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An Integration of Transcriptomic Data and Modular Gene Co-Expression Network Analysis Uncovers Drought Stress-Related Hub Genes in Transgenic Rice Overexpressing *OsAbp57*

Muhammad-Redha Abdullah-Zawawi ¹ , Lay-Wen Tan ², Zuraida Ab Rahman ³, Ismanizan Ismail ^{2,4} and Zamri Zainal ^{2,4,*}

- ¹ UKM Medical Molecular Biology Institute (UMBI), Universiti Kebangsaan Malaysia, Kuala Lumpur 56000, Malaysia
² Institute of Systems Biology (INBIOSIS), Universiti Kebangsaan Malaysia, Bangi 43600, Malaysia
³ Malaysian Agricultural Research and Development Institute (MARDI), Serdang 43400, Malaysia
⁴ Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi 43600, Malaysia
* Correspondence: zz@ukm.edu.my; Tel.: +60-389-213-936



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Abstract: Auxin receptor plays a significant role in the plant auxin signalling pathway in response to abiotic stress. Recently, we found that transgenic rice overexpressing *ABP57* had higher drought tolerance than the wild-type cultivar, MR219, due to the fact of its enhanced leaf photosynthetic rate and yields under drought stress. We performed a microarray study on this line to investigate the underlying mechanisms contributing to the observed phenotype. After microarray data filtering, 3596 genes were subjected to modular gene co-expression network (mGCN) development using CEMiTool, an R package. We identified highly related genes in 12 modules that could act to specific responses towards drought or any of the abiotic stress types. Gene set enrichment and overrepresentation analyses for modules extracted two highly upregulated modules that are involved in drought-related biological processes such as transmembrane transport of metal ions and response to oxidative stress. Finally, 123 hub genes were identified in all modules after integrating co-expression information with physical interaction data. In addition, the interplay of significant pathways between the metabolism of chlorophyll and flavonoid and the signalling pathways of MAPK, IAA, and SA inferred the concurrent involvement of stress tolerance response. Collectively, our findings seek new future directions for breeding strategies in rice tolerant improvements.

Keywords: transcriptomics; co-expression network; modular analysis; drought stress; hub gene; *Abp57*; rice improvement; bioinformatics

1. Introduction

Rice (*Oryza sativa* L.) is one of the primary staple food sources that contributes to over 20% of the daily calorie intake of more than 3.5 billion people. Hence, considerable effort has been made to ensure a sufficient rice yield supply to meet the rising population's demand. However, a recent study suggested that rice's global average yield improvement is still far behind the required rate to achieve the projected global demands by 2050 [1]. The slower pace of yield improvement is partly due to the diminishing returns to further intensification of irrigated rice systems [2]. In addition, the growing competition for water resources from other crops and industrialisation have also limited the expansion of irrigated rice areas.

In contrast to irrigated rice systems, which have almost achieved the full genetic potential of the high-yielding rice cultivars, the yield of rainfed rice remains low and inconsistent. This indicates an enormous upside potential for the yield of rice grown under a rainfed ecosystem. Numerous studies have pointed out that frequent occurrences of

drought and the lack of drought-tolerant high-yielding rice cultivars are two of the major factors contributing to the suboptimal rice production in rainfed ecosystems [3,4]. Therefore, the development of drought-tolerant high-yielding rice is of paramount importance to ramping up the production of rainfed rice.

Auxin is a phytohormone that functions as a major coordinator in virtually all of the biological processes in plant growth and development. These biological processes include a plant's tropic response towards gravity and light, organ patterning, cell differentiation, seed dormancy, and vascular development [5]. Recent mounting evidence shows that auxin may also play a role in plant response to drought stress. Much of the evidence comes from the realisation that the gene expression of many auxins-related genes was significantly altered under drought conditions. In rice, an expression analysis on 31 rice indole-3-acetic acid (*OsIAA*) genes showed that more than 15 were upregulated under drought stress [6]. In addition, a genome-level microarray analysis in rice also demonstrated that more than 204 auxin-responsive genes exhibited altered expression under desiccation conditions [7]. Given the response of auxin-related genes towards drought stress, numerous studies have been conducted to overexpress these genes in various plant species to enhance their drought tolerance. In potatoes, the overexpression of *YUCCA6*, a flavin monooxygenase of tryptophan-dependent auxin biosynthetic pathways, has resulted in elevated endogenous auxin levels and enhanced drought resistance [8]. In addition, the overexpression of auxin-related genes, such as *OsPIN3t*, *TLD1/GH3.13*, and *OsIAA6*, have also been found to confer higher tolerance of rice towards drought conditions [9–11]. Taken together, auxin-related genes show great potential to be utilised in the attempt to develop rice cultivar with drought tolerance.

Auxin-binding protein (ABP) is a family of low-abundance proteins that bind reversibly to auxin with high specificity and affinity. Owing to their auxin-binding properties, ABP has long been suggested to function as an auxin receptor, mediating diverse cellular responses in response to different endogenous auxin levels. ABP57 is a 57 kDa ABP that was first isolated from the soluble protein fraction of the shoot of rice (*Oryza sativa* L.) seedlings by Kim et al. (1998) [12]. An *in vitro* experiment has proven that *ABP57* functions by activating plasma membrane H^+ -ATPase through direct interaction in response to the concentration of auxin [13]. In contrast to the classical *ABP1*, *ABP57* appears insensitive to naphthaleneacetic acid (NAA) despite having a very high binding affinity with IAA [14]. Numerous overexpression studies have shown that a higher expression level of *OsAbp57* can lead to increased seed size, faster seed germination, and seedling growth [15]. Recently, we found that transgenic rice overexpressing *ABP57* had higher drought tolerance than wild-type cultivar, MR219, due to the fact of an enhanced leaf photosynthetic rate and yields under drought stress [16]. We performed a microarray study on this line to investigate the underlying mechanisms contributing to the observed phenotype.

Co-expression is a well-known biological network technique in predicting the gene function using a myriad of transcriptomics data from RNA sequencing or microarrays technology by connecting the genes based on their similar expression profiles [17,18]. Through the guilt-by-association principle, genes with similar mRNA expression profiles across the tissue, treatment, or developmental stage are predicted to share a similar function and are regulated via similar transcription factors [17,19]. The co-expression relationship can be represented as a network graph that connects nodes (i.e., co-expressed genes) by edges, indicating correlation-based evidence between genes. A group of nodes that are highly interactive with one another is known as a module. Within a module, genes with high connectivity are defined as hub genes. Modules are usually enriched to discover a set of co-expressed genes that are overrepresented in a similar biological process (i.e., gene function such as plant defence or signal transduction), molecular function (i.e., gene activity such as protein kinase or catalytic activity), and cellular component (i.e., gene location such as the cell wall or chloroplast). This information would be essential for understanding the function of a gene and on how genes are produced and operate during response to drought in rice.

In the present study, an assessment of the morpho-physiological traits exhibited by transgenic rice overexpressing *OsAbp57* grown under two watering regimes was performed to identify the possible traits that may contribute to the drought tolerance of transgenic rice. In addition, a microarray analysis was also conducted to identify differentially expressed genes in the transgenic plant compared to its parental line, MR219, under normal conditions. Here, we performed a modular gene co-expression network (mGCN) analysis of this in-house microarray dataset to examine the co-expressed gene modules, evaluate the module activity between samples of MR219 and transgenic rice overexpressing *OsAbp57* (*Abp57*-OE), and enrich a set of co-expressed gene modules to associate them with a particular function, activity, and cellular location. In this study, we represented the phenotypic class of MR219 and transgenic rice as wild-type (WT) and overexpressing *OsAbp57* (*Abp57*-OE), respectively. To grasp an understanding of the transcriptional activity of overexpressed *OsAbp57* under drought conditions, we integrated protein–protein interaction (PPI) information into the constructed modules to identify the most connected genes (i.e., hubs) within the network that may play a function as a critical regulator in response to drought stress. Finally, we conducted an expression analysis on a few selected genes to investigate their role in rice drought tolerance.

2. Materials and Methods

2.1. Sample Preparation and RNA Extraction

The T3 seeds of *Abp57*-overexpressing rice (OE) were obtained from our previous *Agrobacterium*-mediated transformation study [20] and were cultivated on MS media for ten days along with MR219 (WT) seed as a control. Each WT and *Abp57*-OE group consisted of three biological replicates. Total RNA was isolated from seedling tissues of *Abp57*-OE and WT using TRIzol reagent (Life Technology, Waltham, MA, USA), followed by DNase treatment via Ambion TURBO™ to eliminate DNA contamination. The quality of the extracted RNA was measured using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) in which samples with a concentration of 1.8 to 2.1 (A260/A280) were considered. We determined the integrity of the extracted RNA using a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All samples with an RNA Integrity Number (RIN) of 8.0 to 10.0 were subjected to microarray analysis.

2.2. Gene Expression Profiling, Differentially Expressed Genes (DEGs), and Enrichment Analyses

Gene expression profiling was conducted on the three replicates of seedling tissues of WT and *Abp57*-OE using Affymetrix Rice (Chinese Build) Gene 1.0 ST Array that consists of 41,770 transcripts (Affymetrix Inc., Santa Clara, CA, USA). The CEL file was imported into Affymetrix Expression Console™ Software to check for quality control of raw data and data normalisation following the manufacturer's protocol. The robust multiarray analysis (RMA) method was applied to ensure that the relative log expression signal (RLE) was comparable between samples. The microarray experiment was then previously deposited into the NCBI by Tan et al. (2017) [21] and is accessible through the NCBI GEO DataSets (DOI: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE99055>, accessed on 1 June 2017) [22].

The CHP files resulting from normalisation for DEGs analysis were then imported into the Transcriptome Analysis Console (TAC) software version 3.1 (Applied Biosystems, Foster City, CA, USA), following the manufacturer's specifications. Using TAC, a statistical method of one-way analysis of variance (ANOVA) was utilised to obtain the DEGs of WT and *Abp57*-OE with default parameters: a fold change > |2| and an ANOVA *p*-value < 0.05. One-way ANOVA was calculated based on $F = \text{Mean sum of square between group (MSB)} / \text{Mean sum of square within group (MSW)}$, where *F* follows an *F* distribution with degrees of freedom between group (*df*) = *K* – 1 and within group (*DFw*) = *N* – *K*. *K* represents the number of groups, and *N* is the total number of observations across all groups.

For the MSB formula, $\text{MSB} = \frac{\sum(X_i \sum X_t)^2}{K \sum 1}$; where *X_i* is the mean of group *i*, and *X_t* = mean of all observations (all observations from all groups are combined to form a single group, and the mean is subsequently calculated). For the MSW formula,

$SSW = \sum(X_{ij} - X_j)^2 / N - K$; where X_{ij} is an observation within group j , and X_j = mean of group j . Thus, overall, $F = MSW / MSB = (\sum(X_i - X_t)^2 / K - 1) / (\sum(X_{ij} - X_j)^2 / N - K)$.

For functional annotation of DEGs, we searched against several databases, including the National Center for Biotechnology Information version 236 (NCBI; <https://www.ncbi.nlm.nih.gov>, accessed on 15 March 2020) [23], UniProt version 2020_04 (<https://www.uniprot.org>, accessed on 15 March 2020) [24], Rice Genome Annotation Project version 7.0 (RGAP; <http://rice.plantbiology.msu.edu/>, accessed on 15 March 2020) [25], The Rice Annotation Project Database version 1.0 (RAP-DB; <https://rapdb.dna.affrc.go.jp>, accessed on 15 March 2020) [26], and the Beijing Genomics Institute Rise Information System version 1.0 (BGI-RIS; <http://rise.genomics.org.cn/>, accessed on 15 March 2020) [27]. The Gene Ontology (GO) terms were analysed with the Singular Enrichment Analysis (SEA) program available on AgriGO version 2.0 (<http://bioinfo.cau.edu.cn/agriGO/>, accessed on 15 March 2020) [28] with the Affymetrix Genome Array (GPL2025) as background. Genes with 2-fold expression levels and p -values < 0.05 were used for SEA analysis.

2.3. Expression Analyses of Randomly Selected Expressed Genes by qRT-PCR

Six genes were randomly selected for further investigation to validate the reliability of the microarray data as follows: *ATPF1G* (Os07g0513000), *OsCAO1* (Os10g0567400), *OsEno5* (Os06g0136600), *OsCPS4* (Os04g0178300), *NAS1* (Os03g0307300), and *CHI* (Os12g0115700). Total RNA was extracted from one-week-old seedlings of MR219 using TRIzol reagent (Life Technology, Waltham, MA, USA) and then subjected to DNase treatment using Ambion TURBO™ DNase (Thermo Fischer Scientific, Waltham, MA, USA). The total RNA was reverse-transcribed into first-strand cDNA with a Maxima First Strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). An equal amount of cDNA was used as the template for PCR amplification using SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). Genes of interest were amplified using the specific primers listed in Supplementary Materials Table S1. The relative expression level of an individual gene was determined using the $\Delta\Delta$ Ct calculation [29]. The housekeeping genes *U6* and *UBQ5* were used as an internal control. One-way ANOVA was then performed to assess DEGs with a p -value < 0.05. The expression level of DEGs was later compared with gene profiling data to determine the validity of the microarray data.

2.4. Construction of Gene Co-Expression Network Modules

To further support the reliability of our *Abp57*-OE microarray data in drought, the co-expression module analysis was carried out using the CEMiTool R package version 1.20 [30]. The published microarray dataset, under the accession number GSE99055, was employed as a discovery set [21]. In this study, the correlation method of Pearson was selected to transform the gene expression matrix ($m \times n$) into an adjacency matrix, where m denotes as genes and n represents the samples. A soft-threshold power (β) selection algorithm was executed based on a scale-free topology criterion to construct an adjacency matrix, later associated with the relationship between co-expression modules. The adjacency matrices were transformed using the best threshold determined based on the “scale-freeness” of the constructed network generated by the default function of CEMiTool. Unsigned network analysis was conducted to infer whether modules represented general processes of drought stress or any stress-related responses in transgenic rice overexpressing *OsAbp57*. The dissimilarity threshold of 0.8 was employed as a cut-off for hierarchical clustering by the agglomerative method, where genes with common expression levels were grouped into clusters. The following criteria were used to build the network module: coefficient of determination for linear regression fit, R^2 ($R^2 > 0.8$); the number of gene modules ($\text{min_ngen} > 20$); threshold similarity of eigengene ($\text{diss_thresh} > 0.8$); the number of high-connectivity genes in each module, hub genes ($n = 5$).

2.5. Gene Set Enrichment and Overrepresentation Analysis of Modules

The gene set analysis was performed for each module using the mean rank method implemented in the Fast Gene Set Enrichment Analysis (FSGEA) R package to identify modules of interest from constructed gene co-expression network [31]. The activity of the modules was evaluated by determining which modules were upregulated or downregulated in the samples of the wild-type MR219 and the *OsAbp57* overexpressing lines. The size of the gene set was generated by default in a range between 15 and 1000. To enrich the associated function for each module, the hypergeometric distribution test (overrepresentation analysis) was performed using the clusterProfiler R package [32]. The Gene Ontology (GO) annotation of gene modules was retrieved and downloaded from Phytozome version 12.0 (<https://phytozome.jgi.doe.gov/>, accessed on 10 April 2021) [33]. The significance cut-off of p -value < 0.05 was used to assign overrepresented biological process (BP), molecular function (MF), and cellular component (CC) of genes within modules.

2.6. Identification and Validation of Hub Genes

The hub genes were determined by identifying genes with higher connectivity within the modules. The hub genes were ranked based on the top 10 genes within the network modules. For instance, if the module size was 200, then 20 genes with the largest number of connectivity could be considered hub genes [34]. Integration between co-expression information and protein–protein interactions (PPIs) may help discover important hubs in the module. The rice PPI dataset from the STRING database version 11.5 was retrieved by the Cytoscape plug-in, StringApp version 1.7.0 [35,36], using the list of gene modules as search queries with the default confidence cut-off of 0.4 and above. A single protein with no interaction was discarded, and only interactions with evidence of known interaction (i.e., curated databases and experimentally determined) were kept for further analysis. Module graphs for gene-encoded protein networks from the rice PPI dataset on STRING were constructed by merging PPI information to visualise gene interaction in co-expression modules and identify potential hubs.

2.7. KEGG Pathway Mapping Analysis

Each module was used to conduct pathway mapping analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Mapper tool version 4.3 (<https://www.genome.jp/kegg/mapper.html>, accessed on 1 June 2021) [37]. The KEGG Orthology identifier (KO ID) for genes was retrieved from the Phytozome v12 database using the BioMart tool [33,38]. All genes in each module were then categorised into the following KEGG pathways: (i) metabolism, (ii) genetic, (iii) environmental information processing, (iv) cellular processes, and (v) organismal systems. The interactions between hub genes were then manually connected as red arrows on the pathway maps.

3. Results

3.1. Global Gene Expression Profiling of the *Abp57*-OE Line

To gain insight into the transcriptional changes triggered by the *OsAbp57* overexpression, a global gene expression analysis on WT and transgenic line (*Abp57*-OE) grown under normal conditions was performed through DNA microarrays. The microarray data analysis revealed that a total of 131 genes (90 upregulated and 41 downregulated) were differentially expressed (fold change > 2 , p -value < 0.05) in transgenic lines as compared to MR219. The reliability of the microarray data was then assessed by quantitative real-time PCR on six randomly selected DEGs, and the differences were validated (Figure 1). The expression level of DEGs was largely consistent with the microarray result, implying that the data were highly reliable.

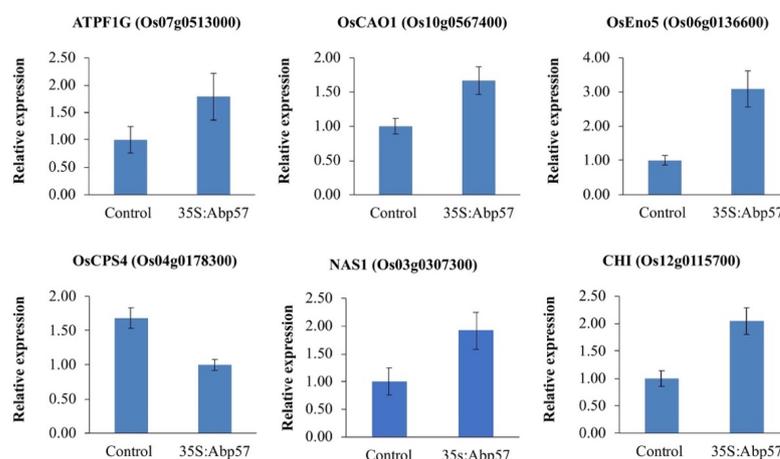


Figure 1. qPCR validations on six randomly selected DEGs. The bar plot represents the mean \pm standard error for two biological replicates and three technical replicates. The difference in gene expression between the control (WT) and *Abp57*-OE was determined by a one-way ANOVA method with a cut-off *p*-value < 0.05 . The error bars indicate the standard error.

Gene Ontology (GO) enrichment analysis revealed that response to stimulus, homeostasis process, transport, oxidation-reduction, lipid metabolic process, and biosynthetic process were among the significantly altered biological processes in transgenic rice. Meanwhile, for molecular function, DEGs were enriched considerably in metal ion binding, ATP binding, ion transmembrane transporter activity, antiporter activity, peroxidase activity, electron carrier activity, and catalytic activity. For cell components, we identified that the DEGs were significantly regulated in the membrane, chloroplast, and vacuole. The result of the GO enrichment analysis is summarised in Table 1.

Table 1. Functional classification of the differentially regulated genes (DEGs) in *OsAbp57*-overexpressing transgenic rice. GO term enrichment analysis was performed using the AgriGO analysis tool—Single Enrichment Analysis (<http://bioinfo.cau.edu.cn/agriGO/analysis.php>, accessed on 15 March 2020). The GO enrichment of biological process (B), molecular function (F), and cellular component (C) was generated based on a false discovery rate (FDR) and adjusted *p*-value cut-off of 1.0×10^{-5} .

GO Term	Ontology	Description	Number of DEGs	FDR
GO:0050896	P	Response to stimulus	32	2.20×10^{-24}
GO:0051234	P	Establishment of localisation	30	6.00×10^{-21}
GO:0051179	P	Localisation	30	6.00×10^{-21}
GO:0006810	P	Transport	29	4.60×10^{-20}
GO:0009987	P	Cellular process	51	8.40×10^{-18}
GO:0009607	P	Response to biotic stimulus	14	8.50×10^{-18}
GO:0006811	P	Ion transport	17	3.20×10^{-16}
GO:0008152	P	Metabolic process	47	1.70×10^{-15}
GO:0006950	P	Response to stress	17	5.80×10^{-12}
GO:0042592	P	Homeostatic process	10	1.00×10^{-11}
GO:0051704	P	Multi-organism process	10	2.30×10^{-10}
GO:0003824	F	Catalytic activity	50	6.30×10^{-14}
GO:0022857	F	Transmembrane transporter activity	17	7.60×10^{-14}
GO:0005488	F	Binding	48	2.60×10^{-12}
GO:0015075	F	Ion transmembrane transporter activity	13	2.60×10^{-12}
GO:0005215	F	Transporter activity	17	5.00×10^{-11}
GO:0022891	F	Substrate-specific transmembrane transporter activity	13	5.00×10^{-11}
GO:0046872	F	Metal ion binding	25	6.60×10^{-11}
GO:0043167	F	Ion binding	25	1.20×10^{-10}
GO:0043169	F	Cation binding	25	1.20×10^{-10}
GO:0022892	F	Substrate-specific transporter activity	13	2.80×10^{-10}

3.2. Modular Gene Co-Expression Analyses of the *OsAbp57*-OE Line

To further support the reliability of our *Abp57*-OE microarray data in drought stress, we performed the mGCN analysis. In total, 41,770 genes across six samples were used to construct an mGCN to determine key modules of drought-tolerant transgenic rice, *OsAbp57*-OE. In Figure 2A, the dendrogram demonstrates the clustering of six samples, whereby the samples were clustered based on wild-type samples (turquoise) and *OsAbp57*-OE samples (red). Using the default parameters, the soft-threshold value (β) of 14 was selected by a scale-free topology fit (R^2) of 0.824, indicating the best threshold for a scale-free network model (Figure 2B). A total of 13 co-expressed modules containing 3596 genes (i.e., M1 to M12, including one noncorrelated module, M13) were identified using the dissimilarity threshold of 0.8 as a hierarchical clustering cut-off. Module M1 holds the highest number of co-expressed genes (2004), followed by other modules in the following order, and the smallest number of genes (41) was discovered in M11 and M12. Of these, only 11 co-expressed modules (M2 to M12) with a p -value less than 0.05 were subjected to significant module activity. All module enrichment plots discovered in co-expression analysis were shown to be upregulated in the class of *OsAbp57*-OE samples (OE) compared with the wild-type samples (WT), except for module M6 of *Abp57*-OE, which featured a general downregulation when compared with the WT sample (Figure 2C). The upregulation in *Abp57*-OE class and downregulation in WT class has suggested the sensitivity of transgenic rice, *OsAbp57*, towards drought stress.

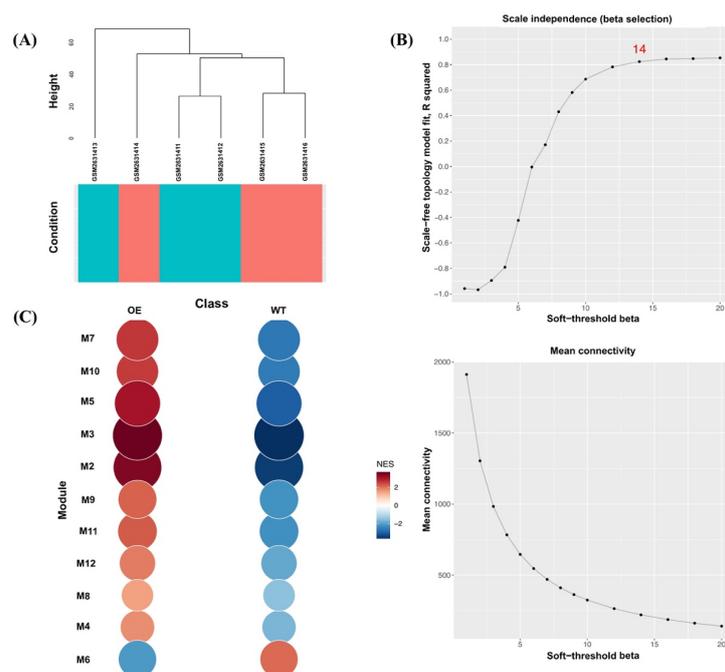


Figure 2. CEMiTool outputs for the *OsAbp57*-OE line microarray dataset. (A) Clustering dendrogram of genes based on the expression profiles. The turquoise colour indicates MR219, and the red colour represents the *OsAbp57*-OE line. (B) Scale-free topology (R^2) and mean connectivity to identify the soft-threshold power (β) between 1 and 20. The scale-freeness of the network was determined at a soft threshold of 14, above the R^2 threshold of 0.8. (C) Gene set enrichment analysis for module activity of the *OsAbp57*-OE line (OE) and MR219 (WT). The size and colour of the modules represent the normalised enrichment score (NES). All modules were upregulated in OE except M6, with downregulation in OE and upregulation in WT.

In OE, modules M2 (OE; NES = 3.39; WT; NES = -3.46) and M3 (OE; NES = 3.58; WT; NES = -3.63) featured strong upregulation and downregulation in module activity (Figure 2C). The enrichment analysis of each module demonstrated that co-expressed genes in modules M2 and M3 were involved in GO terms transmembrane transport including

metal ion transport and response to oxidative stress (Figure 3A,B). Module M2 was enriched in defence response (adjusted $p = 0.30099$; 11/1117 genes), cell redox homeostasis (adjusted $p = 0.37956$; 10/1117 genes), and proteolysis (adjusted $p = 0.3183$; 51/1117 genes). However, genes in module M3 were involved in the fatty acid biosynthetic process (adjusted $p = 0.88612$; 18/1117 genes) and signal transduction (adjusted $p = 0.88612$; 13/1117 genes). Overall, other modules were also strongly enriched in response to oxidative stress, such as modules M4, M5, M7, M8, M10, and M11 (Supplementary Materials Table S2).

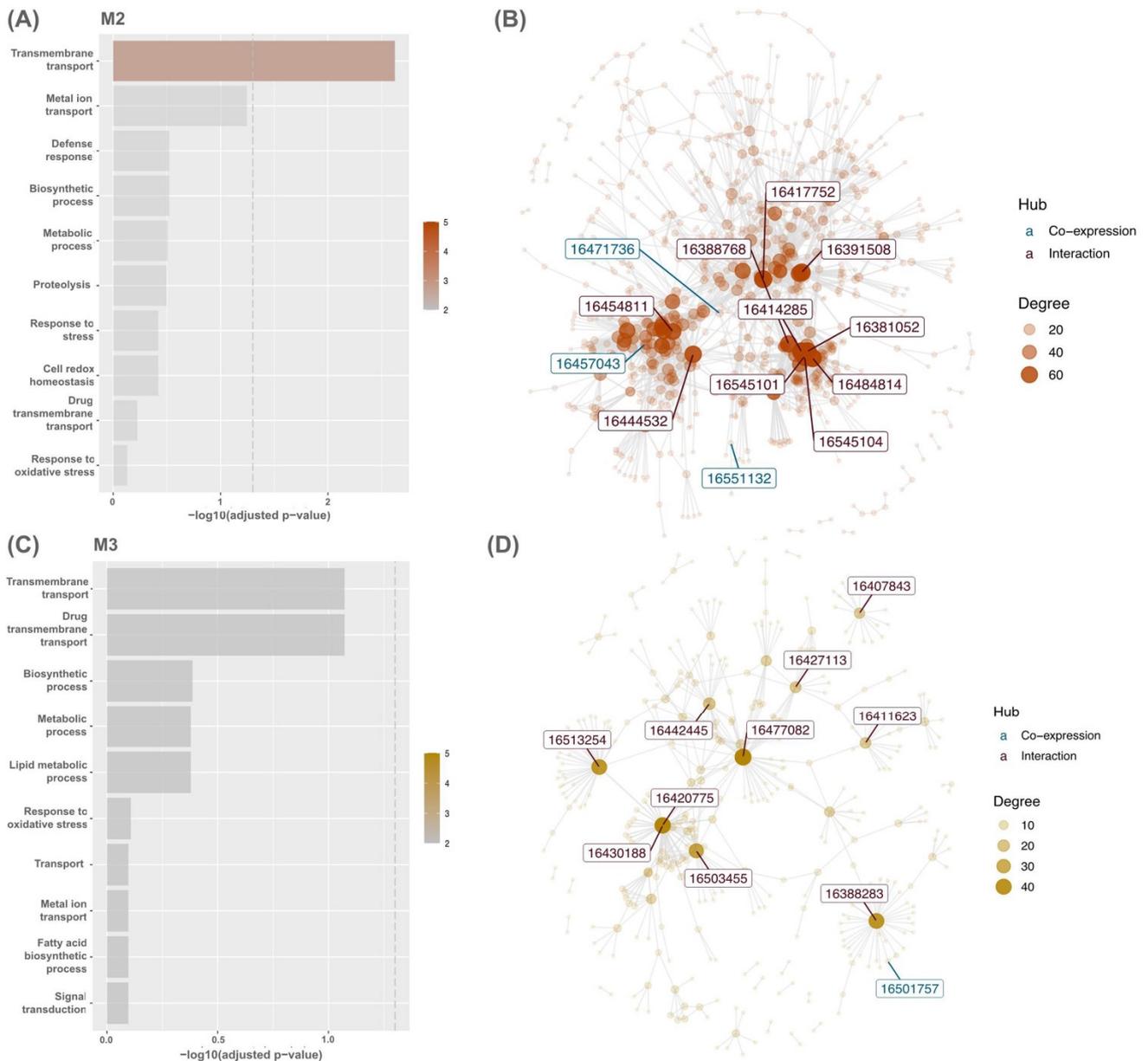


Figure 3. Bar graph for the top ten GO terms enriched between genes in modules (A) M2 and (C) M3, respectively, and gene sets from the Phytozome database. The dashed line represents the $-\log_{10}$ adjusted p -value of 0.01. Interaction network of modules (B) M2 and (D) M3. The top ten hubs' colours are shown based on the originality of hubs present in the CEMiTool co-expression module (blue) or rice PPI dataset (red) on the STRING database.

3.3. Hub Genes' Identification in *OsAbp57*-OE Associated with Drought Stress

A hub gene is a high-degree gene in the module and is presumed to be a vital candidate that controls other genes in the network. The top 10 most connected genes in each module

were determined using the abovementioned methods. We identified 123 hub genes in 12 modules (i.e., M1 to M12). Detailed information on the hub genes of each module is listed in Supplementary Materials File S1. The hub gene analysis discovered 15 origin hubs, which were initially found in the CEMiTool module, including *UGDH* and *ONI3* in module M1; *KINUA*, *TPR*, and *ABC-1* in M2; *HRZ2* in M3; *SUPM1* and *BAK1* in M5; *AAO2* and *4CL1* in M7; *PEPC-2* and *NPR5* in M10; *PPR* and *OAT* in M11; *OsMutY* in M12. Moreover, we found that origin hubs associated with drought stress mechanisms were upregulated in the network, particularly *HRZ2* in response to Fe³⁺ starvation and JA-mediated signalling pathway, *AAO2* in response to water distress, and *OAT* in various responses to abiotic stress tolerance such as to water distress, osmotic and salt stress, hormonal stimuli of ABA, JA, BR, and IAA.

Figure 3B,D demonstrate the gene network for interesting modules M2 and M3, respectively. We discovered several upregulated genes in enriched *OsAbp57*-OE modules M2 and M3 such as *HSP70*, *KINUA*, *TPR*, *ABC-1*, *PK*, *Os01g0655500*, *PRR1*, *MDH*, *HRZ2*, *TIG*, *PTR*, *PRF1*, and *KAR* (Table 2). Interestingly, the Gene Ontology annotation shows that some hub genes are significantly involved in stress-related biological processes, for instance, in response to the metal ion, Cd²⁺ (i.e., *HSP70*), Fe³⁺ starvation and JA-mediated signalling pathway (i.e., *HRZ2*), and reactive oxygen species (ROS) (i.e., *MDH*), including *PRR1*, are reported to play a role in the phosphorelay signal transduction system and the regulation of circadian rhythm, *PK* in glycolysis, *KAR* in elongation of fatty acid, and *TIG* and *PTR* in transport function of protein and oligopeptide, respectively. However, although several defence-related biological processes have been reported through the GO annotation, the potential mechanisms underlying the regulation of drought tolerance genes by *OsAbp57* are not well understood. A further experiment must be conducted to determine how these hub genes may navigate the function of defence during drought conditions in rice.

Table 2. Hub genes from interesting modules M2 and M3.

Probe ID	Gene ID	Gene Name	Description	Gene Ontology	Trait Ontology	Log2FC	Up or Down
16381052	Os01g0100700	<i>RPS5-1</i>	40S ribosomal protein 55-1	Defence response to fungus, response to H ₂ O ₂ , ABA, salt stress, water deprivation	Oxidative stress, sheath blight disease resistance, drought and salt tolerance, ABA sensitivity	0.04197709	Down
16388768	Os01g0686800	<i>RACK1A</i>	WD repeat-containing protein	Shoot and root development, seed germination, circadian rhythm, detection of redox state, defence response to fungus, ET- and SA-activated signalling pathway, SA-mediated signalling pathway, photoperiodism, response to SA, ET, salt stress, H ₂ O ₂ , and Cd ²⁺	ABA content, blast disease, disease resistance, ET sensitivity, photoperiod sensitivity, salt tolerance	0.0606787	Down
16391508	Os01g0840100	<i>HSP70</i>	Heat shock protein Hsp70	Response to Cd ²⁺ and stress	–	0.09918908	Up
16454811	Os02g0797400	<i>MCM5</i>	MCM family protein	Cell cycle, DNA replication	–	0.0735361	Down
16444532	Os02g0805200	<i>PCNA</i>	Proliferating cell nuclear antigen	Regulation of DNA replication, double-stranded break repair, response to gamma ray, DNA damage stimulus, UV, radiation, and H ₂ O ₂	UV light sensitivity	0.0467894	Down
16457043	Os03g0152900	<i>KINUA</i>	Kinesin-like protein KIN-UA	Microtubule-based movement	–	0.13444718	Up
16471736	Os03g0308800	<i>TPR</i>	Tetratricopeptide-like helical domain containing protein	RNA processing, regulation of translation	–	0.1440686	Up
16484814	Os04g0598200	<i>RPL12</i>	60S ribosomal protein L12	Translation, ribosomal large subunit assembly, defence response to bacterium, response to heat, cold, SA, JA, H ₂ O ₂ , and water deprivation	Heat, cold & drought tolerance, JA sensitivity, bacterial blight disease resistance, oxidative stress	0.1768968	Down
16545101	Os08g0117200	<i>RPS13</i>	40S ribosomal protein S13	Translation, defence response to fungus, response to water deprivation, salt stress, ABA, H ₂ O ₂	Drought & salt tolerance, oxidative stress, sheath blight disease resistance, ABA sensitivity.	0.93240096	Down

Table 2. Cont.

Probe ID	Gene ID	Gene Name	Description	Gene Ontology	Trait Ontology	Log2FC	Up or Down
16545104	Os08g0117300	<i>RPS13</i>	40S ribosomal protein S13	Translation	–	0.0503529	Down
16551132	Os09g0250700	<i>ABC-1</i>	ABC-1 domain containing protein.	–	–	0.1204065	Up
16414285	Os10g0466700	<i>RPL17</i>	60S ribosomal protein L17	Translation	–	0.0262824	Down
16417752	Os11g0216000	<i>PK</i>	Pyruvate kinase family protein	Glycolysis	–	0.09241819	Up
16388283	Os01g0655500	<i>Os01g0655500</i>	Protein kinase, core domain containing protein.	Protein phosphorylation	–	0.27208653	Up
16442445	Os02g0618200	<i>PRR1</i>	Two-component response regulator-like PRR1 (Pseudo-response regulator 1)	Phosphorelay signal transduction system, regulation of circadian rhythm	–	0.10612373	Up
16477082	Os03g0773800	<i>MDH</i>	Malate dehydrogenase	Response to ROS, metabolic processes of malate and carbohydrate, cell redox homeostasis, TCA cycle	–	0.11776116	Up
16503455	Os05g0143800	<i>GH3.6</i>	GH3 auxin-responsive promoter domain containing protein	Response to IAA, light stimulus, salt stress	Salt tolerance	0.2140365	Down
16501757	Os05g0551000	<i>HRZ2</i>	Hemerythrin motif-containing RING- and Zn-finger protein2	Response to Fe ³⁺ starvation, JA-mediated signalling pathway	Fe ³⁺ sensitivity	0.24291249	Up
16513254	Os06g0308000	<i>TIG</i>	Trigger factor-like protein	Protein folding and transport	–	0.09959792	Up
16411623	Os10g0111700	<i>PTR</i>	POT family protein, peptide transporter	Oligopeptide transport	–	0.20793006	Up
16407843	Os10g0323600	<i>PRF1</i>	Profilin A	Cytoskeleton organisation, sequestering of actin monomers	–	0.16372392	Up
16420775	Os11g0106700	<i>FER1</i>	FERRITIN 1	Response to Fe ³⁺ , Zn ²⁺ , H ₂ O ₂ , ABA, bacteridium and cold; leaf and flower development; Fe ³⁺ transport and homeostasis; photosynthesis	–	0.1615892	Down
16430188	Os12g0106000	<i>FER2</i>	FERRITIN 2	Response to Fe ³⁺ , Zn ²⁺ , Cu ²⁺ , H ₂ O ₂ , cold, bacterium, ABA; leaf and flower development; Fe ³⁺ transport and homeostasis; photosynthesis	Temperature response trait, Cu ²⁺ sensitivity	0.1529454	Down
16427113	Os12g0242700	<i>KAR</i>	Beta-ketoacyl reductase	Fatty acid elongation	–	0.48300481	Up

To validate the hub gene, Figure 4 demonstrates the interaction network for all hub gene modules: M1 to M12. The network consisted of 125 nodes and 584 edges of which 14 were identified as single nodes. Figure 4B showed β -ketoacyl reductase (*KAR*), a fatty acid biosynthetic gene, which was highly upregulated in *OsAbp57*-OE. We found that stress-related hub genes, including *HSP70*, *HRZ2*, and *MDH*, significantly interacted with other upregulated hub genes (Figure 4B). For instance, *HSP70* with *EF2*; *PK*, *PPR*, and *MDH*, *HRZ2* with *Os01g0655500*; and *MDH* with *CFR*, *ACP*, *ACP-1*, *ACP3*, *ppc4*, *PK*, *cytME2*, *HSP70*, and *PPR1*. Several downregulated genes were also identified to interact with *HSP70* and *MDH*, suggesting its potential negative regulation of drought responses in *OsAbp57*-OE.

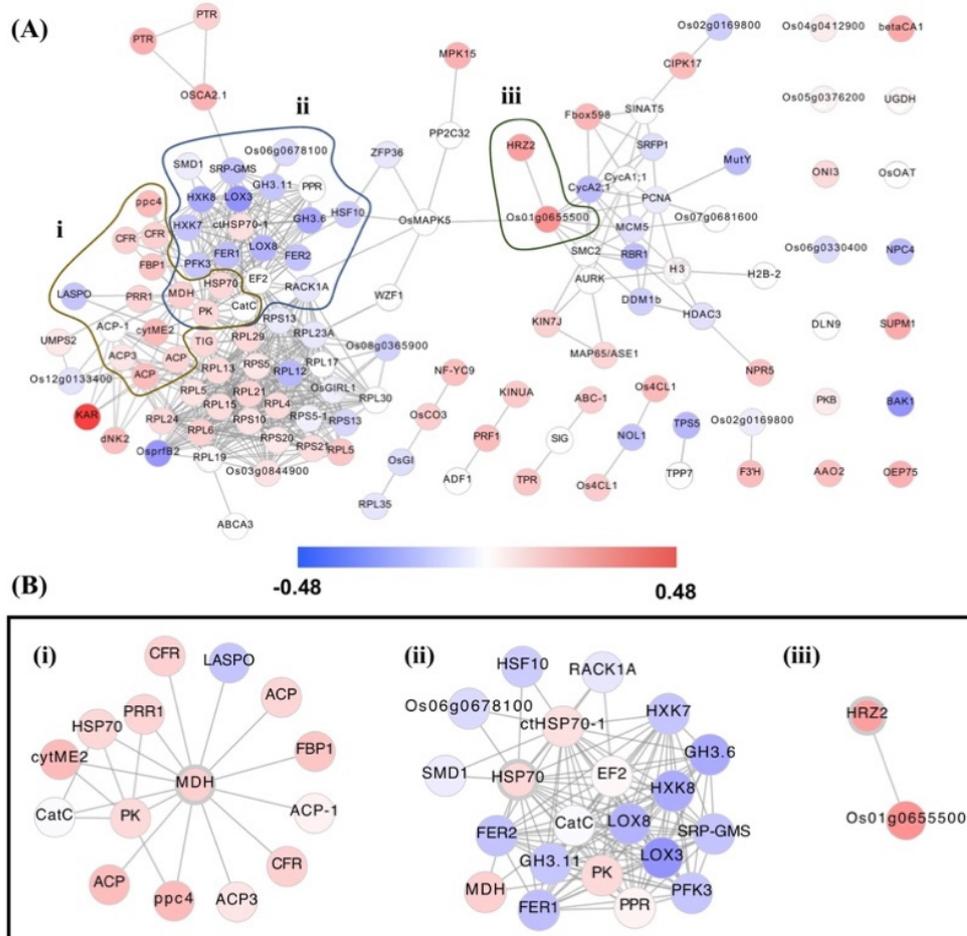


Figure 4. Interaction network for all hub genes in *OsAbp57*-OE: (A) modules M1–M12; (B) stress-related hub genes: (i) MDH, (ii) HSP70, and (iii) HRZ2.

3.4. Pathway Mapping Analysis of *OsAbp57*-OE

The number of genes from network modules M1 to M12 was assigned according to their presence in the KEGG pathway maps. The pathway maps are categorised into metabolism, organismal systems, cellular processes, and environmental and genetic information processing. The modules M1 to M11 demonstrated active involvement in metabolism (Supplementary File S2). Genes with a putative function in cellular processes were absent in modules M5 to M7, M9, M10, and M12, including the absence of environmental information processing in M4, M5, M7 to M9, and M12, organismal systems in M7 to M12, and genetic and environmental processing in M3, M4, M8, and M10. The hub genes showed high participation in the genetic information process (30 genes) and metabolism (29 genes) including participation in the environmental information process (4 genes), cellular processes (3 genes), and organismal systems (2 genes) (Figure 5A). In addition, integration with protein–protein interaction data validated the correlation of hubs between the pathway maps. The hubs under the phenylpropanoid biosynthesis were correlated to the porphyrin and chlorophyll metabolism map in module M7 (Figure 5B).

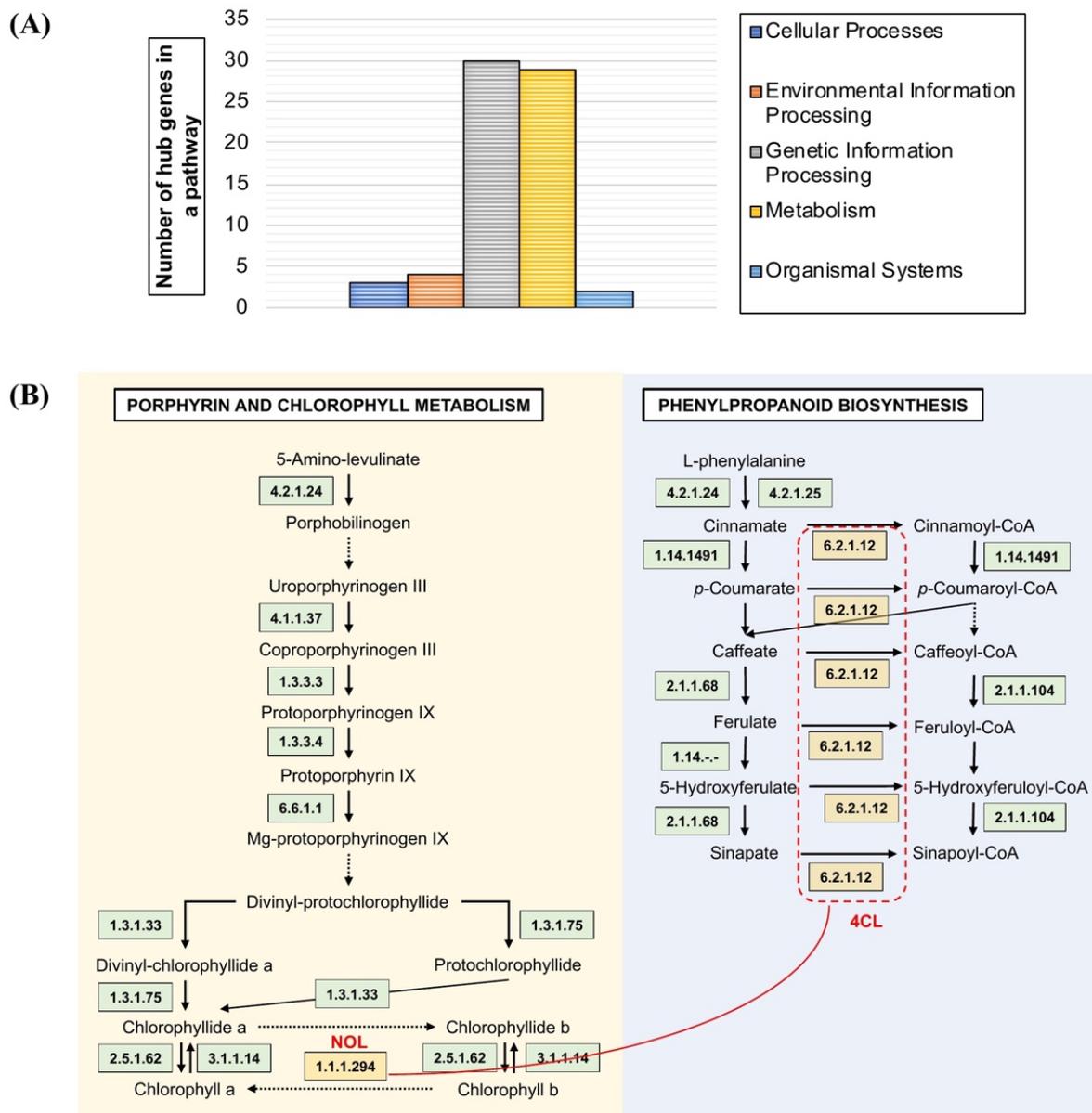


Figure 5. Pathway mapping analysis of *OsAbp57-OE*. **(A)** Classification of hub genes in the modular network into five main pathway maps: cellular processes, environmental information processing, genetic information processing, and organismal systems; **(B)** interaction between M7 hubs in the porphyrin and chlorophyll metabolism pathway, and phenylpropanoid biosynthesis maps are represented with red lines.

No correlation of hubs within other modules was identified between the pathway maps. Pathway analysis of several hubs under plant hormone signal transduction showed interaction with the MAPK signalling pathway map. Two hubs of *GH3* in M3 and M6, *GH3.6* and *GH3.11* under plant hormone signal transduction for plant growth, showed interplay to disease resistance *NPR5* and stress-tolerant response *CatC* under the MAPK signalling pathway map (Figure 6).

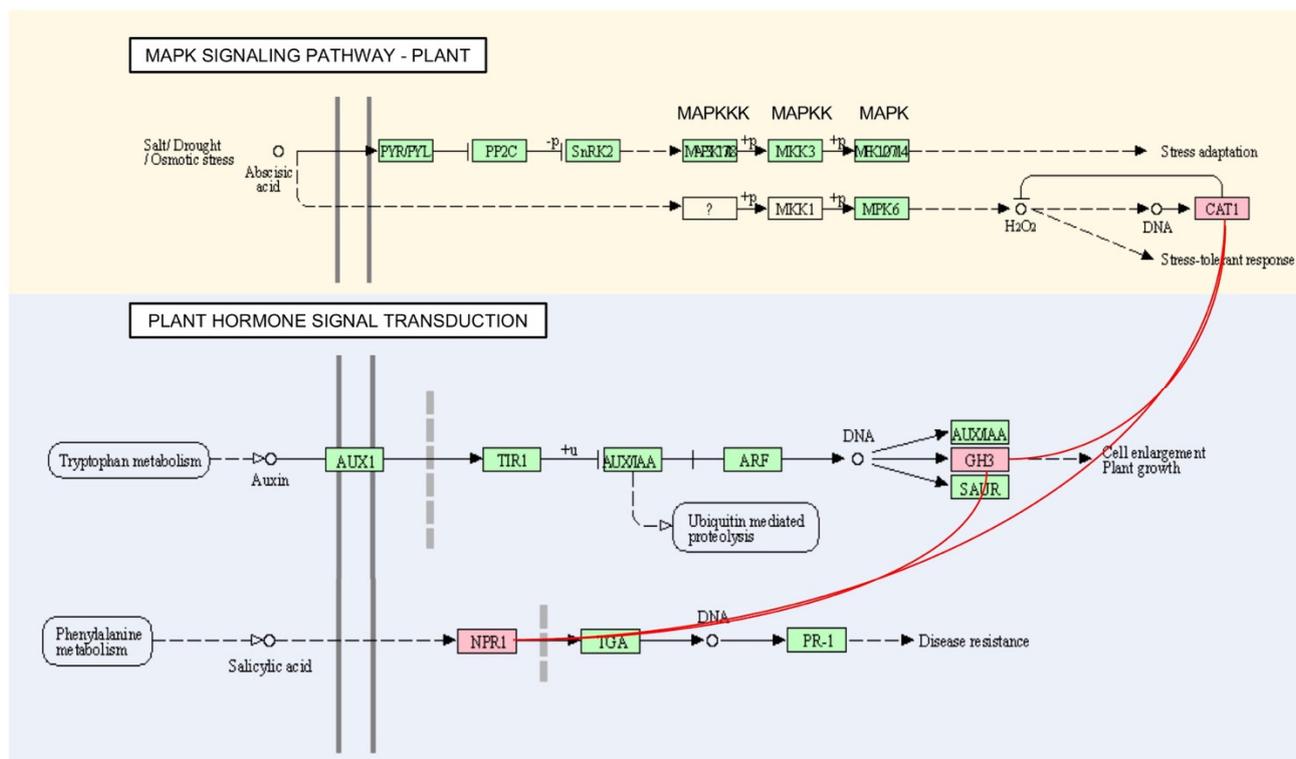


Figure 6. Interaction between hub genes in MAPK signalling pathway and plant hormone signal transduction maps. Interaction is represented in red lines.

4. Discussion

In the present study, we demonstrated that the overexpression of an auxin-binding protein, *OsAbp57*, can enhance the drought tolerance of a popular Malaysian rice cultivar, MR219. A comparative analysis of drought tolerance among two *OsAbp57* overexpressing lines and MR219 revealed a much delayed drought-induced leaf senescence in *OsAbp57* overexpressing lines compared to MR219. Numerous previous studies have reported that delayed drought-induced leaf senescence is one of the important traits that can enhance the tolerance of a plant toward drought stress [8,39,40]. This has been further supported by Liu et al. (2016) [41], who reported that transgenic rice with accelerated drought-induced senescence exhibited higher sensitivity to drought stress than its nontransgenic parental line.

Further examination of the physio-morphological traits also revealed that the performance of both *OsAbp57* overexpressing lines was superior to MR219 in most of the traits evaluated under both watering regimes. We noticed that the root dry weight of *OsAbp57* overexpressing exhibited the greatest responsiveness towards drought conditions. Compared to its counterparts grown under the normal condition, the root dry weight of *OsAbp57* overexpressing lines grown under drought conditions and the root weight of both *OsAbp57* overexpressing lines grown under drought conditions exhibited more than 47% of increments (OE1: 47%; OE2: 52%). Being the primary organ involved in plant water uptake and the first organ to perceive drought stress, the root traits have long been known as one of the determining factors in plant productivity under drought stress [42]. This is particularly true for the plant species employing drought avoidance mechanisms, such as rice. Previous studies on rice have also reported that the root mass and length are good predictors of rice yield under drought due to improved contact between the root and the shrinking soil water [43]. Therefore, higher responsiveness of the root weight of *OsAbp57* overexpressed in response to drought stress may be one of the factors contributing to the drought resistance of transgenic rice, which allows the transgenic plant to avoid a drastic reduction of plant water potential despite a shortage of soil moisture.

Although *OsAbp57* overexpressing lines exhibited a higher drought tolerance than MR219, we also observed that the overexpression of *OsAbp57* led to approximately a 23–48% reduction in the leaf photosynthetic rate compared to MR219. This implies that the *OsAbp57* overexpression may have a negative effect on the photosynthetic activities of rice. Suboptimal performance of transgenic rice under well-irrigated conditions has also been reported in many previous studies. One of the examples was the transgenic plant overexpressing *OsPYL/RCAR5* [44], whereby the overexpression had resulted in dwarf phenotype and yield reduction. In addition, the transgenic plant overexpressing *OsNAC6* produced shoot growth retardation under control conditions [45]. Therefore, employing drought-inducible promoters may avoid the adverse effects imposed by the overexpression of *OsAbp57* under well-irrigated conditions.

Our transcriptome data showed that *OsAbp57* regulated redox metabolism in rice. The overexpression of *OsAbp57* led to changes in redox activity and increased iron ion uptake in transgenic rice. Iron is essential for plant growth, but excess Fe^{2+} can generate ROS via the Fenton reaction. These results signified the involvement of ROS metabolism in plants. *OsAbp57* has been proven to bind to IAA and directly activate plasma membrane H^+ -ATPase. These findings agree with previous studies, whereby plant growth and development activities governed by auxin are closely associated with ROS. The application of auxin to cells rapidly induced ROS generation. Although ROS caused considerable cellular damage, these molecules are also important signalling molecules for stress tolerance. The possibility of utilising a gene involved in Fe^{2+} homeostasis in establishing drought-tolerant rice has been demonstrated by a recent journal. According to the author, the expression of the nicotianamine biosynthetic genes (*OsNAS1* and *OsNAS2*) was upregulated significantly in several drought-tolerant transgenic rice. Previous ChIP-seq analysis showed that *OsNAS1* and *OsNAS2* are the direct targets of a stress-responsive transcription factor called *OsNAC6*, which is necessary for drought response. The overexpression of *OsNAS1* and *OsNAS2* has also demonstrated the importance of the accumulation of NA for drought tolerance in rice [46].

The ROS in plants must be kept at a safe level to avoid cellular damage and death. Previous studies showed that drought stress would lead to a burst of different ROS in different cellular compartments, such as mitochondria, chloroplast, and peroxisome [47]. Increasing evidence indicates that enhanced production of ROS may play a role in plant stress signalling, which can facilitate plants to perceive stress levels in different organs. However, the unregulated production of ROS is detrimental to the cell. Therefore, an increase in ROS production must be accompanied by upregulation of the ROS scavenging system. Our microarray data shows that biosynthesis of flavonoids was upregulated in transgenic rice overexpressing *OsAbp57*. Flavonoid has solid antioxidative properties by reducing the production and quenching ROS, especially those derived from photosynthetic apparatus. This is done through several mechanisms, such as suppression of singlet oxygen, inhibiting enzymes that generate ROS, and chelate ions of transition metals that may catalyse ROS production.

Decreased plant height and increased branching are essential characteristics for rice crop improvement and are also part of a plant's acclimatisation strategy to diminish stress exposure (stress-induced morphogenic responses, SIMRs). The interactive network of auxin, ROS, and antioxidants has been proposed to form a redox signalling module that links plant development and environmental cues. According to Xia et al. (2020) [48], *OsWUS* plays a significant function in tiller development and weak apical dominance, and the loss of *OsWUS* function influenced rice plant morphology. Furthermore, auxin response is significantly enhanced due to the inhibition of auxin-associated gene *ASP1*, a physical interactor of *OsWUS* that suppressed the formation of tiller buds in *OsWUS* loss-of-function mutant, decreased culm number 1 (*dc1*).

Our results showed that overexpression of *OsAbp57* enhanced drought tolerance in transgenic rice. This might be due to the altered auxin homeostasis in the plant. Previous studies indicated that endogenous and exogenous auxin positively regulated ROS

metabolism and antioxidant activity. Likewise, transgenic potato overexpressing *AtYUC6* (member of the *YUCCA* family of flavin-containing monooxygenase) showed high auxin and enhanced drought-tolerant phenotype through a regulated ROS homeostasis [8]. Enhanced ROS production or ROS signalling is also associated with various abiotic stresses, such as drought, salt stress, oxidative stress, UV-radiation stress, and heavy metal stress. The disturbed ROS homeostasis during stress conditions could act as a signal to activate a stress response pathway, such as mitogen-activated protein kinase (MAPK) cascades. Moreover, our microarray analysis also showed downregulation of gibberellin biosynthesis activity. According to Zawaski and Busov (2014) [49], gibberellin (GA) catabolism and repressive signalling mediate shoot growth inhibition and physiological adaptation in response to drought. The transgenic plants with GA-deficiency or GA-insensitive displayed more excellent resistance to drought. In addition, GA-deficiency in response to Fe homeostasis could also lead to dwarfism of the shoot, where Fe-deficient causes foliar chlorosis and decreased leaf biomass. The concentrations of Fe and CHL, an indicator of Fe status, were higher in the leaves of dwarfed transgenic rice [50].

The modular co-expression network (mGCN) constructed based on our microarray data, showed strong upregulation of module activity in *OsAbp57*. Modules M2 and M3 refer to transmembrane transport, which significantly adjusts water scarcity by allocating various molecules through the root, stomata, and cuticle [51]. Although the transport of several metal ions, including K^+ , Na^+ , and Cl^- , has been reported to be critical in counteracting drought stress [51], the role of Fe^{3+} transport in rice is still not well understood. In fact, the presence of Fe causes the activation of reactive oxygen species (ROS) scavenging enzymes, such as catalase and peroxidase, which are known to regulate the expression of the stress-responsive gene to confer tolerance to environmental stress [52,53]. Also, GSEA revealed promising insights into the tolerance mechanisms of *OsAbp57* against drought. For instance, the activity of most of the modules, which are associated with oxidative stress, suggests an intense regulation of ROS and ROS-mediated signalling pathways during drought. Our mGCN study revealed the dynamics of genes involved in drought tolerance due to the possible increase of reactive oxygen species (ROS) levels when oxidative stress is induced during water depletion. This leads to redox homeostasis, thus activating redox-dependent signalling that could initiate the adaptive plant response. To escape such water scarcity, membrane transport will take place to transfer molecules, metal ions, and water in the context of root response to drought. However, further experiments are much needed to assess the importance of Fe^{3+} transport during a water shortage.

Integration of mGCN with protein–protein interaction (PPI) data revealed the putative involvement of *OsAbp57* hubs in drought stress tolerance. Interestingly, the origin hub that is unlikely present in the PPI data has shown to be upregulated under drought stress. Under drought conditions, Fe micronutrients play a key role in enhancing stress tolerance as it produces assimilates [52]. *HRZ2* functions to negatively regulate the response of Fe deficiency and activate JA signalling at the early stages of Fe sufficiency [54,55]. The *OsAbp57* tolerance of Fe-sufficient conditions may have incurred as manifest in module M3 by origin hub *HRZ2*, which was reported previously to increase the expression of genes involved in Fe uptake and translocation in the *HRZ*-knockdown shoots and roots [56]. The other predicted origin hub, *AAO2*, is specifically expressed in shoot and shows drastic changes in transcript accumulation under drought and salinity stress [57].

Meanwhile, *OAT*, controlled by a stress-responsive transcription factor, *SNAC2*, confers good tolerance to drought and osmotic stress through activation of ROS-scavenging enzymes and ABA-mediated pathways [58]. The regulation of origin hubs identifies new potential genes that may have functioned to increase drought tolerance in *OsAbp57*. The presence of *MDH* and *PK* further explains the possible occurrence of carbohydrate synthesis and glycolysis, respectively [59,60]. In contrast, *TIG*, a distant FKBP family, contains a targeting region that binds to ribosomes and helps to determine the subcellular localisation of mature protein during water deficit [61]. The presence of *PTR* infers the oligopeptide transport events [62] and explains possibilities for nutrient uptake and transport in rice,

such as nitrogen and arsenic [63,64]. In rice, *PRR1*, a component of the circadian clock, plays a vital role in regulating the photoperiod of flowering response. However, a recent study by Wei et al. (2021) [65] reported that *PRR73* positively regulated salt tolerance by co-joint with *HDAC10* to repress the transcription of Na^+ transporter *HKT2;1* in transgenic rice.

We noticed several important hubs physically interacting with each other based on the PPI data. From the interesting modules, we discussed the results based on the upregulated hub associated with drought stress and hub interaction between the pathway maps.

In module M2, *HSP70* functions in protein folding and preventing DNA degradation or fragmentation under stressed and unstressed conditions [66,67]. We found *HSP70* to be upregulated in *OsAbp57*, considering this gene confers drought stress tolerance by maintaining the protein structure or DNA of the plant cell. The interaction of *HSP70* with *MDH* and *PK*, which are involved in carbohydrate synthesis and glycolysis, may be regarded as a crucial trait for plant-life-sustaining activities. Drought causes changes in sucrose and amino acid content, which was revealed by an increased phosphoenolpyruvate carboxylase (PEPC) expression in starch and sucrose metabolism [68]. In poplar, the *PPR* gene may be involved in environmental adaptation as it was confirmed to respond to cold, salinity, and JA conditions [69] and under drought in *Arabidopsis* [70]. In M3, the interacted genes, *HRZ2* and *Os01g0655500*, are related in function. As *HRZ2* plays a dominant role in Fe uptake and translocation, *Os01g0655500*, a protein kinase-containing domain gene, is regulated in response to Fe deficiency and excess [71]. Among the *MDH* neighbours, *ACP* may suggest an essential role in type II fatty acid synthesis and mitochondrial protection against drought stress [72]. In mitochondrial protection, *ACP* has been identified as a transmitter for nutrient status concerning mitochondria biogenesis [73]. We found *ACPs*, including *ACP1* and *ACP3*, were upregulated in *OsAbp57*, which has previously been reported to improve salt tolerance through alterations of fatty acid composition and control the concentration of Na^+/K^+ [74]. In addition, by scavenging ROS at higher concentrations of Na^+ , another interesting hub, *CFR*, may enable the protection of chloroplasts from chlorophyll degradation and photodamage of photosystem II [75].

Of all interesting hubs, the flavonoid biosynthesis pathway seems to be functionally correlated to porphyrin and chlorophyll metabolism through the correlation between *4CL1* and *NOL* genes. The relationship between these two pathways is likely to co-exist during adverse environmental conditions that cause leaf colour to shift due to changes in pigment ratios. In a study by Shen et al. (2018) [76], the synergistic effect of flavonoid reduction and porphyrin and chlorophyll enhancement resulted in a change in the leaf colour of tea plants. Hub genes encoding proteins of the MAPK signalling pathway were also associated with plant hormone signal transduction maps. Upon water scarcity or drought conditions, the accumulation of ABA and H_2O_2 activates the MAPK signalling pathway, thereby promoting the catalase activity, which is responsible for maintaining the optimal level of H_2O_2 in plant cells [77]. Our findings found that *GH3* genes (i.e., *GH3.11* and *GH3.6*) were involved in plant growth and development via the IAA signal transduction pathway. However, a *GH3* family gene, *GH3-2*, modulated ET-IAA crosstalk to confer drought and cold tolerance in rice [78]. The interplay of the SA signal transduction pathway with the MAPK signalling pathway and IAA signal transduction pathway inferred the possible event of combined biotic and abiotic stresses. Abiotic stress weakens plant immunity and enhances plant susceptibility to pathogenic organisms [79].

5. Conclusions

This work provided new insight into the emergence of drought tolerance in *OsAbp57*. We presented a solid integration study that can analyse transcriptomics data to discover potential candidates for the drought-responsive gene via modular gene correlations. Integration of our present results and the literature search portrayed the hub gene function of mGCN involved in important biological processes associated with drought stress tolerance. We demonstrated possible hub genes underpinning potential drought tolerance by increased reactive oxygen species (ROS) level at the state of water depletion, therefore

causing the occurrence of metal ion transport, redox homeostasis, and activation of redox-dependent signalling that may trigger the adaptive response in rice. The hub genes also link the chlorophyll and flavonoid metabolism pathways and feature the interplay between the MAPK signalling pathway with IAA and SA signal transduction pathways. These concurrent events might have occurred in *OsAbp57* due to the effect of colour changes in plant cells, stimulated by *4CL* and *NOL* genes, and the possible occurrence of combined stresses activated by *GH3*, *CATC*, and *NPR* genes through the pathogenic susceptibility via weakened immunity during drought stress. The hub genes discovered in our mGCN are crucial in rice breeding strategies to enhance yield and produce drought-tolerant rice varieties. Some of the hub genes and pathways indicated in this study are established candidates associated with drought stress, confirming the functional validity of these findings. However, other poorly investigated genes could necessitate novel mechanisms in *OsAbp57*, worthwhile of further investigations in future research.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy12081959/s1>, Table S1: Primers used in this study for qPCR validation; Table S2: List of overrepresented biological processes in modules (M1–M12) determined by hypergeometric test; File S1: Detailed information on the hub genes of each module; File S2: Detailed information on modular genes mapped on KEGG pathways.

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