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Co-expression network analysis for identification of candidate genes regulating phosphorus use efficiency in wheat (*Triticum aestivum* L.)

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Abstract

Phosphorus (P) is a crucial nutrient for plants, but its deficiency can significantly reduce crop yields, especially in wheat. To understand the genetic basis of Phosphorus Use Efficiency (PUE) in wheat, we analyzed the gene expression patterns of plants under phosphorus stress. We identified and analyzed 1194 differentially expressed genes, constructing a network of these genes through cytoscape. We have extracted 26 hub genes from this network, which are key players in PUE. These hub genes are involved in various biological processes related to phosphorus uptake, transport and utilization, as revealed by KEGG pathway analysis. Our findings provide valuable insights into the genetic mechanisms underlying PUE in wheat and may contribute to the development of strategies for improving crop yields in phosphorus-deficient environments.

Keywords

phosphorus; wheat; phosphorus use efficiency; cytoscape; hub genes; KEGG

Introduction

Wheat (*Triticum aestivum* L.), the world's most cultivated cereal crop, belonging to the Poaceae family, widely accounts for more than 30 % of global cereal production and 50 % of global cereal trade (1). It is one of the world's most important staple crops and a widely cultivated grain in terms of both cultivated area and grain production. Pan wheat, also known as hexaploid wheat (2n = 6 = 42; AABBDD genomes), arose from the fusion of three diploid genomes that were spliced together and had repetitive sequences that accounted for more than 80 % of the entire genome (17 GB) (2). Wheat constitutes more than a third of the food crops for the world's population (3). Not long ago, wheat production in India (2022-23) was estimated at 112.18 million tonnes according to the fourth advance estimate of food production released by the Ministry of Agriculture and Farmers Welfare. In Uttar Pradesh, the total wheat production for the successive year 2021-2022 was esti-

mated at approximately 359 lakh metric tonnes (3). Wheat is a rich source of starch, protein, vitamins, phytochemicals, sugars, fats and fiber. Since wheat contains a fraction of gluten protein, it is used to make breads, pastries, pasta, noodles and other functional ingredients (4).

Minerals that are vital to growth include phosphorus (P), potassium (K), iron (Fe), calcium (Ca), zinc (Zn) and nitrogen (N). The ideal ratio of nutrients is provided to promote plants' growth and increase their maximum yield output. Plants require large amounts of phosphorus and nitrogen because they are essential nutrients and building blocks of basic biological molecules (5). Phosphorus (P) is a key substance that plays an important role in sugar metabolism, energy, photosynthesis and enzymatic reactions. Together with the nucleic acid component, it is also present in plant hormones and also increases crop yield and quality (5). Plants absorb phosphorus as phosphate, but due to its high reactivity with some metal ions, it naturally precipitates, resulting in low availability of phosphorus to crops (6). Assimilation of phosphorus by plant roots becomes difficult because organic material in the soil binds to phosphorus in the form of phytate (inositol compound) (7). Phosphorus diffuses slowly in soil and soil minerals show strong fixation. Therefore, phosphorus has low mobility and compared to other nutrients, its availability to plants is low (8, 9). Phosphorus is found in soil in both organic and inorganic forms. Phosphorus reacts with iron, aluminium and manganese oxides when the soil is acidic and when the soil is alkaline, it forms a compound with calcium carbonate. Plants experience adaptive changes in the distribution, topology and morphology of the roots due to phosphorus deficiency, as well as declines in plant growth and yield (10, 39, 40).

Crop plants enhance phosphorus efficiency by amplifying Phosphorus Use Efficiency (PUE). Phosphorus utilization of plant performance refers to the ability of the plant to use the received phosphorus to produce yield and biomass (11, 42). Phosphorus use efficiency (PUE) is the net result of phosphorus uptake efficiency (PUpE) and phosphorus utilization efficiency (PUtE). PUpE represents the ability of the root system to absorb inorganic Pi from the soil. The main mechanisms underlying PUpE improvement are elongation of the entire root system, slowing of primary root growth, increased root-to-shoot ratio and increased root lateral area, lower axial root growth and increased root thickness and root hair length (12). The ability of plants to convert absorbed P into yield is represented by PUtE. Therefore, it is important to ensure that plants use P from the soil as efficiently as possible, especially in systems with low P input (12). Improving the phosphorus use efficiency (PUE) is related to the following aspects: (i) Root system architecture, the increase of root density with the increase of lateral roots makes the roots able to consume more phosphorus from the upper soil layer (13), Phosphorus Starvation Tolerance1 (PSTOL1) genes present on chromosomes 1A, 1B, 2B, 2D, 3A, 3B, 3D, 5B, 5D, 6A and 6D have been reported for early root growth, effective phosphorus uptake, deeper roots and high yield (3). (ii) increasing secretion of enzymes, protons and organic anions from roots to facilitate phosphorus dissolution in soil (13), for acid phosphatases, mainly purple phosphatase functions to facilitate the hydrolysis of phosphoric-monoester complexes to provide an alcohol and a free inorganic phosphate (14); (iii) Microorganism interactions that increase soil volume and increase soil phosphorus availability (15). Expression of mycorrhizal specific phosphate transporters (mainly Phosphate Transporter 1 (TaPHT1)) in wheat is enhanced during phosphorus stress and improves phosphate (Pi) use efficiency by increasing Pi acquisition (16). Besides, the Phosphate1 (PHO1) genes on chromosomes 1A, 1B and 1D are responsible for responses in Pi stress and they improve wheat yield with less phosphorus fertilizer (16). Phosphate transporters (PHT2, PHT3 and PHT4) genes detected in the roots are responsible for phosphate uptake under phosphate deprivation conditions. In the wheat genome, these genes were located on chromosomes 2A, 4A, 4B, 4D, 5B and 5D (17).

Interoperable and complex networks connecting common DEGs can be constructed *in silico* using omics data and mathematical principles (18, 19). These biological entities can interact with each other physically, genetically, functionally, or protein-protein, each performing a specific analytical function (18, 41, 43). The idea behind these biological networks is that genes with similar expression profiles are more likely to interact with each other (20). Since these genes have important effects on the formation of the entire biological network, it is necessary to infer the master regulators of such networks. Through mathematical iterations, this can be narrowed down to our target genes using several topological considerations (21). Attributes of gene centrality can be inferred from parameters such as degree, closeness and proportion. The term "hub genes" refers to genes with the highest centrality (22). A complex biological network is a collection of multiple subnetworks or modules and it is entirely conceivable that multiple hubs exist within and between these subnetworks (23).

Materials and Methods

The complete workflow consisted of the identification of differentially expressed genes for phosphorous stress tolerance in wheat (*Triticum aestivum* L.).

Identification of differentially expressed genes for phosphorus stress tolerance

Data resources and mining from published data

Gene Id's of genes under phosphorus stress were collected from various scientific journals (24, 25). A total of 7009 differentially expressed genes were collected, which have been further separated on the basis of common gene id's to remove redundant genes. A dataset of 7009 differentially expressed genes (DEGs) with log fold change values was collected from various research journals. These DEGs were compared with another dataset containing 20000 DEGs. By cross-referencing these 2 datasets, we identified 1194 DEGs that were common to both and are likely involved in phosphorus use efficiency. Thus, a total of 1194 differen-

tially expressed genes were obtained and utilized for network analysis (26, 27, 44). These specific genes were identified in the wheat (*Triticum aestivum* L.) root tissues and exhibited unique patterns of activity in response to a phosphorus deficiency.

Network construction for phosphorus stress responsive genes

Using the STRING database, a stress network was constructed of 1194 differentially expressed genes obtained from the transcriptome dataset (28). The network that emerged consisted of 277 nodes (which symbolize genes) linked by 473 edges connecting these nodes. Nodes (or genes) not connected to this network were eliminated, refining it to include only nodes that demonstrated connections. This refined network was exported to Cytoscape for further analysis (29).

Identification of modules and hub genes from the constructed network

In Cytoscape, disconnected nodes were hidden from the network. The network comprises 277 nodes (representing genes) and 473 edges, a confidence level of 0.7 was set for the network. Following, MCODE Cluster (plug in cytoscape) was conducted for the network at degree cut-off values 2, 5, 7 and 9. Significant numbers of clusters obtained were 10, 11, 10 and 8 at threshold values 2, 5, 7 and 9 respectively. Clusters with a substantial number of nodes and edges connections were picked from the number of clusters obtained from different threshold values. Following specific criteria, 6, 1, 2 and 2 clusters were chosen at threshold values 2, 5, 7 and 9 respectively. These resulting clusters were analyzed to identify hub genes, focusing on their highest connectivity (nodes with high edges connectivity >3 edges).

Identification of degree and betweenness centrality of hub genes

An integrated application, Cytohubba of Cytoscape was utilized to determine the degree and betweenness of the top 10 differentially expressed genes from the whole network. The degree from the network was calculated only for the top 10 genes. To know the betweenness of the genes from the network, betweenness of the top 10 genes was calculated using Cytohubba.

Identification of degree and eccentricity of hub genes

An integrated application, Cytohubba of Cytoscape was utilized to determine the degree and eccentricity of the top 10 differentially expressed genes from the whole network. The degree from the network was calculated only for the top 10 genes. To know the eccentricity of the genes from the network, eccentricity of the top 10 genes was calculated using Cytohubba.

Recognition of the top 10 bottleneck genes and their involvement in KEGG pathway

Cytohubba was utilized to find out the topmost 10 bottleneck genes from the network of phosphorus use efficiency genes, which includes 277 genes as nodes and 473 edges. Bottleneck from Cytohubba was applied on the network to find the top 10 bottleneck genes. Further, the bottleneck genes were analyzed to know their involvement in KEGG pathways by applying string enrichment on the network.

Analysis of hub genes for the association to KEGG pathways and gene ontologies terms

The hub genes retrieved were further screened out, aiming to determine their association with KEGG pathways and gene ontology terms (molecular function, cellular component and biological process). Functional annotation data enriched was acquired in Excel format by retrieving it from the string functional annotation section within Cytoscape. After conducting this analysis, the resulting data was collected in a tabular format to explore hub genes association with specific GO terms and KEGG pathways. From the tabular format, relevant KEGG pathways and GO terms associated with hub genes were chosen and structured into a distinct tabular format.

Identification of hub genes linked to phosphorus use efficiency

Retrieved hub genes were further analyzed to recognize their role and function in phosphorus use efficiency and how they help crop plants (wheat) to cope against phosphorus stress and increase their efficiency and yield.

SRPlot software utilization for setting up different plots and graphs through integrated SR Plotting modules

The SR Plot software, an accessible online tool designed for scientific plotting (available at https://www. bioinformatics.com.cn/aboutus_en), was employed to produce diverse plot types. This included bar plots to visualize Gene Ontology terms, GO Chord diagrams, bubble plots illustrating enriched KEGG pathways and category plots for pathway enrichment in KEGG. The essential parameters for crafting these plots were sourced from the enriched terms provided by the STRING database.

Gene expression analysis of hub genes

Analysis of hub-gene expression was carried out through the Wheat Expression Browser (expVIP), accessible at http://www.wheat-expression.com/. This platform is a valuable resource for gathering and examining hub-gene expression data. The data present in the platform was stemmed from the IWGSC2.26 gene models and offers comprehensive insights into gene expression, encompassing details like timing, location (tissue or organ) and expression levels for all identified hub genes. The expression browser provides visual representations of the expressed genes, consolidating data from 32 RNA-seq studies and detailing expressions in organs, tissues and developmental phases. Genes were chosen based on the criterion of having the highest log count value.

Results

Identification of clusters from parent network

Network of 1194 differentially expressed genes was constructed in cytoscape. Network consists of 277 nodes and 473 edges connectivity (Fig. 1). A total of 39 modules/ clusters were retrieved from the parent network through the MCODE Cluster. Out of 39 clusters, only 11 clusters

were considered as they are populated with high nodes and edge connectivity (Fig. 2). The most populated cluster among these 11 clusters contains 14 nodes (genes) and 24 edges, while the least populated cluster comprises 5 nodes (genes) and 6 edges.

The hub genes retrieval from clusters

A total of 26 hub genes were identified across 11 clusters/ modules (Fig. 3, Table 1). Cluster 1 (score 4.5) contributed 3 hub genes with a maximum edge count of 4. Cluster 2 (score 4.5) provided 3 hub genes with each node having an edge connectivity of 4. In Cluster 3 (score 4.25), 3 hub genes were found, exhibiting a range of edges from 4 to 7. Cluster 4 (score 3.33) contributed 2 hub-genes, each with an edge connectivity of 5. Cluster 5 yielded 2 hub genes with an edge connectivity of 3. Cluster 6 (score 2.8) contributed 1 hub gene with an edge connectivity of 4. In Cluster 7 (score 3.5), 3 hub genes were identified with an edge connectivity of 3 for each node. Cluster 8 (score 3.6) provided 3 hub genes, with edge connectivity ranging from 4 to 6. Cluster 9 (score 3.5) contributed 4 hub genes with an edge connectivity of 3. From Cluster 10 (score 3.69), 4 hub genes were identified with edge connectivity between 4 and 6. Lastly, Cluster 11 (score 2.83) contained 6 hub

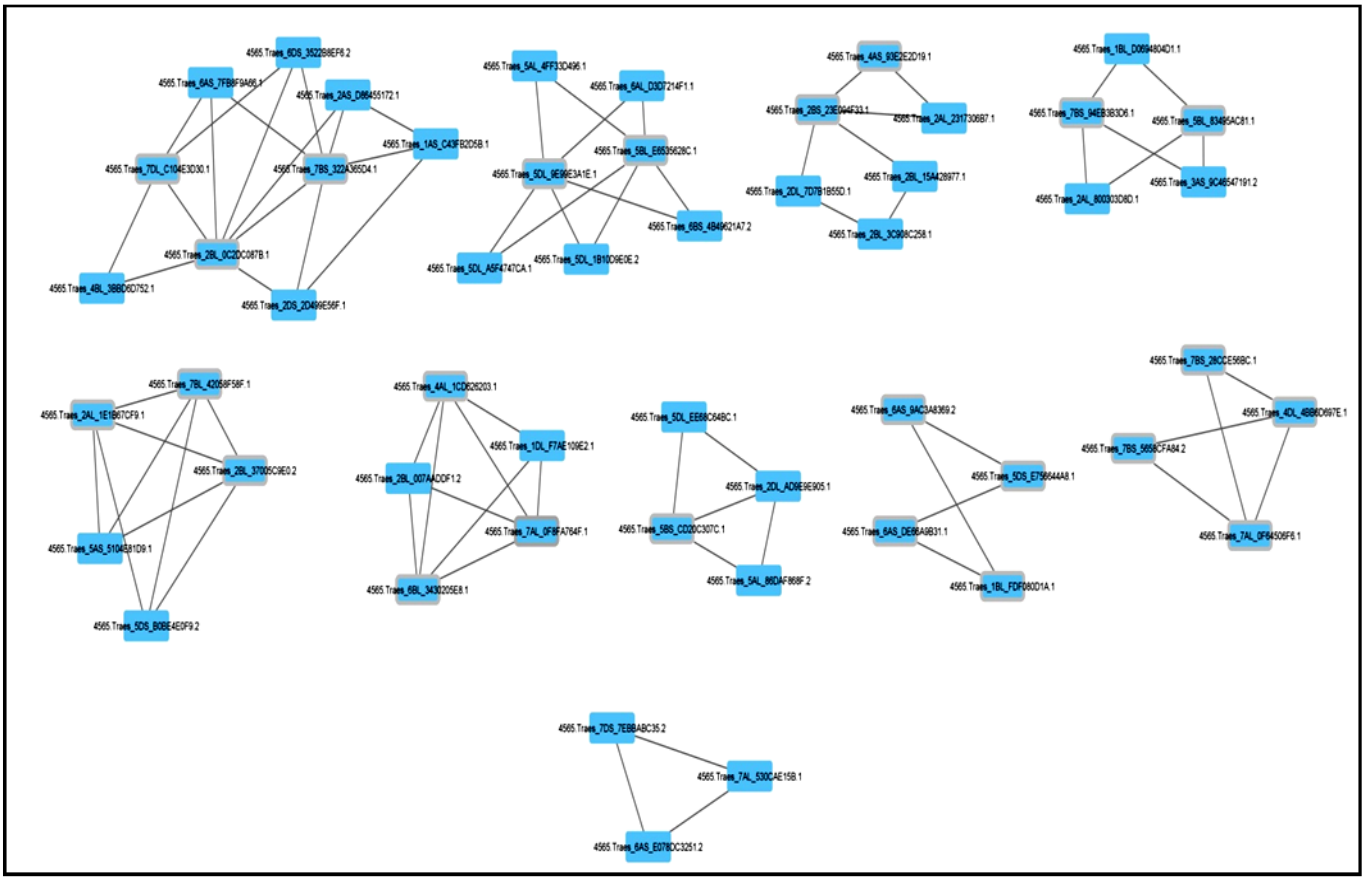
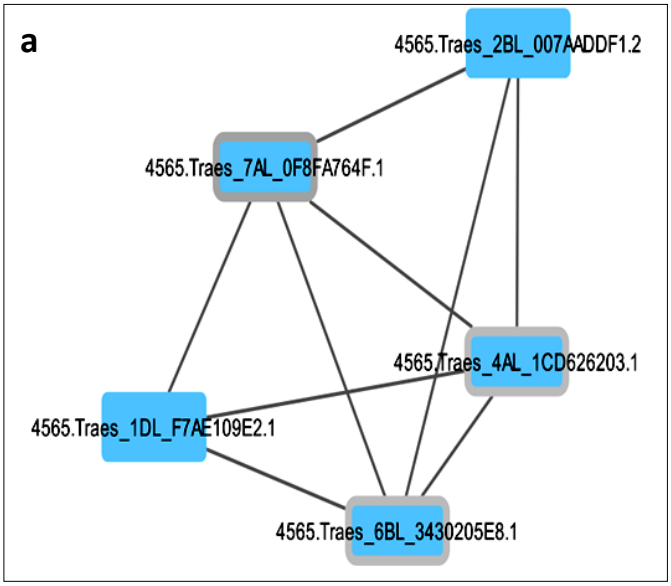
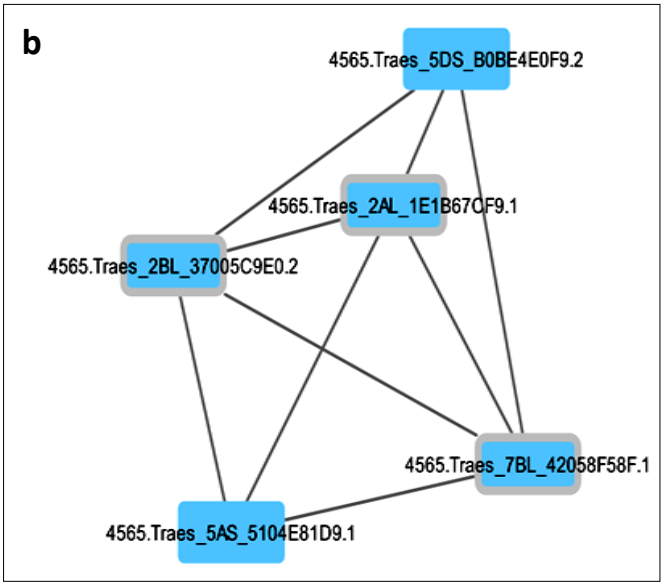


Fig. 1. Cytoscape network for phosphorus stress differentially expressed genes. Network consists of 277 nodes connected with 473 edges.





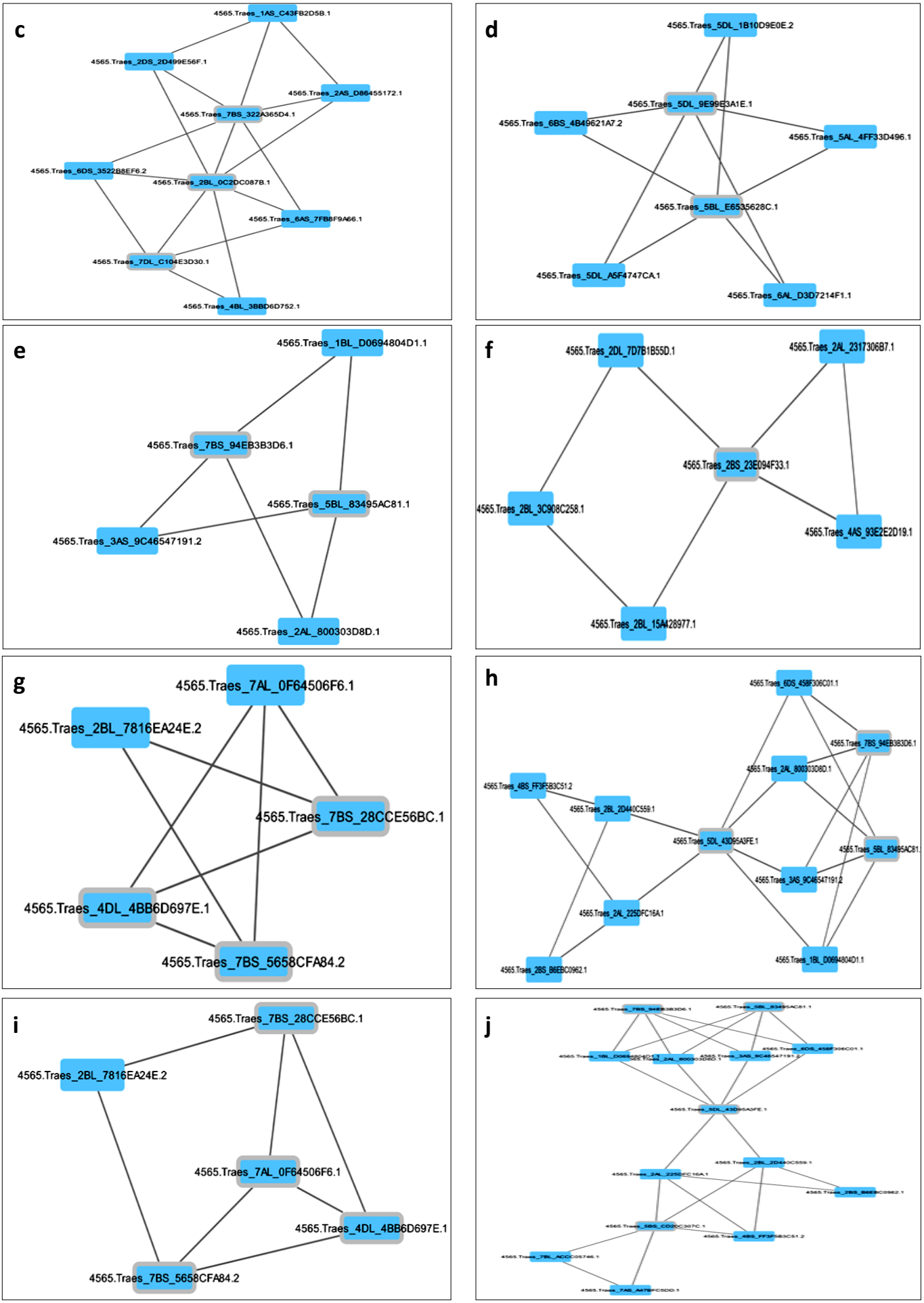


Fig. 2 (a - j). Top 10 modules/clusters obtained through MCODE cluster from Cytoscape network.

genes, each having an edge connection of 3. The identified

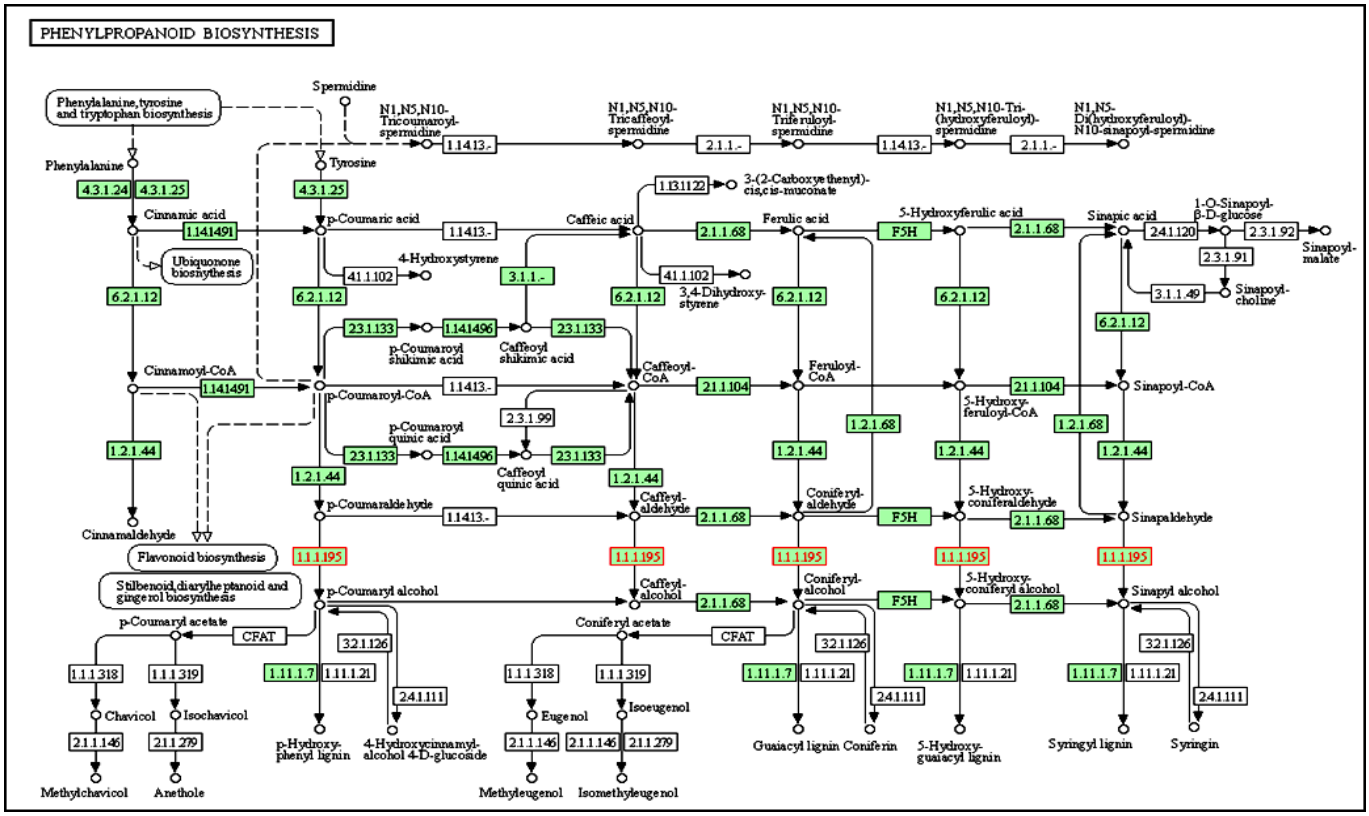


Fig. 3. 10 clusters obtained from MCODE cluster, highlighted nodes (grey border) indicated as hub genes.

Table 1. List of hub genes of phosphorus use efficiency with their description, accession number and locus ID.

Sl. No.	Gene ID	Description	Accession no.	Locus ID
1	Traes_1BL_FDF080D1A.1	Cinnamyl-alcohol dehydrogenase, FAD-binding Berberine family protein	A0A3B5Y7F7	
2	Traes_2AL_1E1B67CF9.1	Cytochrome P450 99A2-like	A0A3B6B3H6	LOC123185949
3	Traes_2BL_0C2DC087B.1	Momilactone A synthase-like	A0A3B6C6X5	LOC123041362
4	Traes_2BL_37005C9E0.2	Flavonoid 3',5'-hydroxylase 2-like	A0A3B6CEI4	LOC123047038
5	Traes_2BS_23E094F33.1	Ribulose bisphosphate carboxylase small chain	A0A1D5U6W5	
6	Traes_4AL_1CD626203.1	Glutamine synthetase	A0A1D5WYB9	
7	Traes_4AS_93E2E2D19.1	Protein SPA, chloroplastic-like	A0A3B6HSV6	LOC123085288
8	Traes_4DL_4BB6D697E.1	Uncharacterized protein; Belongs to the major facilitator superfamily. Sugar transporter family		
9	Traes_5BL_83495AC81.1	Uncharacterized protein; Belongs to the CRISP family		
10	Traes_5DL_43D95A3FE.1	Pathogenesis-related protein 1, PR-1.1; Pr-1-3	A0A3B6KF02	LOC543437
11	Traes_5DL_9E99E3A1E.1	CEN-like protein 2, TFL1-D	W5FWM0	LOC123124558
12	Traes_5BL_E6535628C.1	TFL1-A	W5EXP9	
13	Traes_5BS_CD20C307C.1	probable glucan 1,3-beta-glucosidase A	A0A3B6LH00	LOC123110481
14	Traes_5DS_E756644A8.1	Uncharacterized protein		
15	Traes_6AS_9AC3A8369.2	Uncharacterized protein		
16	Traes_6AS_DE66A9B31.1	Thiosulfate sulfur transferase 16, chloroplastic-like	A0A3B6NH51	LOC123131572
17	Traes_6BL_3430205E8.1	Uncharacterized protein		
18	Traes_7AL_0F8FA764F.1	Tryptophan decarboxylase 1-like	A0A3B6RJ22	LOC123152137
19	Traes_7AL_0F64506F6.1	Uncharacterized protein		
20	Traes_7BS_322A365D4.1	Uncharacterized protein		
21	Traes_7BS_28CCE56BC.1	Uncharacterized protein		
22	Traes_7BS_5658CFA84.2	ABC transporter C family member 10	A0A3B6SD72	
23	Traes_7BS_94EB3B3D6.1	Uncharacterized protein; Belongs to the CRISP family		
24	Traes_7BL_42058F58F.1	Obtusifoliol 14-alpha demethylase-like	A0A3B6SM77	LOC123160214
25	Traes_7DL_21A260D0C.1	RNA polymerase sigma factor sig A	A0A3B6TK54	LOC542980
26	Traes_7DL_C104E3D30.1	Polyamine oxidase 7-like	A0A3B6TV37	LOC123168039

hub-genes retrieved from the network are as follows: Traes_1BL_FDF080D1A.1, Traes_2AL_1E1B67CF9.1,

Traes_2BL_0C2DC087B.1, Traes_2BL_37005C9E0.2, Traes_2BS_23E094F33.1, Traes_4AL_1CD626203.1, Traes_4AS_93E2E2D19.1, Traes_4DL_4BB6D697E.1, Traes_5BL_83495AC81.1, Traes_5DL_43D95A3FE.1, Traes_5DL_9E99E3A1E.1, Traes_5BL_E6535628C.1, Traes_5BS_CD20C307C.1, Traes_5DS_E756644A8.1, Traes_6AS_9AC3A8369.2, Traes_6AS_DE66A9B31.1, Traes_6BL_3430205E8.1, Traes_7AL_0F8FA764F.1, Traes_7AL_0F64506F6.1, Traes_7BS_322A365D4.1, Traes_7BS_28CCE56BC.1, Traes_7BS_5658CFA84.2, Traes_7BS_94EB3B3D6.1, Traes_7BL_42058F58F.1, Traes_7DL_21A260D0C.1, Traes_7DL_C104E3D30.1.

Genes on the basis of degree and betweenness from the network

A degree and betweenness of top 10 genes from the network containing 277 genes as nodes and 473 edges was calculated using Cytohubba. Degree score of the top 10 genes was ranges from 28 to 17 (Table 2, Fig. 4). The highest degree score was 28 for gene id Traes_6DS_3522B8EF6.2 and the lowest degree score was 17 for gene id Traes_2DS_11ADF414D.1. Two hub genes were also included in the range of degree scores. These hub genes were Traes_1DL_F7AE109E2.1 with a degree score of 24 and Traes_7AL_0F8FA764F.1 with a degree score of 18. A degree score indicates that the number of connections of a particular node with other nodes in the network.

Table 2. Top 10 genes from network on the basis of degree. Highlighted gene ids are of hub genes.

Sl. No.	Gene ID	Gene rank	Score
1	Traes_6DS_3522B8EF6.2	1	28
2	Traes_6AS_7FB8F9A66.1	1	28
3	**Traes_1DL_F7AE109E2.1**	3	24
4	Traes_3DL_3E215D878.2	3	24
5	Traes_2AS_D86455172.1	5	20
6	Traes_2DS_2D499E56F.1	5	20
7	Traes_4BS_78741E0A2.1	7	19
8	Traes_7AL_530CAE15B.1	7	19
9	**Traes_7AL_0F8FA764F.1**	9	18
10	Traes_2DS_11ADF414D.1	10	17

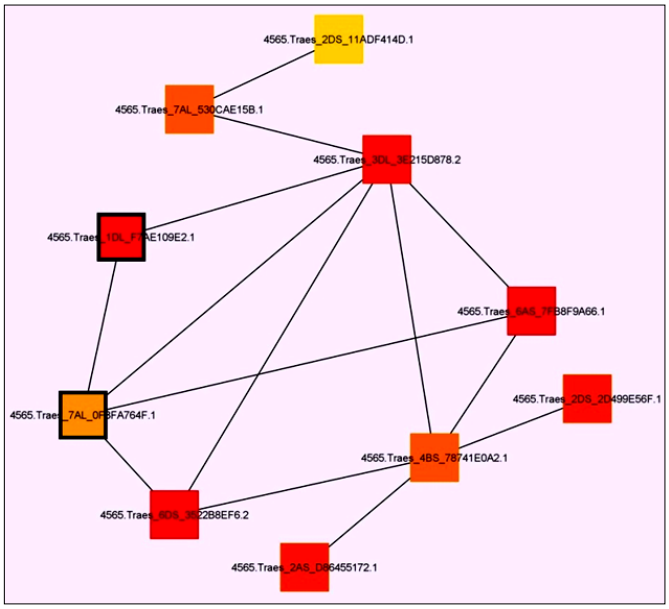


Fig. 4. Top 10 genes network on the basis of degree. Highlighted ones are hub genes in the network. Colour of nodes indicates their ranks. Dark orange- Rank 1 and Yellow- Rank 10.

For betweenness the score ranges between 6264.34 to 1727.36 (Table 3, Fig. 5). Gene Traes_3DL_3E215D878.2 has the highest betweenness score of 6264.34 and the lowest betweenness score of 1727.36 for gene Traes_4BS_78741E0A2. Two hub genes were also included in the range of betweenness score. These hub genes were Traes_7AL_0F8FA764F.1 with a betweenness score of 1849.37 and Traes_1DL_F7AE109E2.1 with a betweenness score of 1779.40. The betweenness score of any nodes (genes) indicates the shortest path of a particular node between all pairs of nodes in a network.

Genes on the basis of degree and eccentricity from the network

Table 3. Top 10 genes regarding eccentricity. Highlighted gene ids are of hub genes.

Sl. No.	Gene ID	Rank	Score
1	Traes_7BS_B2B2B5D1A.1	1	0.115523
2	Traes_2BL_007AADDF1.2	1	0.115523
3	**Traes_4AL_1CD626203.1**	1	0.115523
4	Traes_6AS_7FB8F9A66.1	1	0.115523
5	Traes_3DL_3E215D878.2	1	0.115523
6	Traes_6DS_3522B8EF6.2	1	0.115523
7	**Traes_5BL_E16C03227.1**	7	0.09627
8	Traes_5DS_E756644A8.1	7	0.09627
9	Traes_4BS_39E23C67A.1	7	0.09627
10	Traes_2BL_3C908C258.1	7	0.09627

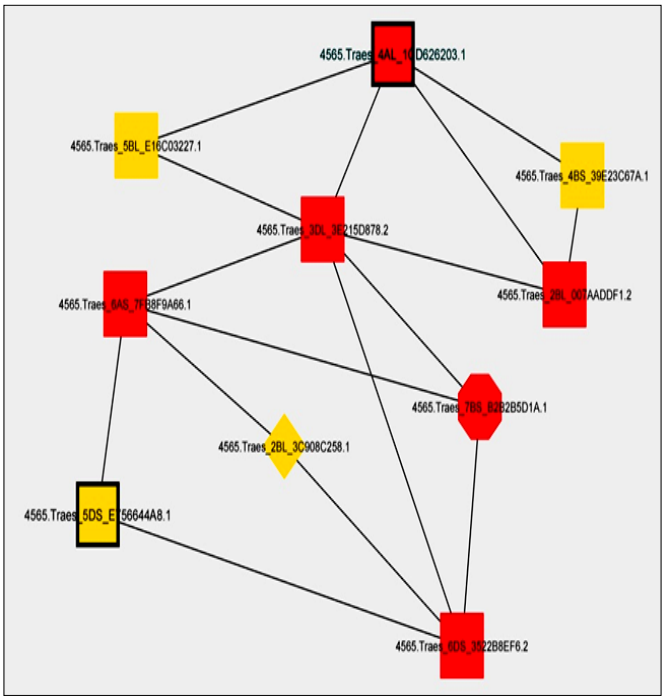


Fig. 5. Top 10 genes in network related to eccentricity. Highlighted background nodes are hub genes. Colour of nodes indicates their ranks. Red- Rank 1 and Yellow- Rank 7. Hexagonal shape node high rank 1 and diamond shaped node has rank 7.

A degree and betweenness of the top 10 genes from the network containing 277 genes as nodes and 473 edges was calculated using Cytohubba. Degree score of the top 10 genes was ranged from 28 to 17 (Table 2, Fig. 4). The highest degree score was 28 for gene id Traes_6DS_3522B8EF6.2 and the lowest degree score was 17 for gene id Traes_2DS_11ADF414D.1. Two hub genes were also included in the range of degree scores. These hub genes were Traes_1DL_F7AE109E2.1 with degree score of 24 and Traes_7AL_0F8FA764F.1 with degree score 18. A degree score indicates the number of connections of a particular node with other nodes in the network.

For eccentricity, the score ranges between 0.1155 to 0.09627(Table 3, Fig. 5). Gene Traes_7BS_B2B2B5D1A.1 has the highest eccentricity score of 0.1155 and the lowest eccentricity score of 0.09627 for gene Traes_2BL_3C90-8C258.1. Two hub genes were also included in the range of eccentricity scores. These hub genes were Traes_4AL_1CD-626203.1 with an eccentricity score 0.1155 and rank 1 and Traes_5DS_E756644A8.1 with an eccentricity score of 0.09627 and rank 7. The eccentricity score of any nodes (genes) indicates the maximum distance to the other nodes of that node.

Top 10 bottleneck genes and their involvement in KEGG pathways

A total of 10 bottleneck genes were extracted from the network of genes in the cytoscape. Top 10 bottle neck genes were Traes_3DL_3E215D878.2, Traes_6DS_3522B8EF6.2, Traes_2DS_2D499E56F.1, Traes_2BL_007AADDF1.2, Traes_5BL_E6535628C.1, Traes_7BS_B2B2B5D1A.1, Traes_1DL_F7AE109E2.1, Traes_7DS_7EBBABC35.2, Traes_7AL_530CAE15B.1 and Traes_5DL_D974E5AD1.1 (Table 4, Fig. 6). The ranking score of the bottleneck genes started from 48 and ends at 9. Highest ranking score of bottleneck gene Traes_3DL_3E215D878.2 was 48 and lowest ranking score of bottleneck gene Traes_5DL_D-974E5AD1.1 was 9.

Table 4. Top 10 Bottle neck genes with their score and description. Highlighted gene ids are of hub genes.

Sl. No.	Gene ID	Gene rank	Score
1	Traes_3DL_3E215D878.2	1	48
2	Traes_6DS_3522B8EF6.2	2	42
3	Traes_2DS_2D499E56F.1	3	20
4	Traes_2BL_007AADDF1.2	4	18
5	**Traes_5BL_E6535628C.1**	4	18
6	Traes_7BS_B2B2B5D1A.1	6	11
7	**Traes_1DL_F7AE109E2.1**	6	11
8	Traes_7DS_7EBBABC35.2	6	11
9	Traes_7AL_530CAE15B.1	6	11
10	Traes_5DL_D974E5AD1.1	10	9

Two hub genes were also involved in bottleneck genes. The genes were Traes_5BL_E6535628C.1 and Traes_1DL_F7AE109E2.1. Scores of bottleneck genes, also

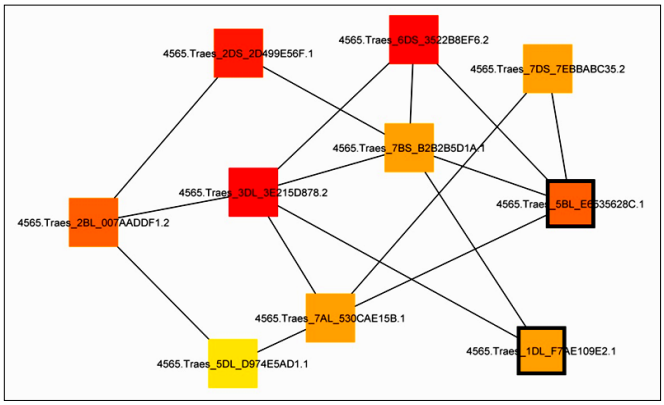


Fig. 6. Top 10 bottle neck genes. Highlighted ones are hub genes involved in bottle neck. Colour of nodes indicates their ranks. Dark orange- Rank 1 and Yellow- Rank 10.

hub genes were 18 for Traes_5BL_E6535628C.1 and 11 for Traes_1DL_F7AE109E2.1.

Analysis of the involvement of bottleneck genes in KEGG pathways showed that only 7 bottleneck genes were involved in KEGG pathways, such as phenylpropenoid biosynthesis, biosynthesis of secondary metabolites, phenylalanine metabolism, metabolic pathways, MAPK signalling pathway and circadian rhythm (Fig. 7). 3 bottleneck genes involved in 3 KEGG pathways, 2 bottleneck genes in 2 KEGG pathways and 2 bottleneck genes in 1 KEGG pathway.

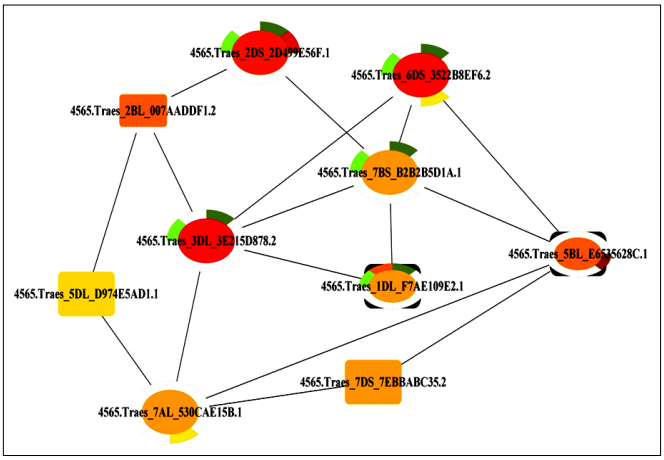


Fig. 7. Bottle neck genes with involvement in KEGG pathways. Colour background indicates different KEGG pathways. Red – Phenylpropenoid biosynthesis, Dark green- Biosynthesis of secondary metabolites, Brown- Circadian rhythm, Yellow- MAPK signaling pathway, Orange- Phenyl Alanine metabolism, Magenta- Diterpenoid biosynthesis, Purple- Starch and sucrose metabolism and Parrot green- Metabolic pathway. Colour of nodes indicates their ranks. Dark orange- Rank 1 and Yellow- Rank 10.

KEGG pathways and gene ontology terms correlated to hub genes

The hub genes were linked with diverse terms across all 3 gene ontology categories: molecular function, cellular component and biological process. In the molecular function category, hub genes were correlated with a total of 6 gene ontology terms: heme binding (GO: 0020037), metal ion binding (GO: 0046872), oxidoreductase activity (GO: 0016491), lyase activity (GO: 0016829), catalytic activity (GO: 0003824) and ion binding (GO: 0043167). The distribution among these terms was 3, 6, 9, 2, 17 and 15 hub genes associated with heme binding, metal ion binding, oxidore-

ductase activity, lyase activity, catalytic activity and ion binding respectively.

In the cellular component category, hub genes were associated with a total of 5 gene ontology terms: cellular anatomical entity (GO: 0110165), extracellular region (GO: 0005576), apoplast (GO: 0048046), cell wall (GO: 005618) and plant type cell wall (GO: 0009505). All 26 hub genes were linked with the cellular anatomical entity, while 6 hub genes were related to the extracellular region. Additionally, 1 hub gene for each was connected to the apoplast, plant-type cell wall and cell wall. Regarding the biological process, hub genes were associated with 22 gene ontology terms.

Regarding KEGG pathway enrichment, a total of 8 KEGG pathways were identified to which hub genes are linked (Table 5). These pathways include: phenylpropanoid biosynthesis (taes00940), biosynthesis of secondary metabolites (taes01110), circadian rhythm plant (taes04712), MAPK signaling pathway (taes04016), phenylalanine metabolism (taes00360), diterpenoid biosynthesis (taes00904), starch and sucrose metabolism (taes00500) and metabolic pathway (taes01100). Specifically, the gene Traes_1BL_FDF080D1A.1 is associated with phenylpropanoid biosynthesis (Fig. 8a). Additionally, 6 hub genes are involved in various aspects of biosynthesis of secondary metabolites, encompassing flavanone biosynthesis, flavonoid biosynthesis, monolignol biosynthesis and mugineic acid biosynthesis (Fig. 8b). Traes_5BL_E6535628C.1 and Traes_5DL_9E99E3A1E.1 hub genes are associated with Circadian rhythm (Fig. 8c (i and ii). Furthermore, Traes_5BL_83495AC81.1 and Traes_7BS_94EB3B3D6.1 play roles in the MAPK signaling pathway (Fig. 8d). Traes_7AL_0F8FA764F.1, Traes_2AL_1E1B67CF9.1 and Traes_5BS_CD20C307C.1 hub genes are linked with phenylalanine metabolism, diterpenoid biosynthesis and starch and sucrose metabolism respectively (Fig. 8e, f and g). Additionally, metabolic pathway involves 10 hub genes. Modules were labelled with different colours for each KEGG pathway that indicated the following nodes (genes) involved in the respective KEGG pathways (Fig. 9).

Directly linked hub genes to phosphorus use efficiency

Further investigation into the 26 hub genes revealed that among them, only 10 hub genes were directly involved in enhancing phosphorus use efficiency in wheat crops (Table 6). For instance, the gene Traes_1BL_FDF080D1A.1, known for FAD binding Berberine, although its function in wheat is unclear, is an ortholog to *Arabidopsis thaliana*, aiding root growth and enabling plants to adapt to phosphorus deficiency (30). Traes_2BL_37005C9E0.2, identified in wheat, plays a role in increasing root length and stimulating mycorrhizal spore germination (31). Traes_2BL_OC2DC087B.1, a momilactone A synthase gene similar to rice (*Orzya sativa*), contributes to phosphorus uptake and utilization. Traes_4AS_93E2E2D19.1, an ortho-log of *Arabidopsis thaliana* (At1G75690), aids in phosphorus uptake and allocation to different plant parts.

Furthermore, Traes_6AS_DE66A9B31.1, akin to *Arabidopsis thaliana* (At2G17850), responds to phosphorus deficiency and aids in mobilizing organic phosphate to plants (32). Traes_7DL_C104E3D30.1 (polyamine oxidase)

Table 5. The hub genes (PUE) associated with KEGG pathway enrichment.

Sl. No.	Gene ID	KEGG ID	KEGG pathway
1	Traes_1BL_FDF080D1A.1	taes00940	Phenyl propenoid biosynthesis
		taes01110	Biosynthesis of secondary metabolites
		taes01100	Metabolic pathway
2	Traes_2AL_1E1B67CF9.1	Taes01110	Biosynthesis of secondary metabolites
		taes00904	Diterpenoid biosynthesis
3	Traes_2BL_37005C9E0.2	taes01110	Biosynthesis of secondary metabolites
4	Traes_4AL_1CD626203.1	taes01100	Metabolic pathway
5	Traes_5BL_E6535628C.1	taes04712	Circadian rhythm
6	Traes_5BL_83495AC81.1	taes04016	MAPK signaling pathway
7	Traes_5BS_CD20C307C.1	taes00500	Starch and sucrose metabolism
		taes01100	Metabolic pathway
8	Traes_5DL_9E99E3A1E.1	taes04712	Circadian rhythm
9	Traes_5DS_E756644A8.1	taes01100	Metabolic pathway
10	Traes_6BL_3430205E8.1	taes01110	Biosynthesis of secondary metabolites
		taes01100	Metabolic pathway
11	Traes_7AL_0F8FA764F.1	taes01110	Biosynthesis of secondary metabolites
		taes00360	Phenylalanine metabolism
		taes01100	Metabolic pathway
12	Traes_7BL_42058F58F.1	taes01110	Biosynthesis of secondary metabolites
		taes01100	Metabolic pathway
13	Traes_7BS_322A365D4.1	taes01100	Metabolic pathway
14	Traes_7BS_94EB3B3D6.1	taes04016	MAPK signaling pathway
15	Traes_7DL_C104E3D30.1	taes01100	Metabolic pathway

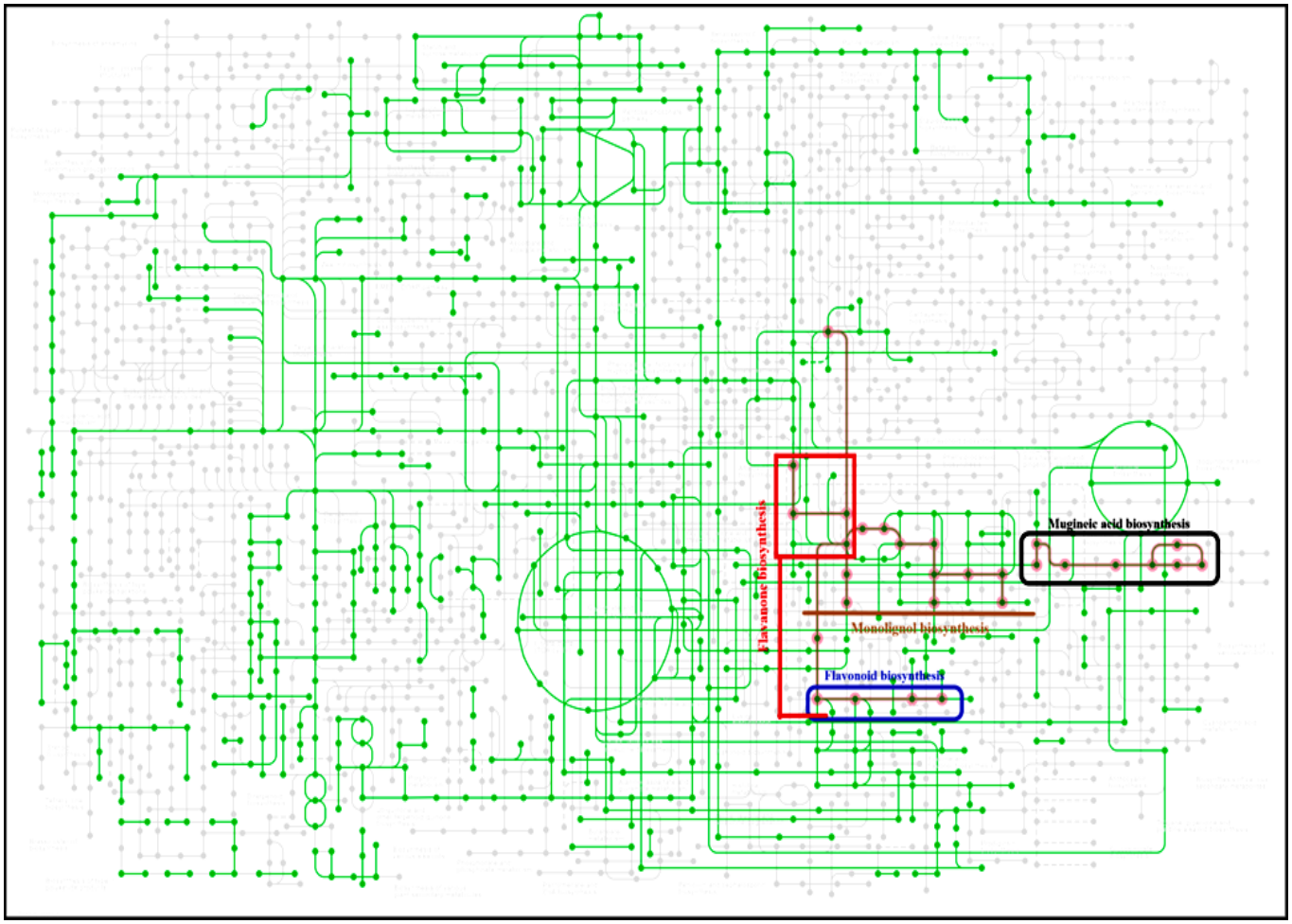


Fig. 8a. Phenylpropenoid biosynthesis, hub gene highlighted in red, Traes_1BL_FDF080D1A.1 (K00083).

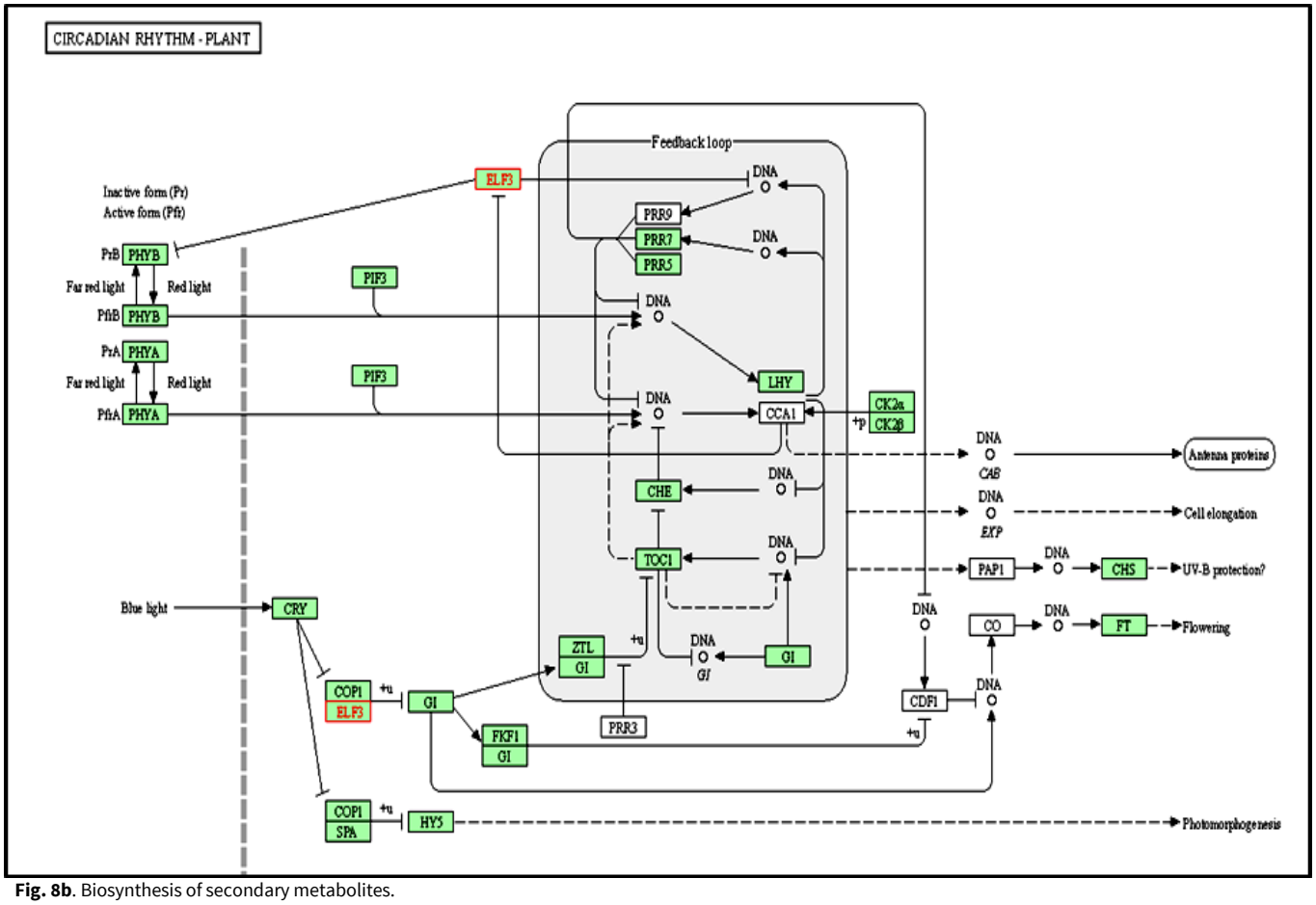
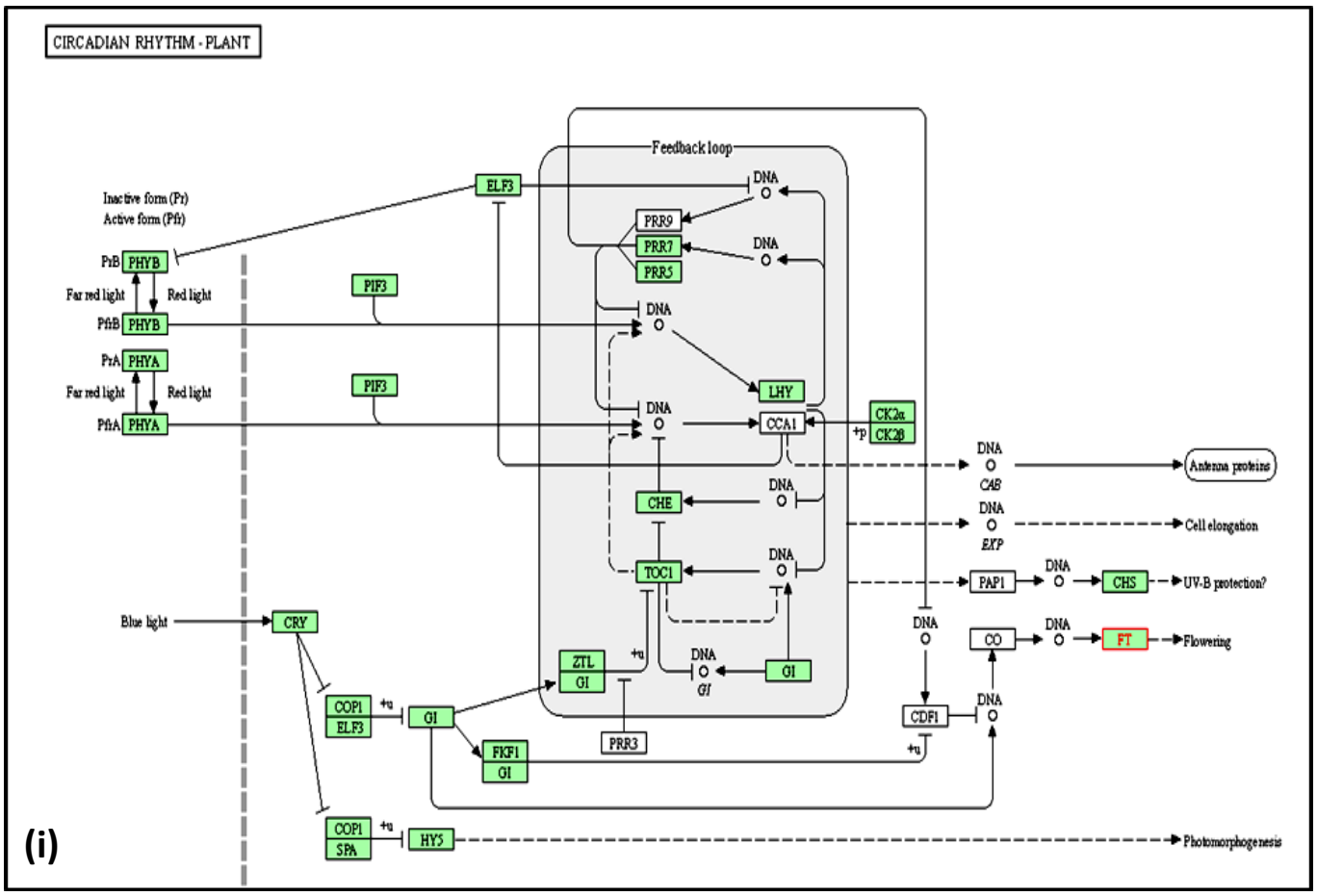


Fig. 8b. Biosynthesis of secondary metabolites.



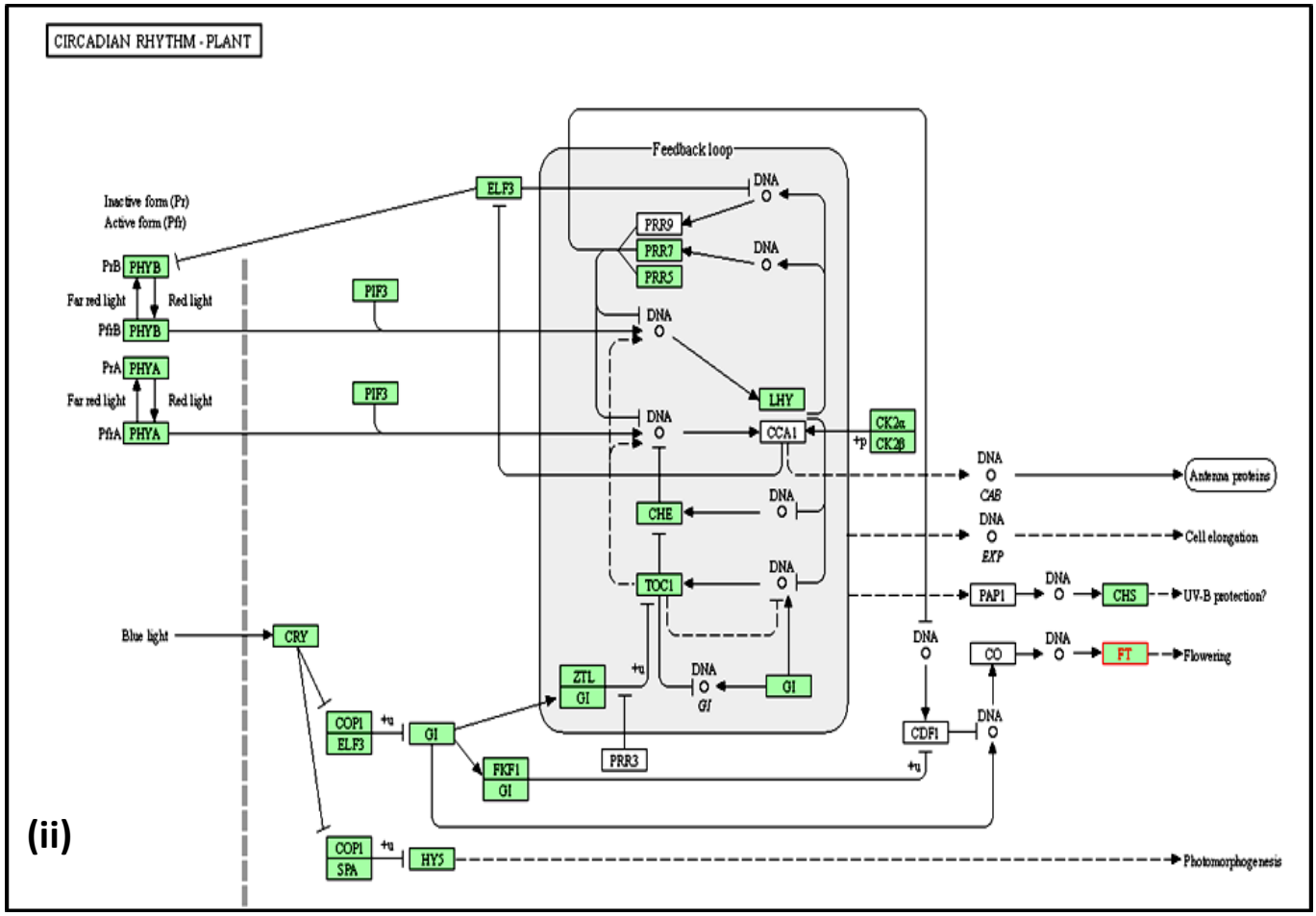


Fig. 8c (i & ii). Circadian rhythm, hub genes highlighted in red, Traes_5BL_E6535628C.1 and Traes_5BL_E6535628C.1.

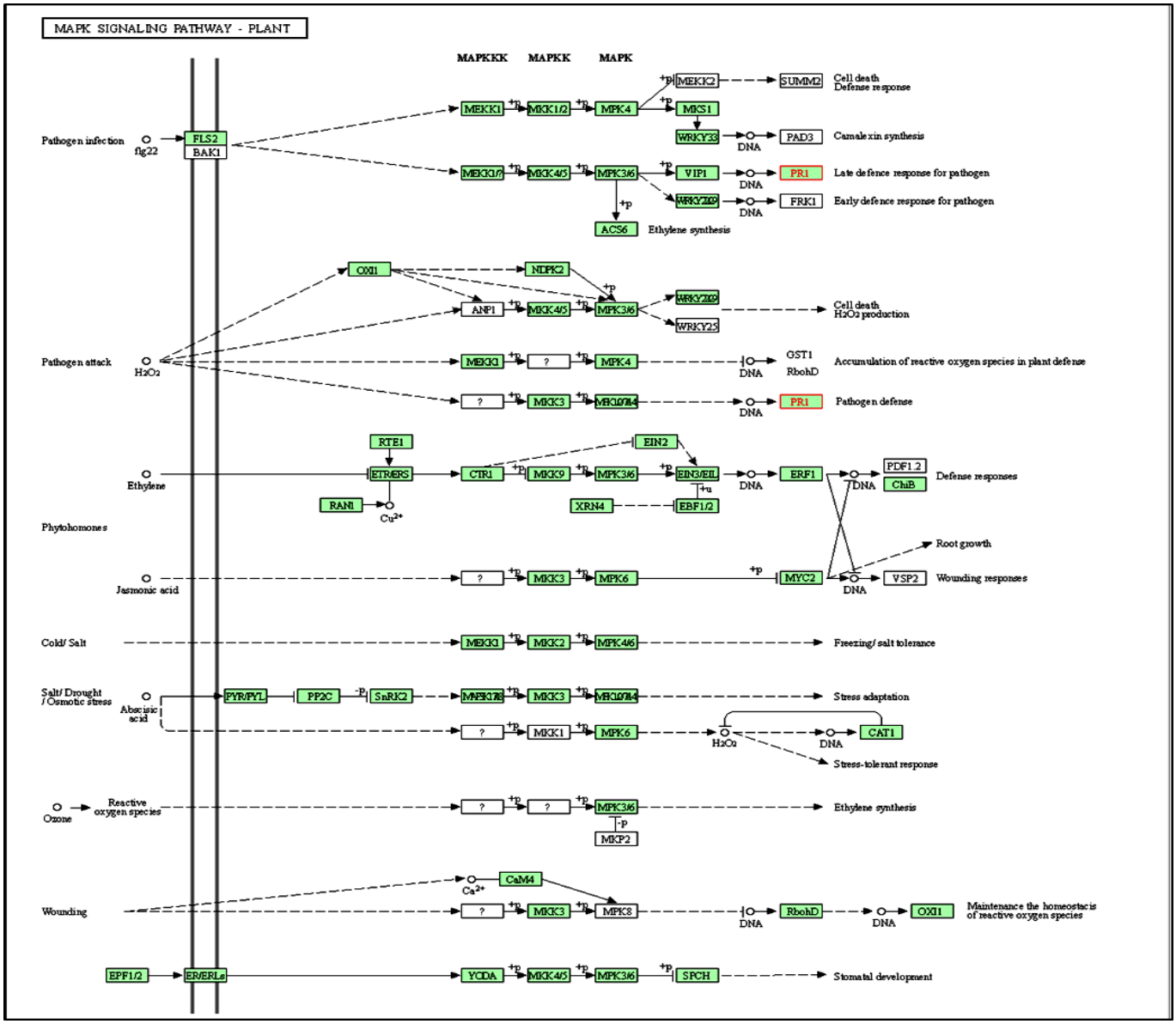


Fig. 8d. MAPK signaling pathway, hub genes highlighted in red, Traes_5BL_83495AC81.1 and Traes_7BS_94EB3B3D6.1.

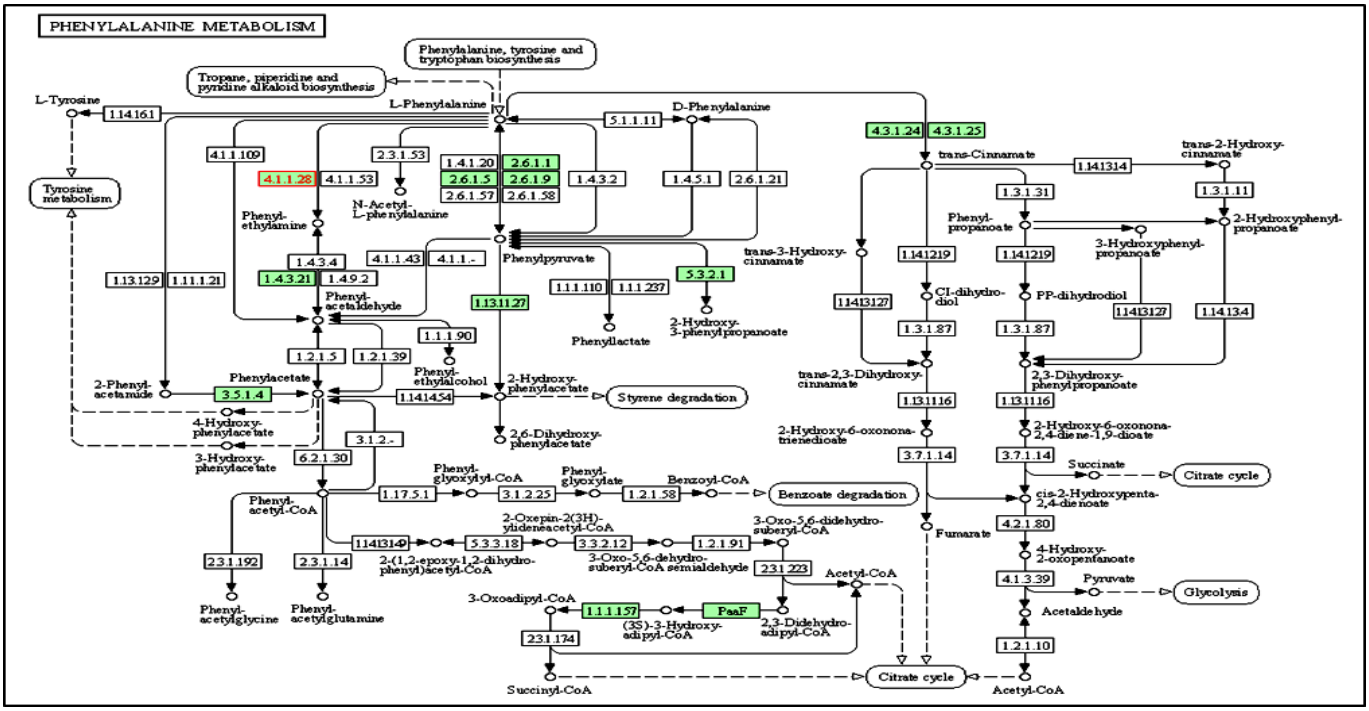


Fig. 8e. Phenyl alanine metabolism, hub gene highlighted in red, Traes_7AL_0F8FA764F.1 (K01593) (EC4.1.1.28).

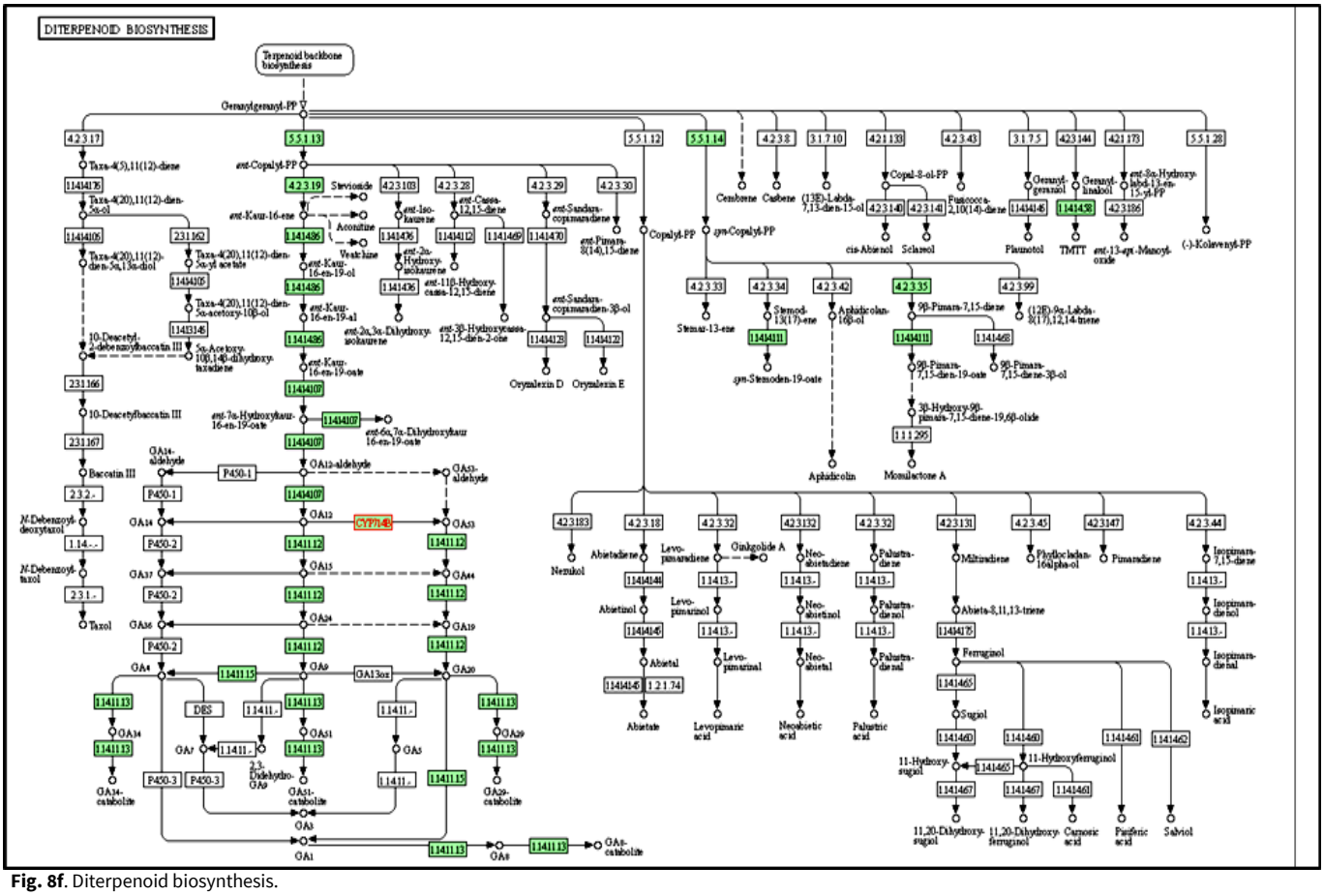


Fig. 8f. Diterpenoid biosynthesis.

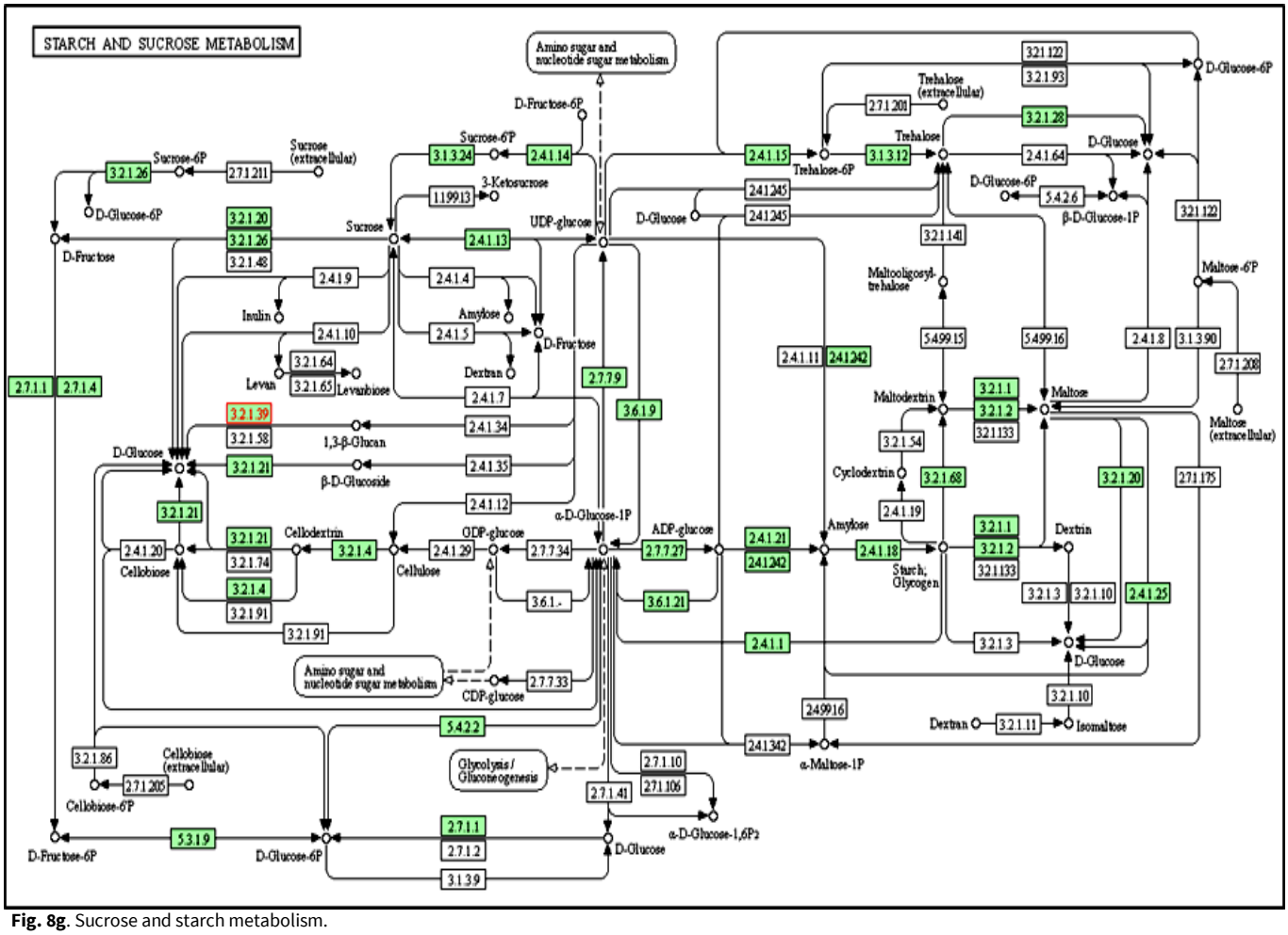


Fig. 8g. Sucrose and starch metabolism.

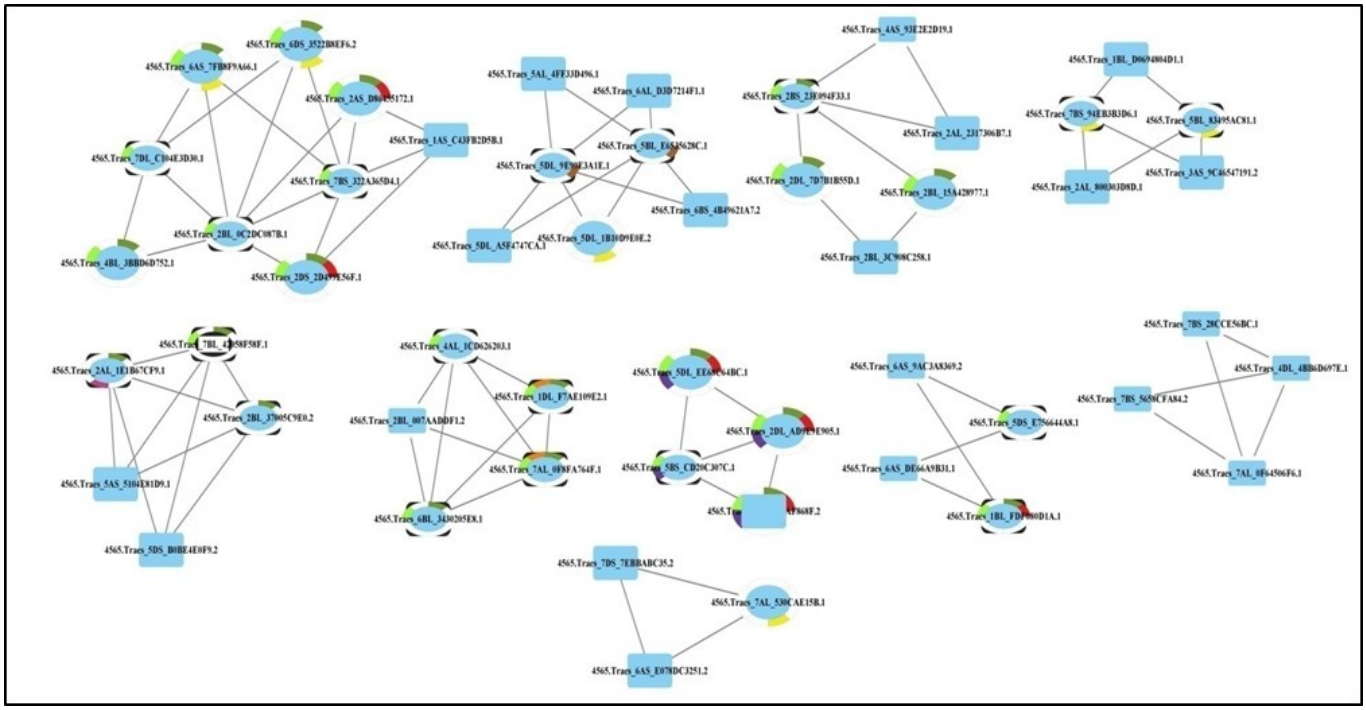


Fig. 9. Clusters labelled with KEGG pathway indications including hub genes. Red-Phenylproponoid biosynthesis, Dark green- Biosynthesis of secondary metabolites, Brown- Circadian rhythm, Yellow- MAPK signaling pathway, Orange- Phenyl alanine metabolism, Magenta- Diterpenoid biosynthesis, Purple- Starch and sucrose metabolism and Parrot green- Metabolic pathway.

Table 6. The hub genes associated directly with phosphorus use efficiency in wheat.

Gene ID	Description	Function	Organism	Ortholog	Ortholog gene ID	Reference
Traes_1BL_FDF080D1A.1	FAD Binding Berberine protein	Helps in root growth Co-ordinates adaptation to phosphorus deficiency		*Arabidopsis thaliana*		(14)
Traes_2BL_37005C9E0.2	Flavanoid 3' hydroxylase	Increase root length, stimulates mycorrhizal spore germination	Wheat (*Triticum aestivum*)			(3)
Traes_2BL_OC2DC087B.1	Momilactone A synthase like	Phosphorus uptake and Utilization		Rice *(Orzya sativa)*		(32)
Traes_4AS_93E2E2D19.1	Protein SPA	Phosphorus Uptake and allocation		*Arabidopsis thaliana*	At1G75690	(38)
Traes_6AS_DE66A9B31.1	Rhodanese related sulfur transferase	Response to P deficiency Mobilization of organic P		*Arabidopsis thaliana*	At2G17850	(22)
Traes_7DL_C104E3D30.1	Polyamine oxidase	Involve in nutrient tolerance Root development	Wheat (*Triticum aestivum*)			(11)
Traes_2AL_1E1B67CF9.1	Cytochrome P450 99A-2 like	Defence against abiotic and biotic stress	Monocot crops			(34)
Traes_7AL_OF8FA764F.1	Tryptophan decarboxylase 1-like	Adaptation and resistance to plant in biotic and abiotic condition	Wheat (*Triticum aestivum*)			(27)
Traes_1BL_FDF080D1A.1	FAD Binding Berberine protein	Improves P uptake by stimulation of microbial growth	Wheat (*Triticum aestivum*)			(36)
Traes_5BL_83495AC81.1 Traes_7BS_94EB3B3D6.1	Uncharacterized protein; Belongs to the CRISP family	Act as master switch regulating nutrient stress by triggering transcription factors and stress-associated genes	Wheat (*Triticum aestivum*)			(37)

in wheat (*Triticum aestivum*) assists in wheat crop tolerance to nutrient stress and root system development (33). Both Traes_2AL_1E1B67CF9.1 and Traes_7AL_OF8FA764F.1 genes in wheat (*Triticum aestivum* L.) contributes to defense against abiotic and biotic stress, enhancing plant adaptability and resistance (34, 35). Additionally, Traes_1BL_FDF080D1A.1, Traes_5BL_83495AC81.1 and Traes_7BS_94EB3B3D6.1, actively present in wheat, facilitate microbial growth to improve phosphorus uptake efficiency (36). Traes_5BL_83495AC81.1 and Traes_7BS_94EB3-B3D6.1 act as master regulators, triggering transcription factors and stress-related genes under nutrient stress conditions (37).

The hub genes categorized under phosphorus gene family

Further analysis revealed that some of the identified hub genes also fall within the phosphorus gene family, specifically classified as phosphate tolerance genes (PSTOL1), phosphate (PHO1-B1), phosphate transporters (PHT 2, 3, 4), purple acid phosphatases and phosphate transporters associated with mycorrhizal associations (PHT-myc). Among these, 8 hub genes (Traes_1BL_FDF080D1A.1, Traes_2BL_37005C9E0.2, Traes_4AL_1CD626203.1, Traes_4AS_93E2E2D19.1,Traes_5BS_CD20C307C.1, Traes_5DL_9E99E3A1E.1, Traes_6AS_DE66A9B31.1 and Traes_7AL_0F64506F6.1) were assigned to phosphate tolerance genes (PSTOL1), 1 hub gene (Traes_1BL_FDF080-D1A.1) was associated with phosphate (PHO1-B1), phosphate transporters (PHT 2, 3, 4) encompassed 3 hub genes (Traes_5BL_E6535628C.1, Traes_4AL_1CD626203.1 and Traes_5DL_9E99E3A1E.1), purple acid phosphatases accounted for 2 hub genes (Traes_5DL_9E99E3A1E.1 and Traes_7BS_28CCE56BC.1) and 3 hub genes (Traes_2BL_0C-2DC087B.1, Traes_4DL_4BB6D697E.1 and Traes_5BL_834-95AC81.1) were identified as part of phosphate transporters with mycorrhizal associations (PHT-myc) (Table 7).

Graphs and plots through SRPlot software modules

Utilizing gene ontology terms, a bar plot encompassing all three gene ontology categories (MF, BP and CC) was generated via SRPlot. The highlighted colours within the bar plot denote GO terms associated with hub genes. In total, the bar plot comprises 61 gene ontology terms related to biological processes, cellular components and molecular functions. Among these, 23 out of 40 biological process terms, 3 out of 7 cellular component terms and 8 out of 14 molecular function terms were linked with hub genes.

Additionally, the enrichment bubble plot representing KEGG pathways demonstrates the highest counts for the metabolic pathway (above 60) and the lowest counts for diterpenoid biosynthesis (below 20). The maximum log value was observed for biosynthesis of secondary metabolites (taes01110), while the minimum log value was recorded for the MAPK signaling pathway (taes04016). The pathway enrichment category plot for KEGG pathways showcased the metabolism category with the longest bars for metabolic pathway (taes01100) having the highest count

Table 7. The hub genes categorized under different phosphorus related gene family.

Gene ID	Description	Gene family	Reference
Traes_1BS_FDF080D1A.1	Cinnamyl-alcohol dehydrogenase, FAD-Binding Berberine family protein	Phosphate Tolerance Genes (PSTOL1)	(3) (31)
Traes_2BL_37005C9E0.2	Flavonoid 3',5'- hydroxylase 2-like Glutamine Synthetase		
Traes_4AL_1CD626203.1	Protein SPA, Chloroplastic like		
Traes_4AS_93E2E2D19.1	Probable glucan 1,3-beta-glucosidase A		
Traes_5BS_CD20C307C.1	CEN-like protein 2, TFL1-D		
Traes_5DL_9E99E3A1E.1	Thiosulfate sulfurtransferase 16, chloroplastic-like		
Traes_6AS_DE66A9B31.1	Uncharacterized protein		
Traes_7AL_0F64506F6.1			
Traes_1BS_FDF080D1A.1	Cinnamyl-alcohol dehydrogenase, FAD-Binding Berberine family protein	Phosphate1 (PHO1-B1)	(7)
Traes_5BL_E6535628C.1	TFL1-A		
Traes_4AL_1CD626203.1	Glutamine Synthetase	Phosphate Transporters (PHT 2,3,4)	(17)
Traes_5DL_9E99E3A1E.1	CEN-like protein 2, TFL1-D		
Traes_5DL_9E99E3A1E.1	CEN-like protein 2, TFL1-D	Purple Acid Phosphatases	(27)
Traes_7BS_28CCE56BC.1	Uncharacterized protein		
Traes_2BL_0C2DC087B.1	Momilactone A synthase-like		
Traes_4DL_4BB6D697E.1	Belongs to the major facilitator superfamily. Sugar transporter family	Phosphate Transporters Mycorrhizal Association (PHT- myc)	(13)
Traes_5BL_83495AC81.1	Belongs to the CRISP family		

(75), while diterpenoid biosynthesis (taes00904) had the lowest count (4). Within the environmental information processing category, the MAPK signaling pathway (taes04016) appeared with a count of 11. Under the organismal systems category, Circadian rhythm plants (taes04712) had a count of 5.

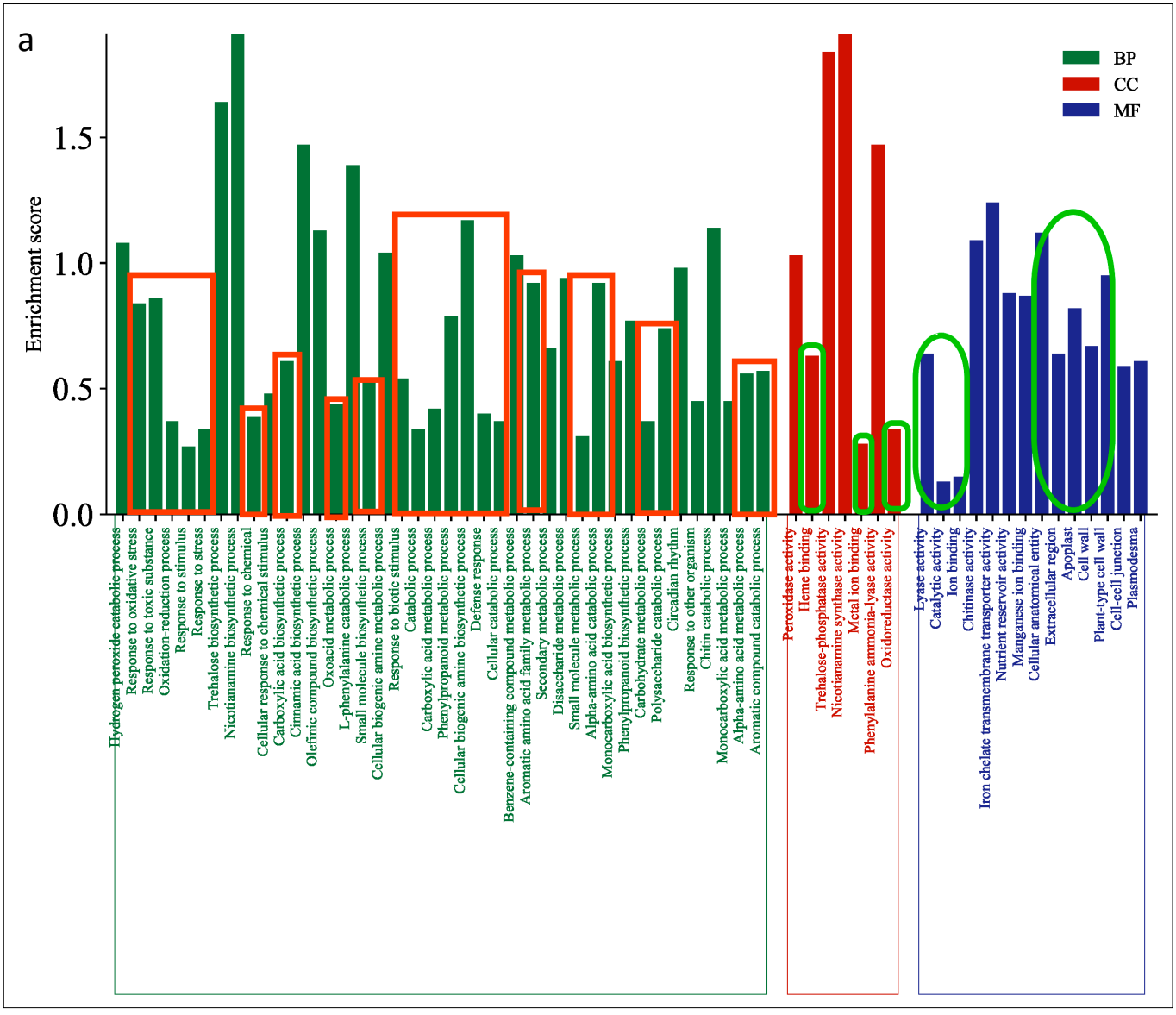
The Gene Ontology Chord (GO Chord) for molecular function illustrated the connectivity between hub genes and molecular functions, forming a ribbon-like structure. Each molecular function term was represented by different colours. Thicker ribbons denoted a higher number of genes associated with that molecular function, whereas thinner ribbons indicated a lower number of genes linked to the respective molecular function (Fig. 10).

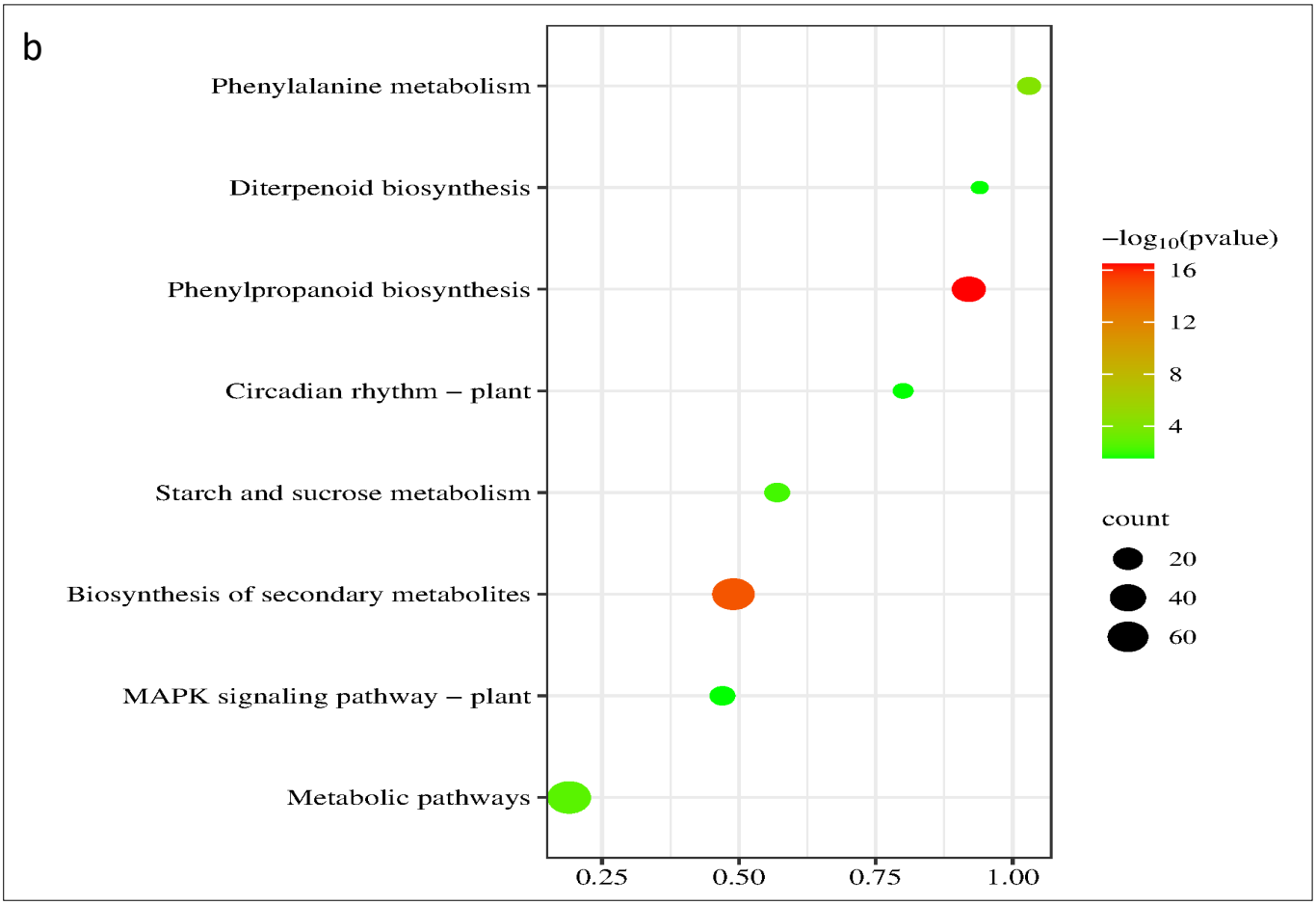
Gene expression behaviour of hub genes

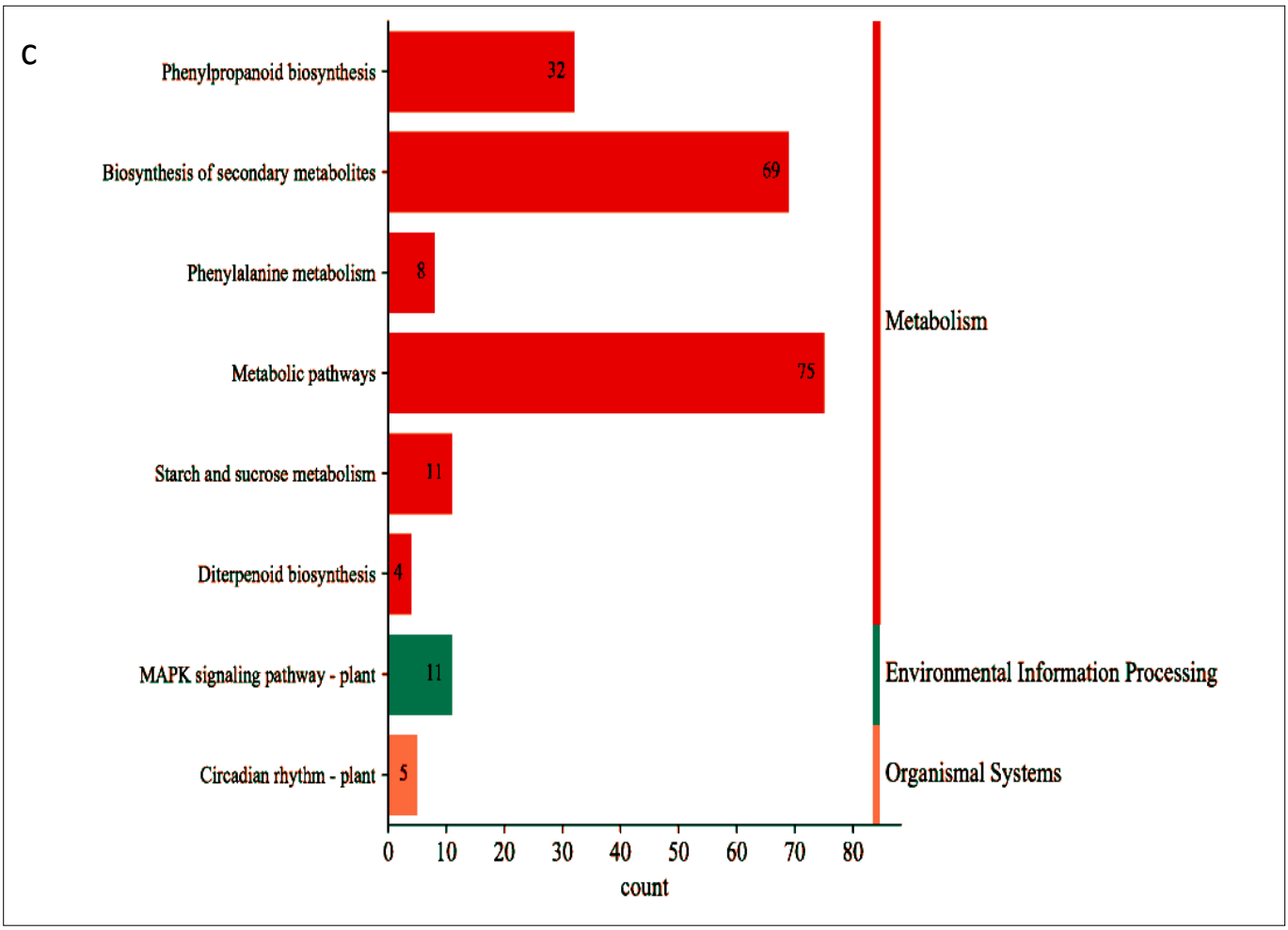
Gene expression behaviour of hub genes was not performed as there were not good resources and suitable conditions for performing RT-PCR for their validation. Substitute to the above issue, a wheat expression browser was utilized to check the expression of 26 hub genes.

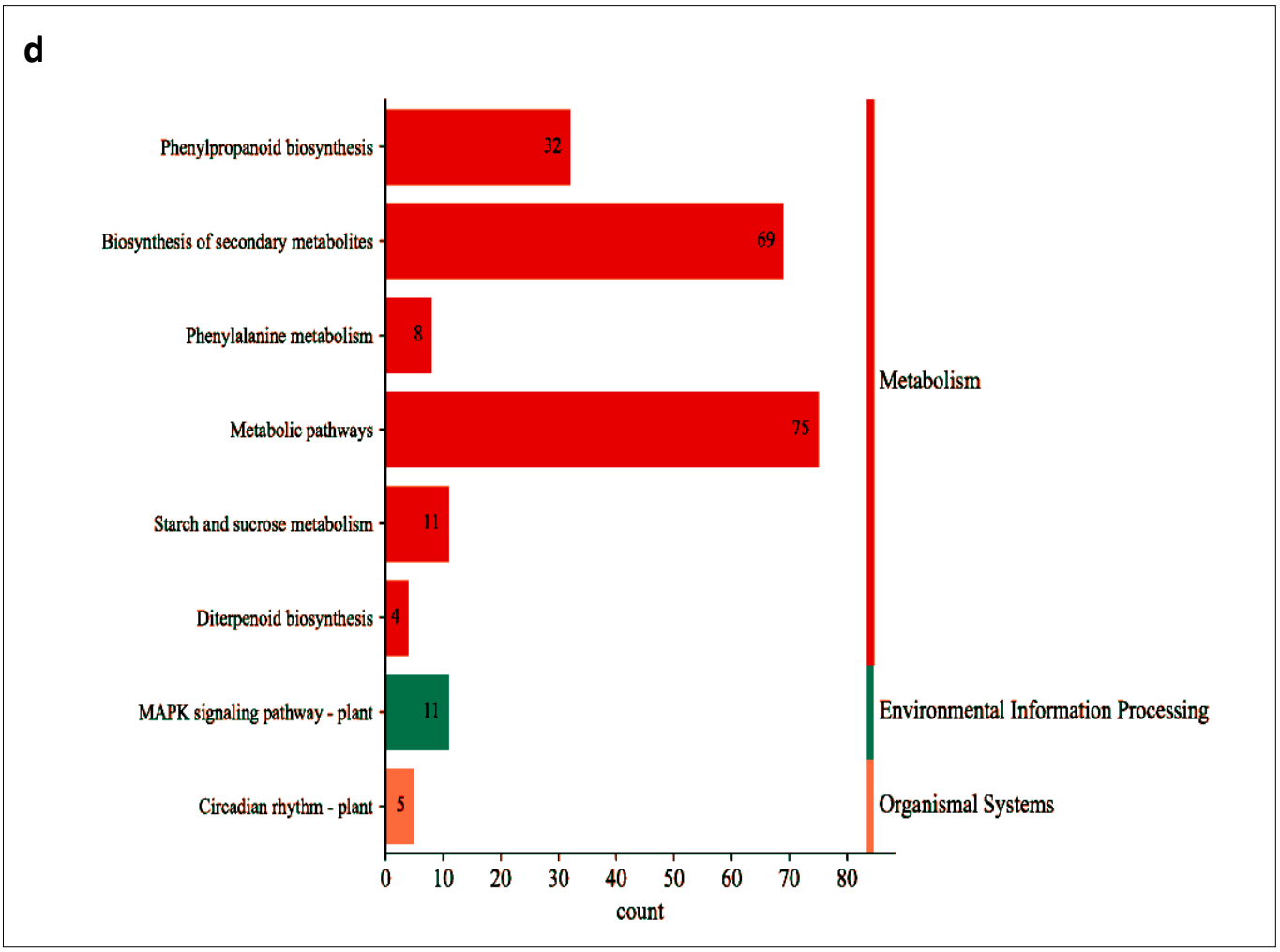
The gene expression level in biological samples serves as a reliable indicator to discern a gene's functional role (38). The Wheat Expression Browser, accessible at http://www.wheat-expression.com/, vividly portrays the expression patterns of hub genes. Among the 26 hub genes, four (Traes_2BS_23E094F33.1, Traes_7DL_C-104E3D30.1, Traes_5BS_CD20C307C.1 and Traes_5-DL_43D95A3FE.1) exhibit high expression levels surpassing the threshold value (expression count of 13, log2) (Fig. 11), while the remaining hub genes have expression values below the threshold. The expression of these 26 hub genes varied across developmental stages and tissue types.

For instance, the gene Traes_2BS_23E094F33.1 demonstrates high expression levels during the seedling and vegetative stages, particularly under disease and abiotic stress conditions, predominantly expressed in leaves and shoots. Its log count value ranges from 13.3 to 15.16, peaking at 15.16 during the vegetative stage in leaves and shoots and reaching its lowest point at 13.34 during the same stage for azhumaya (n=24). Traes_7DL_C104E3D30.1 and Traes_5BS_CD20C307C.1 show expression in leaves and shoots during the vegetative stage with log counts of 14.26 and 13.4 respectively. Traes_5DL_43D95A3FE.1 exhibits expression during the reproductive stage in leaves and shoots.




b
Phenylalanine metabolism
Diterpenoid biosynthesis
Phenylpropanoid biosynthesis
Circadian rhythm – plant
Starch and sucrose metabolism
Biosynthesis of secondary metabolites
MAPK signaling pathway – plant
Metabolic pathways
0.25
0.50
0.75
1.00
−log10(pvalue)
16
12
8
4
count
20
40
60


c
Phenylpropanoid biosynthesis
32
Biosynthesis of secondary metabolites
69
Phenylalanine metabolism
8
Metabolic pathways
75
Starch and sucrose metabolism
11
Diterpenoid biosynthesis
4
MAPK signaling pathway - plant
11
Circadian rhythm - plant
5
0
10
20
30
40
50
60
70
80
count
Metabolism
Environmental Information Processing
Organismal Systems



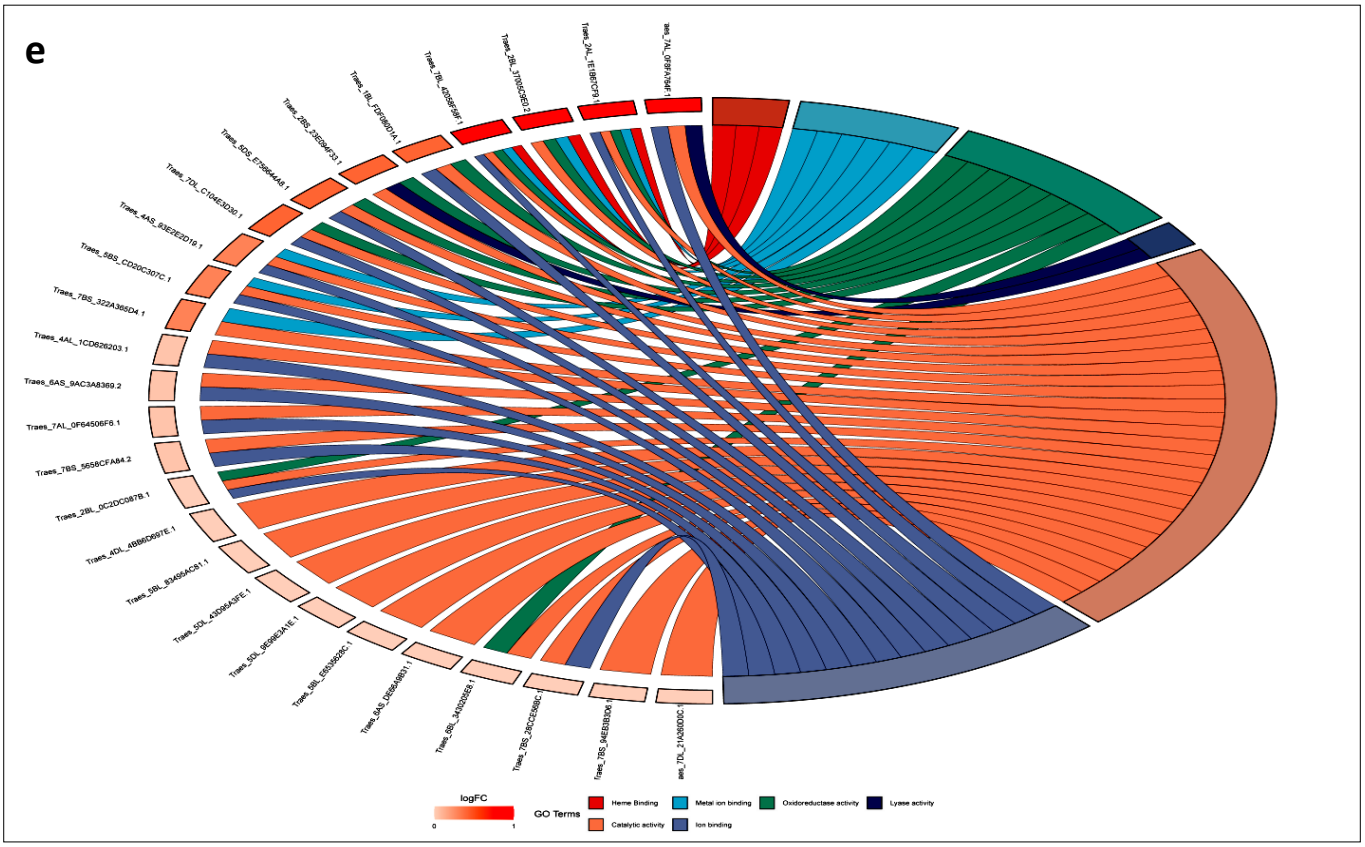


Fig. 10. Plots and graphs related to gene ontology terms and KEGG pathway through SRPlot (Bar Plot, Enrichment bubble, Pathway enrichment and GO Chord) for Phosphorus Use Efficiency.

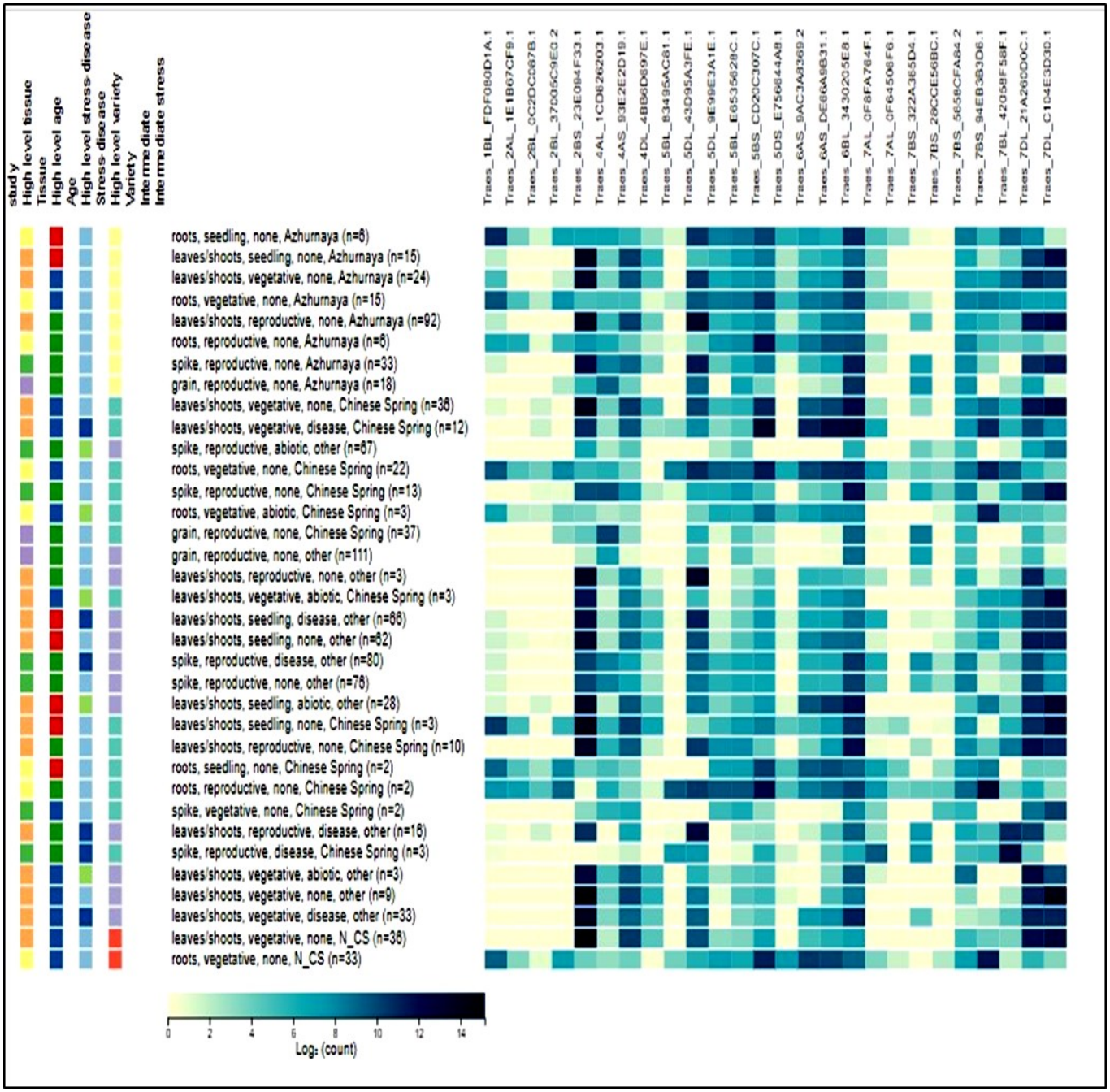


Fig. 11. Level of 29 hub genes (NUE) expression retrieved from Wheat Expression Browser (expVIP).

Discussion

The lack of phosphorus is one of the factors that contribute to the excessive price increase for farmers, because it has to be used as fertilizer, which is not profitable. Plants tend to deal with situations of phosphorus deficiency by increasing phosphorus assimilation, which can be achieved by changing the architecture of the root system, changing the rhizosphere, interacting with microorganisms or internal phosphorus transport and mobilization. Due to the high cost of farmers' fertilizers and the huge environmental damage they cause, it is imperative for current crop production to adopt and develop new P fertilizer management and processing strategies to increase P use efficiency. A lack of P forces plants to adapt by producing more lateral roots that expand the effective root area. If genes that increase PUE are found and their expression is controlled, crops can require less P fertilizer. This prompted the study of genes and signaling systems responsible for plant responses to P deficiency. Plant PUE is inherently complex because it involves phosphorus sensing, uptake, translocation, assimilation and remobilization. It is regulated by multiple interrelated genetic and environmental variables. As our knowledge of the genes and interactions underlying these fundamental processes evolves, so does the refinement of PUE. Therefore, research is needed to develop plants that absorb P more efficiently and use it in the plant.

In this study, we identified 1194 differentially expressed genes from the transcriptome data expressed under phosphorus stress. A network of 1194 expressed genes was constructed through the String database and exported to Cytoscape for further analysis. There were a total of 277 genes in the Cytoscape network with a confidence level of 0.7, a total of 26 hub genes were identified based on edge connectivity, with a minimum number of edges of 3 and a maximum number of edges of 24. In addition, the

MCODE cluster (Integrated Cytoscape Application) was performed to identify clusters in the following network. A total of 39 clusters were obtained. Out of them, only 11 clusters have highly populated and vast networking, and they were considered for finding hub genes. Total 26 hub genes were retrieved from 11 clusters; further analysis was performed to link hub genes that were directly linked to phosphorus use efficiency. Only 10 hub genes were retrieved, which have a role in phosphorus use efficiency in wheat. These 10 hub genes were involved in root growth, coping plants to phosphorus deficiency, phosphorus uptake and utilization, a mycorrhizal association for more phosphorus uptake and also act as switch master regulators against nutrient stress. A total of 8 KEGG pathways were discovered, which shows the association of hub genes.

In summary, our study highlights key genes that were expressed under phosphorus stress to overcome crop phosphorus use efficiency in phosphorus-poor environments. In addition, research or studies can be conducted to increase phosphorus utilization in wheat under abiotic stress environments.

Conclusion

In conclusion, this comprehensive analysis of phosphorus stress tolerance in wheat (*Triticum aestivum* L.) has elucidated several key aspects of its genetic and molecular underpinnings. Through the identification and network analysis of differentially expressed genes (DEGs) associated with phosphorus use efficiency (PUE), a robust network of hub genes was constructed. These hub genes were linked with various KEGG pathways and Gene Ontology (GO) terms, revealing their pivotal roles in biochemical processes such as nutrient metabolism, stress response and root development. The identified hub genes, such as Traes_1BL_FDF080D1A.1 and Traes_4AS_93E2E2D19.1, have been shown to significantly enhance phosphorus uptake and utilization, which is crucial for improving wheat yield and resilience under phosphorus-limited conditions. The investigation also demonstrated these hub genes' potential for genetic improvement tactics by highlighting their participation in particular phosphorus-related gene families, such as phosphate transporters and phosphate tolerance genes. Insights into the intricate regulatory networks influencing phosphorus stress tolerance were obtained by the integration of omics data and mathematical modelling, providing useful targets for further study and agricultural improvement initiatives. Altogether, this study advances our knowledge of how wheat responds to phosphate stress and lays the groundwork for creating plans to increase phosphorus use efficiency, which will support sustainable farming methods and food security.

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Authors' contributions

NC software, validation, formal analysis, investigation, data curation, writing—original draft preparation, writing—review and editing; RD conceptualization, validation, investigation, resources, original draft preparation, writing—review and editing, supervision, project administration, funding acquisition; PK software, resources, writing—review and editing; KT software, validation, writing—original draft preparation, writing—review and editing; NK investigation, writing—review and editing; NS investigation, writing—review and editing, supervision, project administration, funding acquisition; PV software, validation, formal analysis; SM writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Compliance with ethical standards

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