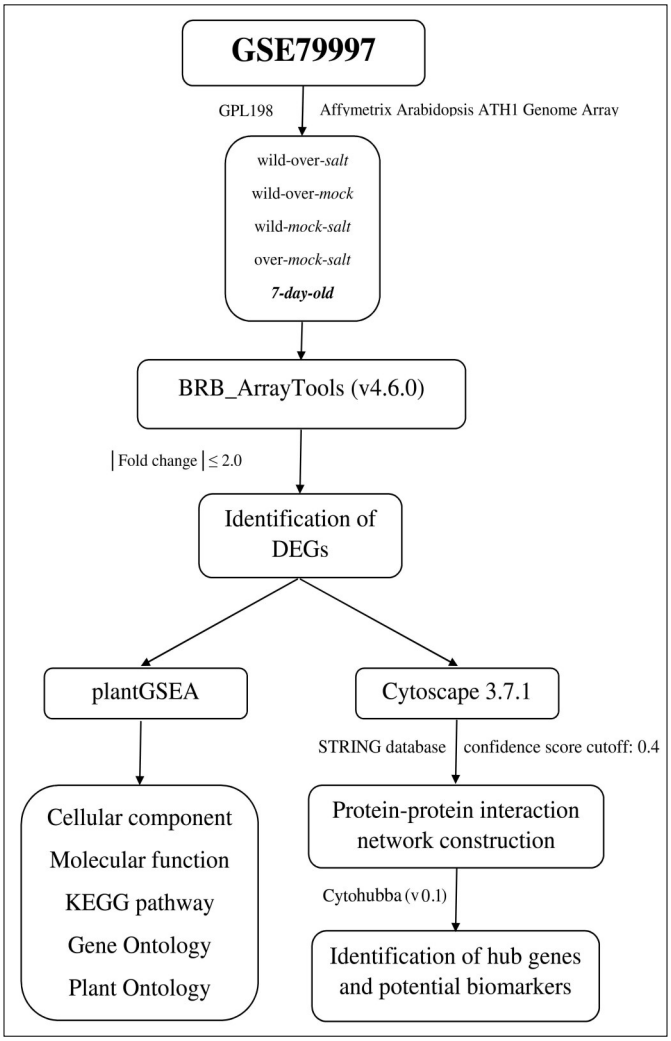

Fig. 1. Flowchart of step by step approaches to achieve the final validated genes in terms of *Arabidopsis* salt stress.

DEGs determination through pre-processing and statistical tests

The BRB-ArrayTools version 4.6.0, developed by Dr. Richard Simon and the BRB-ArrayTools Development Team, was used as a free and non-commercial integrated excel toolbox for microarray analyses. The Affymetrix *Arabidopsis* ATH1 genome array annotation data (chip ath1121501) "ath1121501.db"(16) developed for the R programming environment was also utilized for the corresponding gene symbols retrieval of probe ids. Moreover, for pre-processing procedures, the Microarray Suite version 5.0 (MAS 5.0) algorithm and pre-defined settings for enabling the spot filtering, quantile normalization and gene filtering were considered where the fold change parameter was set to less than 2. To identify the significant differentially expressed genes (DEGs), the "class comparison between groups" menu of BRB-Array Tools was selected by setting the univariate permutation tests and fold change thresholds as 10000 and 2. The additional outputs from the analyses include the illustrations of the volcano plot and box plot based on the microarray data.

Analyses of GO, PO and KEGG pathway and functional enrichment

To determine the molecular and cellular functional processes of the obtained significant gene lists as well as their enriched pathways, the online tool, PlantGSEA (Plant Gene Set Enrichment Analysis) website (i.e., http://structuralbiology.cau.edu.cn/PlantGSEA), was applied (17). This website took advantage of gene ontology (GO) annotation analysis, plant ontology (PO), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) to cover the required assessment properties. Moreover, the results were tested and analyzed with hypergeometric statistical test and Bonferroni multi-test adjustment methods and a significant level of 0.05. The input queries consisted of two gene lists: *(i)* the list of probe ids retrieved from potential significant DEGs and *(ii)* customized background corresponding to anatomy type of TAIR (The *Arabidopsis* Information Resource) genes. However, the temporal type of TAIR gene list was not considered due to the full coverage of genes overlapped with anatomy type related to the salt stress (i.e., with "salt" keyword).

Construction of protein-protein interaction network and identification of significant genes

For further analysis of the identified DEGs in their intra-protein interactions, the STRING database (with 5090 organisms, over 24.6 million proteins and over 2000 million protein-protein interactions) plugin for Cytoscape 3.9.0 was used (updated on October21, 2021). The confidence score cutoff default value was set to 0.4. After, the ClusterOne v.1.0 as one of the well-known graph clustering algorithms was used to generate overlapping clusters of PPI network (18) to determine the modules with p-value ≤ 0.05 where the genes with the highest degrees of connectivity were introduced as the potential biomarkers or the most involved gene in the expression of the differences between two defined groups (e.g., the wild types at mock and salt conditions).

Analysis of DEGs identified from this study and literature

This section includes the identified DEGs from BRB-array tools and those identified from the literature for further analysis and comparison (15). However, the significant DEGs from the literature were available for only 3 groups denoted by wild-*mock-salt*, wild-over-*mock*, over-*mock-salt*. And, the calculation for wild-over-*salt* was separately performed using the data obtained in the current investigation. For achieving a robust comparison procedure, the TAIR IDs were of interest as some of them might not have a corresponding official gene symbol. First, the target DEGs included in the groups mentioned above were used to construct the PPI network based on the STRING database using the Cytoscape 3.9.0. Then, the cytoHubba plugin was utilized to determine the most significant DEGs by performing eleven topological methodologies (19).

Moreover, Maximal Clique Centrality (MCC), Maximum Neighborhood Component (MNC), Degree, Edge Percolated Component (EPC) and EcCentricity were considered to obtain ten top-ranked genes for each ranking score distinctly. After overlapping the top 10 ranked list of genes, 5 ranking score algorithms were extracted for the 4 groups mentioned above. Finally, the shared genes between our study and the literature would be identified and were the target of interest for being analyzed for their functionality using the literature researches.

Results

The numbers of those genes that passed the filtering criteria after the microarray dataset import for wild-*mock-salt*, wild-over-*mock*, wild-over-*salt* and over-*mock-salt* groups are 2216, 86, 234 and 1573 respectively. Additionally, the outcomes of pre-processing procedure obtained from class comparison of BRB-ArrayTools for 4 groups mentioned above demonstrate the significant differentially expressed genes (DEGs) using the two-sample T-test. The numbers of DEGs for wild-*mock-salt*, wild-over-*mock*, wild-over-*salt* and over-*mock-salt* groups are 1288 (730 downregulated genes and 558 upregulated genes), 33 (30 downregulated genes and 3 upregulated genes), 3 (0 downregulated genes and 3 upregulated genes) and 1525 (884 downregulated genes and 641 upregulated genes) respectively. Fig. 2 shows the boxplot, a representation of gene expression levels and volcano plots (i.e., the fold change values) of the microarray data for four groups after pre-processing approach. The list of top upregulated and downregulated genes for 4 groups is shown in Supplementary Table 1.

The output of the plant GSEA online tool provides several gene ontology functional analyses and plant ontology assessments on the DEGs of four groups. The analyses results include gene ontology cellular components (GO-CC), gene ontology molecular functions (GO-MF), the KEGG pathway as well as the plant ontology (PO) assessments (Fig. 3).

According to over-*mock-salt*, several cellular components (e.g. cell, cell part), molecular functions (e.g. catalytic activity, binding), KEGG pathways (e.g. metabolic pathways, biosynthesis of phenylpropanoids) and plant ontology (e.g. fluorescence meristem, petiole) can be significantly enriched by DEGs based on the identified p-values. Moreover, the wild-*mock-salt* group demonstrates the DEGs enrichment by cellular components (e.g. cell, cell part), molecular functions (e.g. binding, catalytic activity), KEGG pathways (e.g. metabolic pathways, biosynthesis of plant hormones) and plant ontology (e.g. petiole, florescence meristem). Finally, the wild-over-*mock* set offers only the plant ontology assessment such as stamen and collective leaf structure.

The construction and visualization of protein-protein interaction networks for the 4 groups have resulted in PPI networks for over-*mock-salt* with 850 nodes and 4895 edges, for wild-*mock--salt* with 667 nodes and 3300 edges and wild-over-*mock* with 20 nodes and 23 edges. However, due to only one DEG for the wild-over-*salt* group, no PPI network has been determined. Supplementary Table 2 shows the list of the top 10 genes which have been ranked based on their interconnectivity degree considering the 4 constructed PPI networks. Among them, JAZ1

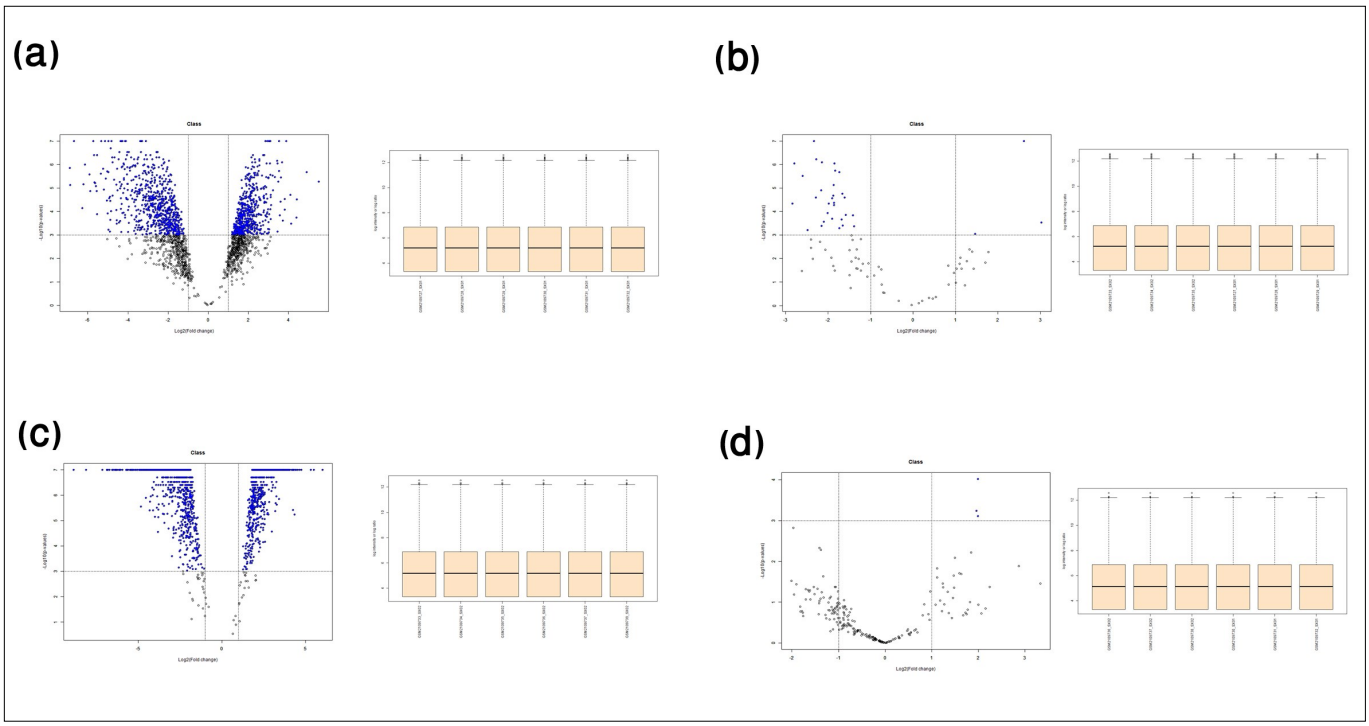

Fig. 2. Boxplot and volcano plots of four groups. (**a**) wild-mock-*salt*, (**b**) wild-over-*mock*, (**c**) over-*mock-salt* and (**d**) wild-over-*salt*.

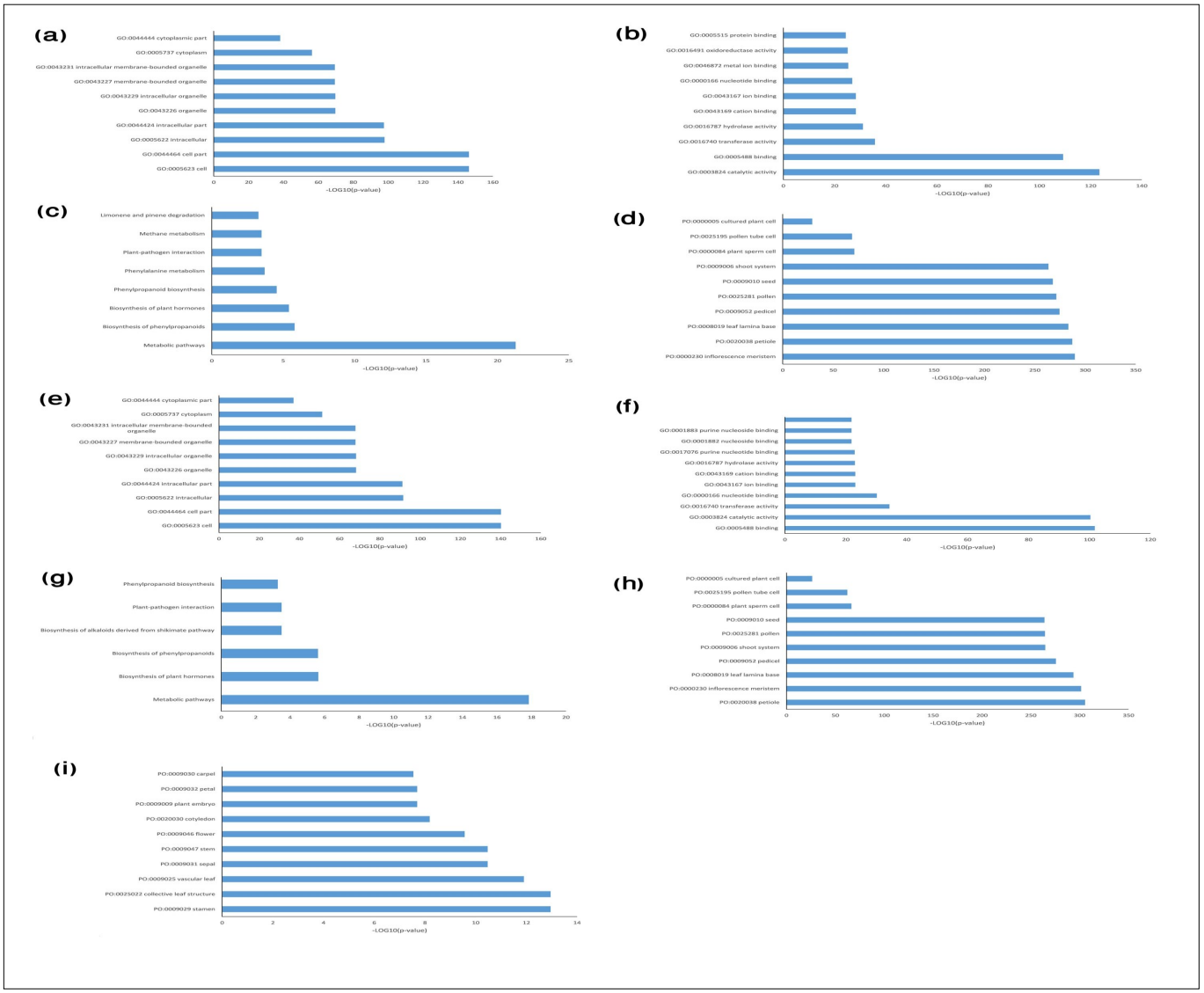

Fig. 3. Gene ontology cellular components (GO-CC), gene ontology molecular functions (GO-MF), the KEGG pathway as well as the plant ontology (PO) assessments.(**a**), (**b**), (**c**) and (**d**) are related to over-*mock-salt* for CC, MF, KEGG and PO respectively. (**e**), (**f**), (**g**) and (**h**) are related to wild-*mock-salt* for CC, MF, KEGG and PO respectively. (**i**) is the PO analysis for wild-over-*mock*.

(degree=89, downregulated), CYP38 (degree=36, upregulated), and ATSDI1 (degree=5, downregulated) have the highest connectivity degrees for the PPI networks, including over-*mock-salt*, wild-*mock--salt* and wild-over-*mock* respectively. The module analyses using the ClusterOne algorithm on the constructed PPI networks demonstrate significant clusters (*p*-value < 0.05) including 11, 9 and 4 protein modules for the over-*mock-salt*, wild-*mock--salt* and wild-over-*mock* groups respectively (Fig. 4). Detailed information on the properties of the potential genes with the highest degree of connectivity within the clustered hub genes is specified in Supplementary Table 2 for the target groups.

The analysis and comparison results of the current study and the literature are depicted as Venn diagrams for three groups: wild-*mock-salt*, wild-over-*mock* and over-*mock-salt* (Fig. 5). Additionally, the properties of over-

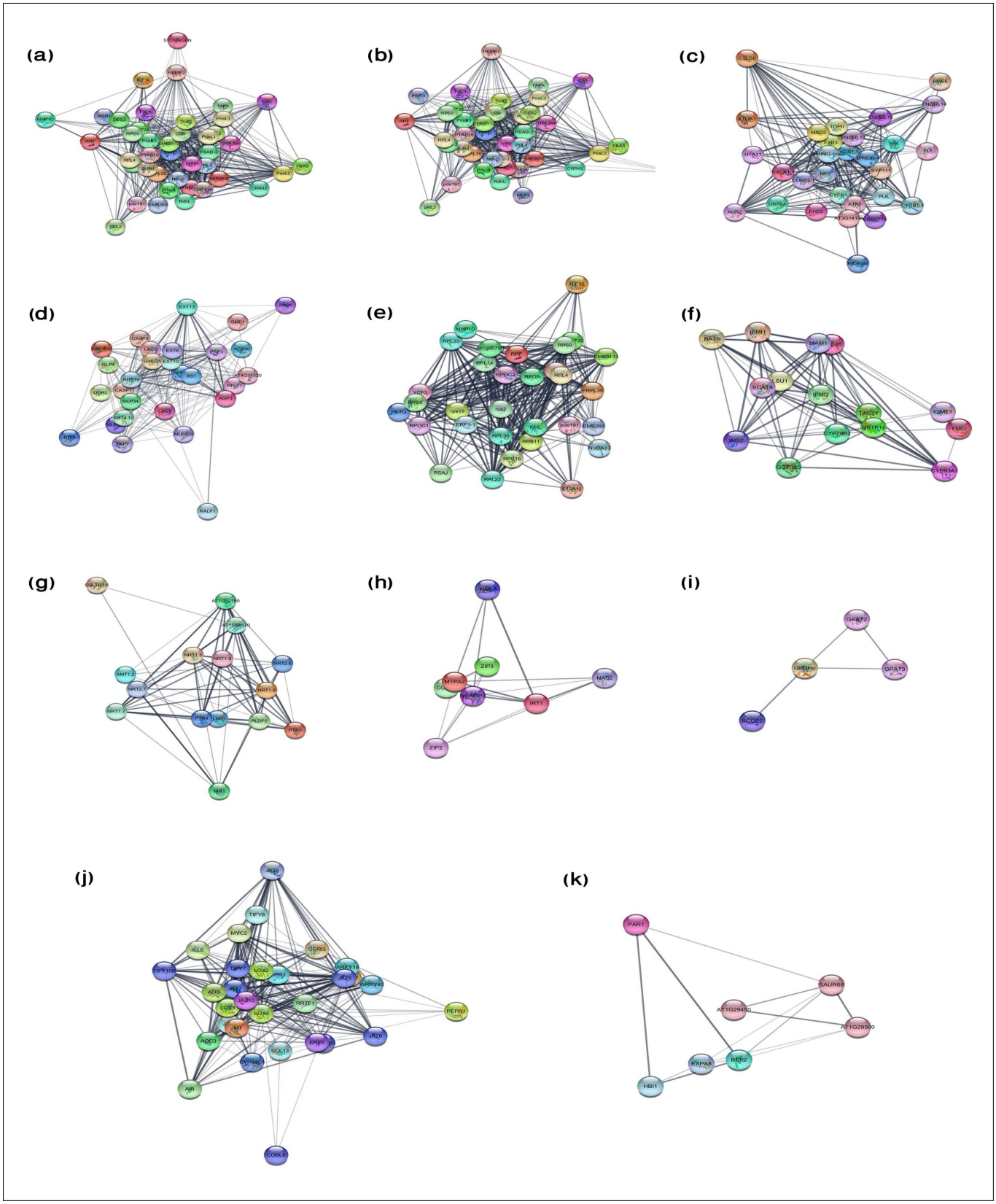

Fig. 4 A. Significant protein modules for over-*mock-salt* (***a***)-(***k***)

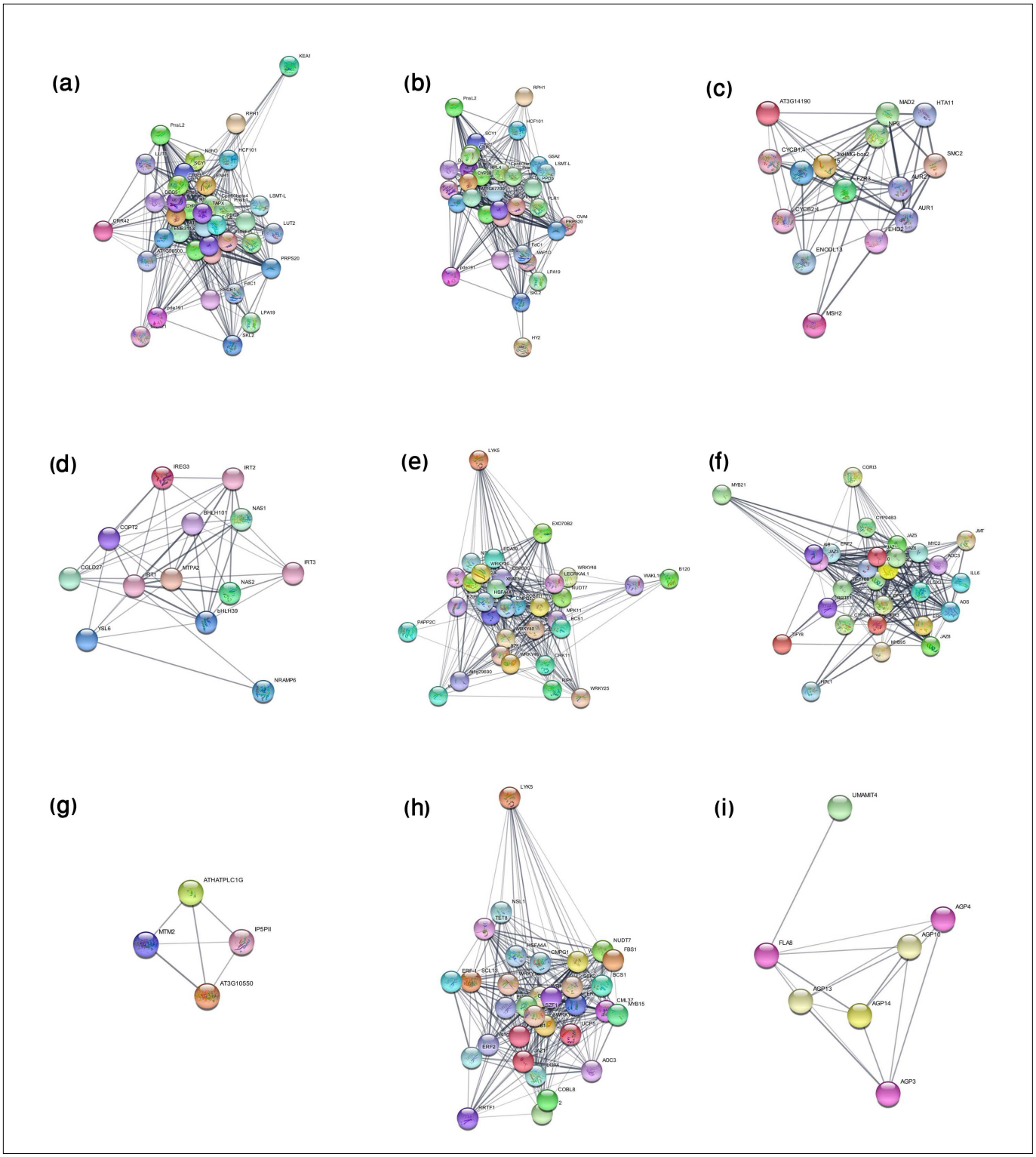

Fig. 4B. Wild-*mock--salt* (***a***)-(***i***) .

lapped genes between current and earlier studies (15), including TAIR IDs, gene symbols, fold changes, up/down regulated status of genes, and the significant *p* values, are listed in Supplementary Table 3. And, the genes listed as the last group in Supplementary Table 3 are those only identified through the BRB-array tool as significant DEGs. However, the PPI network analysis is not applicable due to the low number of DEGs (with only three critical genes).

Discussion

The extensive need for food is directly linked with human life, which can be affected by various environmental risk factors such as abiotic and biotic stresses (20). So, providing food production security for the whole world is of the top-most requirements that policymakers of the countries should be aware of and make thoughtful decisions for increasing the plants' stress tolerance. Because the plants are always immobile and affected mainly by stressful situations, they need more attention in manipulating mechanisms and functionalities through upregulating or downregulating the gene expression levels to achieve optimal productions (21, 22). In the current study, salt stress as one of the substantial abiotic stresses in *Arabidopsis thaliana* was investigated for 2 types, including wild and PtSnRK2.7 over expression.

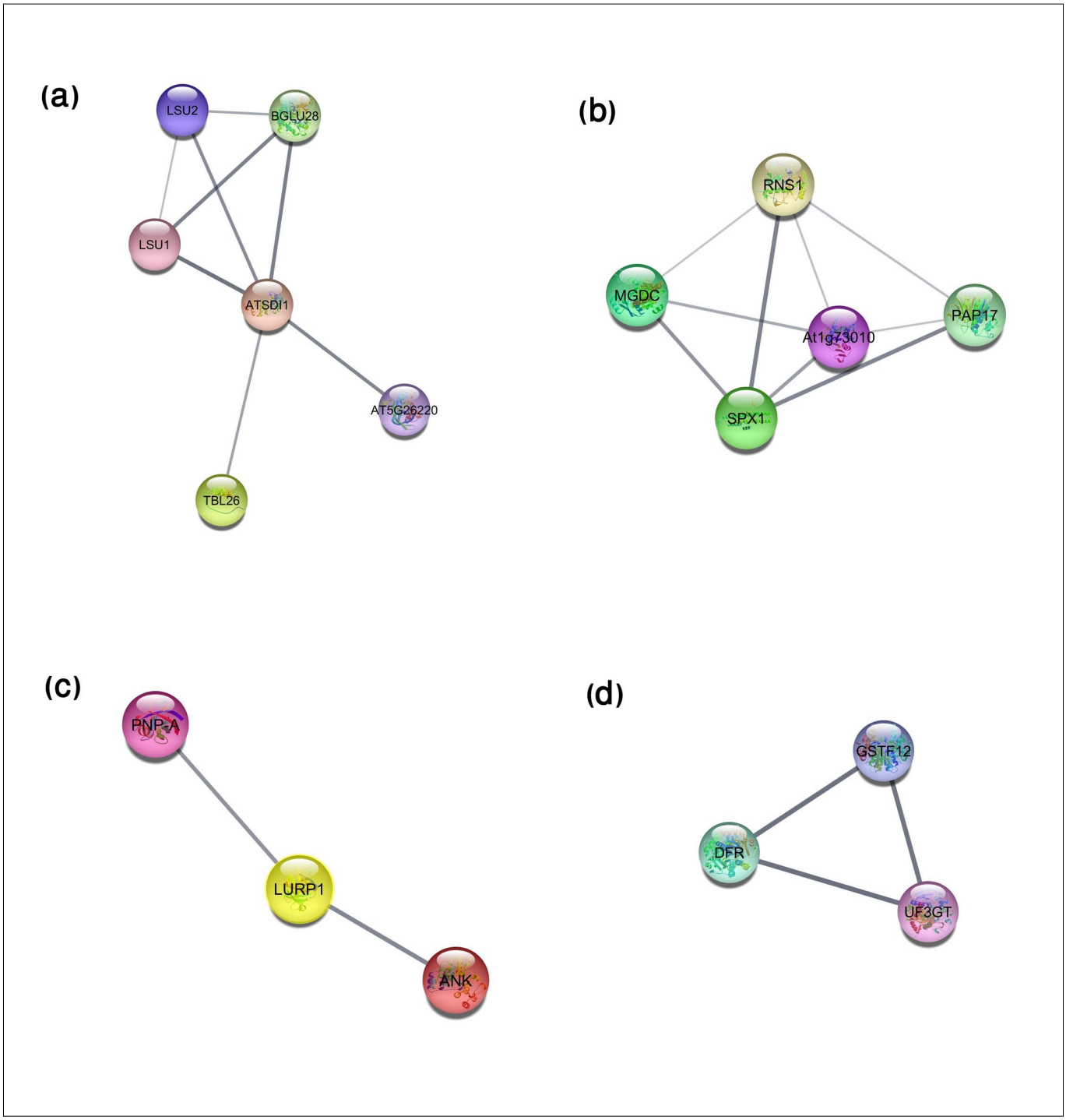

Fig. 4C. Wild-over-*mock* (***a***)-(***d***)

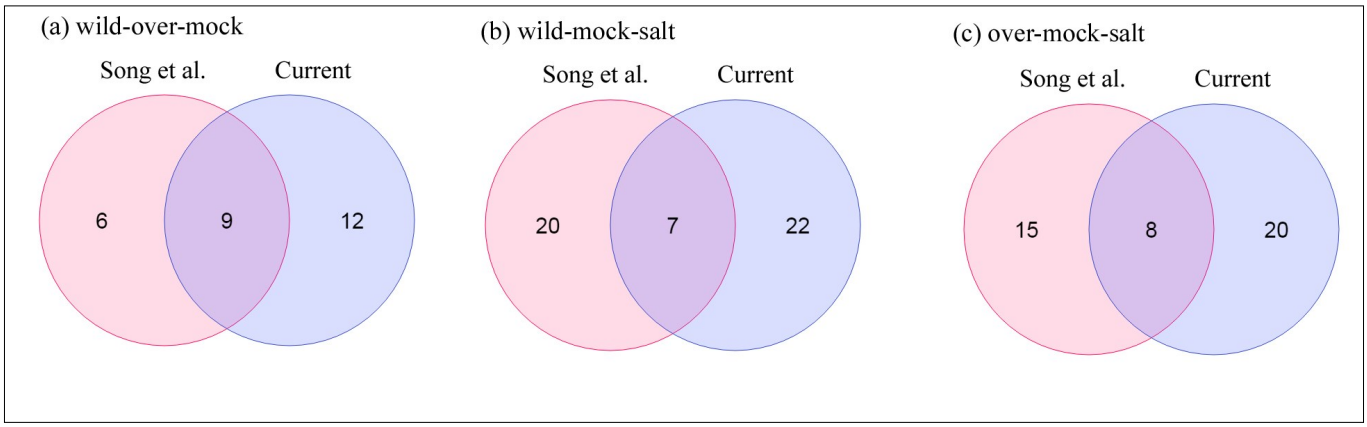

Fig. 5. The number of overlapping genes between current and Song et al. studies.

In the case of wild and PtSnRK2.7 overexpression types (7-day-old seedlings) by 200mM NaCl treatment for two days (wild-over-*salt*), the survival rate of the latter was improved by 2.5 as reported in the literature (15); while the

chlorophyll contents were also higher than that of wild type due to the effects of PtSnRK2.7 over expression on chlorophyll biosynthesis. And, this shows a direct relationship between chlorophyll contents and survival rate. The comparison outcomes of the current study in terms of wild and PtSnRK2.7 overexpression types showed that the only upregulated differentially expressed gene was CYP97B3 (i.e., AT4G15110 at chromosome 4). The 2 others were AT1G15010 (at chromosome 1 affected mostly in transient stress conditions (23)) and AT4G19430 (at chromosome 4 related to inactivation of the chloroplast (24)) without any annotated gene symbols. CYP97B3 as a member of plant cytochrome P450 involved in several biosynthetic reactions that can result in the production of defensive compounds (25). And, this can be almost regarded as no changes between wild and PtSnRK2.7 over expression types while treated by NaCl. Additionally, it induces the fact that the wild type will overexpress the level of PtSnRK2.7 gene to get adapted to the salt tolerance condition by consuming some resources resulting in a lower survival rate and chlorophyll contents.

It has been reported that in the absence of salt stress, significant changes were not observed in the chlorophyll contents of both types (15). This study also demonstrates that the 2 types have only changes in 8 downregulated genes with the highest interconnectivity degree, which can be the reason for the PtSnRK2.7 overexpression processes. It is worth noting that 5 out of 8 identified genes were also determined in a previous study for this condition (15). The other 3 were novel ones At1g73010, ATRNS1 and ATSPX1, which have been reported in the literature for stresses such as hypoxia and reoxygenation (23), plant defensive response (26) and transient stress (23).

Despite the existence of few conflicts in terms of the relationships between fold changes and the over/under-expression status of the overlapped genes, which may be directly related to the nature of the statistics-based algorithms used in the study for analyzing (15) and identifying the initial significant DEGs, the overlapping genes have substantial effects on the 3 compared groups wild-*mock-salt*, wild-over-*mock*, over-*mock-salt* (Supplementary Table 3). A variety of statistical tests are commonly used to identify differentially expressed genes (DEGs), including Welch's t-test, moderated t-test and permutation tests. For parametric tests, accurately estimating intra-sample-group variance is a critical issue; 2 improved variance estimation techniques are the locally pooled error and empirical Bayes methods. Because Omics data analysis typically involves tens of thousands of statistical tests, correcting multiple hypotheses is essential (27). To find DEGs, we usually use the t-test with the ordered set of P values converted to cumulative false discovery rate (FDR) estimates. A typical cutoff would be 10%. Both statistical functions are implemented in BRB-Array Tools (28).

Furthermore, the remaining group wild-over-*salt* was the only group being analyzed in this study and three essential genes (i.e., AT4G15110, AT1G15010, AT4G19430) were determined responsible for salt stress and chloroplast in similar conditions in such that the salt resistance response of SnRK2.7 overexpression can be revealed (Supplementary Table 3). Finally, Supplementary Table 4 lists the overlapping genes, chromosome location and function in agreement with their corresponding literature studies.

Taking in to consideration the wild type exposed to salt condition (wild-*mock-salt*), thirteen unique genes were observed within nine statistically significant modules (p-value < 0.05) from the constructed PPI network. Supplementary Table 5 expounds the genes functions, chromosome position, accession no., as well as their cited references.

Moreover, fifteen genes with the highest interconnectivity with eleven identified gene modules were found statistically significant (p-value < 0.05), which are listed in Supplementary Table 6, along with their specific functionalities mostly related to several stresses such as salt stress.

A system biology approach was applied for studying the responses of *A. thaliana* to salt stress (abiotic stress) in wild and PtSnRK2.7 overexpression types. Furthermore, several analyses in terms of gene ontology (cellular component and molecular function), KEGG enrichment and plant ontology were performed. The outcomes were a set of significant genes identified from the PPI networks module clustering for four conditions mentioned above. Then, the identified genes were further validated through the literature findings considering their relations to abiotic and biotic stresses.

Conclusion

To provide salt tolerance in plants specifically *A. thaliana*, various mechanisms at the cellular, molecular, biological and whole-plant levels are involved. So, suitable strategies are needed to control the plant responses and adaptations to salinity stress. Although many studies have been carried out in this field, it is required to accumulate their results for the plant biologists to identify the responsible genomic modifications accounted for different corresponding mechanisms and critical pathways. Moreover, a gap exists as the investigations are routinely performed on one single gene rather than several potential genes. And, this urges future studies to take advantage of the retrospective computational-based studies to confirm the previously achieved outcomes possibly.

In conclusion, our findings suggest that the overexpressed PtSnRK2.7 gene primarily involved in resistive salt stress responses is, at least partially, responsible for molecular and functional mechanisms of the *A. thaliana* plant model. One of the significant outcomes of the current study, which was not mentioned in previous studies, is the effect of over expression in responsive genes related to unregulated chloroplast contents. Also, it was found out that the number of shared genes between the current study and the previous investigation for the 3 groups wild-*mock-salt*, wild-over-*mock*, wild-over-*salt* were 8, 9, 7. And, for the remaining group, only 3 significant genes were identified. Thus, additional investigation may be essential

to validate the critical role of PtSnRK2.7 overexpressed protein against stress responses to accommodate the required evidence in improving stress tolerance in several plants and trees.

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Authors contributions

EA carried out the gathering and analyzing of studies and drafted the manuscript. SA participated in the design of the study and performed the statistical analysis. BS and SD conceived of the study and participated in its design and coordination as well as the analyses of data. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

Supplementary data

Table 1. Top upregulated and downregulated genes for four groups

Table 2. List of potential gene biomarkers using ClusterOne module analysis.

Table 3. Overlapping genes between curent and Song et al. study along with their properties.

Table 4. Overlapping genes, chromosome location, and literature functional mechanisms.

Table 5. Overlapping genes, chromosome location, and literature functional mechanisms.

Table 6. The details of fifteen significant genes determined from the over-*mock-salt* type.

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