IN-SILICO TRANSCRIPTOME STUDY OF THE RICE (*ORYZA SATIVA*) STRIGOLACTONE-DEFICIENT (*DWARF17*) MUTANT REVEALS A POTENTIAL LINK OF STRIGOLACTONES WITH VARIOUS STRESS-ASSOCIATED PATHWAYS

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Abstract

Strigolactones (SLs), the newest group of phytohormones, are involved in a wide range of functions, including the regulation of plant growth and physiology. Besides, emerging evidence suggests that SLs also participate in the promotion of plant environmental stress resilience through mediation of different metabolic genes/pathways. However, thus far little is known about SL-mediated transcriptional changes in rice (*Oryza sativa*), compared to other model plants. To meet this objective, we analyzed the RNA-seq-based comparative transcriptome data sets of rice SL-deficient *dwarf17* (*d17*) mutant line and its respective wild-type (WT), obtained from the National Center for Biotechnology Information GenBank. Both, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed, in order to identify genes/pathways which function downstream of SLs. With respect to the WT, a large number of functional GO terms, mainly stress-associated terms such as 'response to stress', 'response to stimulus', 'response to chemical', 'response to oxidative stress' and 'reactive oxygen species metabolic process' were significantly suppressed in the *d17* mutant plants. In addition, KEGG metabolic pathways such as 'valine, leucine and isoleucine degradation', 'plant hormone signal transduction', 'galactose metabolism', 'fatty acid degradation' and 'phenylalanine metabolism' were also remarkably undermined in the *d17* lines relative to the WT. These results imply a possible involvement of rice SLs in the regulation of distinct stress-related metabolic genes/pathways, which may function in environmental stress tolerance of plants. Taken together, the study provides new opportunities to broaden our limited understanding of SL-regulated downstream pathways, especially in rice.

Key words: Environmental stresses, Metabolic pathways, *Oryza sativa,* Strigolactones, RNA-sequencing.

Introduction

Strigolactones (SLs), a bunch of phytohormones, for the first time were extracted from the cotton (*Gossypium hirsutum* L.) root exudates (Cook *et al*., 1966). Later studies demonstrated that SLs promote seed germination of different parasitic plant genera, including *Alectra*, *Orobanche* and *Phelipanche* (Mostofa *et al*., 2018; Xie *et al*., 2010). In addition to this, later on, several studies discovered that SLs also promote the hyphal branching of arbuscular mycorrhizal fungi (AMF), leading to the symbiotic interaction among the AMF and the host plant (Akiyama *et al*., 2005). Strikingly, afterward, it was found that SLs also function as plant hormones, and participate in the promotion of phytophysiological and morphological processes, such as early germination of seeds, regulation of the plant secondary growth, development of the plant roots, and senescence of the leaves (Mostofa *et al*., 2018; Pandey *et al*., 2016; Brewer *et al*., 2013).

Furthermore, molecular studies demonstrated that SLs also function in the positively modulation of abiotic stresses resilience, mainly nutrient and drought stresses in different model plant species (Mostofa *et al*., 2018; Saeed *et al*., 2017; Andreo-Jimenez *et al*., 2015; Marzec *et al*., 2013). Besides, current molecular and genetic research confirmed the involvement of SLs in disease resistance against specific fungal and bacterial plant pathogens (Marzec *et al*., 2016). In this regards, a study revealed that tomato (*Solanum lycopersicum*) *carotenoid cleavage dioxygenases 8* (*slccd8*) mutants, impaired in SL biosynthesis, were remarkably susceptible to fungi *Botrytis cinerea* and *Alternaria alternate* attacks compared to the wild-type (WT) (Torres-Vera *et al*., 2014).

To decipher the SL-regulated transcriptional changes, microarray and qRT-PCR-based gene expression analyses have been performed on several model plant species, including that of *Arabidopsis thaliana*, *Medicago truncatula* and tomato (Juan & Harro, 2008; Mashiguchi *et al*., 2009; Mayzlishgati *et al*., 2010: Mdodana., 2012). The results of these studies exhibited the differential expression of considerable number of genes participating in various metabolic pathways, growth regulation and stress response pathways. For instance, various stress-related pathways, including secondary metabolism and

phytohormones (such as abscisic acid, cytokinins and auxins) have been shown to be regulated by SLs (Juan Antonio & Harro, 2008; Mashiguchi *et al*., 2009; Mayzlishgati *et al*., 2010; Mdodana, 2012). Although, these findings shed light on the possible role of the SLs in regulation of distinct downstream stress-associated metabolic pathways, the SL-dependent regulation of gene expression/metabolic pathways in the monocot model and commercially important crop rice (*Oryza sativa* L.) remains poorly explored.

Moreover, compared to microarray, transcriptome data analysis based on the high-throughput RNA-sequencing gives highly reliable and a complete picture of the alterations that occur at gene expression level, and thus allows the accurate identification of metabolic pathway changes that occur in plants during comparison of the two different genotypes/cultivars (Chen *et al*., 2017; Tian *et al*., 2018a; Tian *et al*., 2018b).

In the present *in-silico* study, we studied the SL-biosynthesis-dependent regulation of genes/metabolic pathways in rice. To this end, we conducted gene ontology (GO) and KEGG metabolic pathways analysis (which is based on the leaf RNA-seq data retrieved from National Center for Biotechnology Information, NCBI, GenBank) of the rice SL-deficient (*dwarf17*, *d17*) mutant and the corresponding WT (Nasir *et al*., 2019a). Overall, our findings propose that SL-biosynthetic pathway acts upstream of various stress-associated genes/metabolic pathways, which may function in environmental stress resistance of rice.

Materials and Methods

In-silico **RNA-seq analysis:** In the present study, we used the leaf RNA-seq (transcriptome) data sets of strigolactone-deficient (*d17*) mutant lines and the corresponding WT rice (*Oryza sativa* L. cv. Shiokari), downloaded from the NCBI GenBank (http://www.ncbi.nlm.nih.gov) via the SRA accession number PRJNA545916 (Nasir *et al*., 2019a). The read counts were calculated using HTSeq package. The DESeq package was used for normalization of the read counts and the formula of corrected *P*-value (*q*-value) ≤0.05 and [fold change ≥2] (Nasir *et al*., 2019b) was used in order to identify differentially expressed genes between *d17* vs. WT comparison.

Gene Ontology and KEGG pathway enrichment analyses: Gene Ontology (GO) annotation was performed using Blast2GO program at *P*-value ≤0.05. In addition, KOBAS (2.0) was used for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis by adjusting *P*-value at ≤0.05.

Results and Discussion

The basic aim of the present study was to explore SL-mediated downstream pathways in rice during normal condition. To achieve this, we compared the RNA-seq data sets of SL-deficient (*d17*) mutant and WT rice leaves under normal condition, retrieved from NCBI GenBank (Nasir *et al*., 2019a). We obtained about 264,140,088 raw reads from the 6 samples, having 40,565,114-51,024,242 raw reads per sample (Table 1). After filtering poor-quality sequences, a total of 247,139,775 clean reads were obtained. The percentage of clean reads per sample that could be mapped ranged from 93.12 to 93.99% (Table 1).

Our study shed light on SL-regulated changes in the gene expression/metabolic pathways in rice. We noticed that the expression levels of a wide range of genes differs significantly between the *d17* and WT rice. Importantly, we found a remarkable down-regulation in the transcripts related to various stress-associated GO functional terms and KEGG metabolic pathways.

Identification of differentially expressed genes (DEGs): To obtain the significantly expressed transcripts in *d17* vs. WT comparison, the present criteria of *q*-value ≤ 0.05 and fold change ≥ 2 was used (Fig. 1). A total of 5391 DEGs were identified, among which 2920 were significantly up-regulated, while 2471 genes were being down-regulated in SL-deficient (*d17*) relative to that of WT genotypes (Fig. 1). Fragments Per Kilobase Million (FPKM) value-based cluster and heat-map of the DEGs of *d17* vs. WT comparison are illustrated in Fig. 1.

Gene Ontology (GO) annotation analysis: We performed GO annotation analysis of the DEGs of *d17* and WT rice in order to assess the GO functional terms that were suppressed in *d17* compared to WT. GO enrichment analysis inferred that the large number of genes in 'biological process' category of the GO functional terms including 'response to stress', 'response to stimulus', 'response to chemical', 'response to oxidative stress', 'reactive oxygen metabolic process' and so on, as illustrated in (Fig. 2) in detail, were significantly undermined in the *d17* with respect to the WT. In addition, a considerable number of down-regulated transcripts in *d17* pertaining to 'oxidoreductase activity', 'peroxide activity', 'antioxidant activity' and so on in the GO functional category 'molecular process' as shown in (Fig. 2) in detail.

Table 1. Summary of RNA-sequencing reads mapped to the rice reference genome.

Sample	Raw reads	Clean reads	Percentage of mapped reads	Average percentage of mapped reads	Uniquely mapped reads	Percentage of uniquely mapped reads	Average percentage of uniquely mapped reads
WT-1	44,860,790	41,703,216	92.96		40,447,752	90.16	
WT-2	40,565,114	37,618,244	92.74	93.12	36,472,660	89.91	90.31
WT-3	51,024,242	47,784,319	93.65		46,358,451	90.86	
d17-1	44,742,122	42261,529	94.46		41,214,450	92.12	
d17-2	40,937,078	38,294,973	93.55	93.99	37,388,548	91.33	91.74
d17-3	42,010,742	39,477,494	93.97		32,173,726	91.77	

Note: WT=Wild-type rice, *d17*=Strigolactone-biosynthetic mutant rice. *The RNA-seq data were retrieved from the GenBank (http://www.ncbi.nlm.nih.gov) using the accession number PRJNA545916 (Nasir *et al*., 2019a)
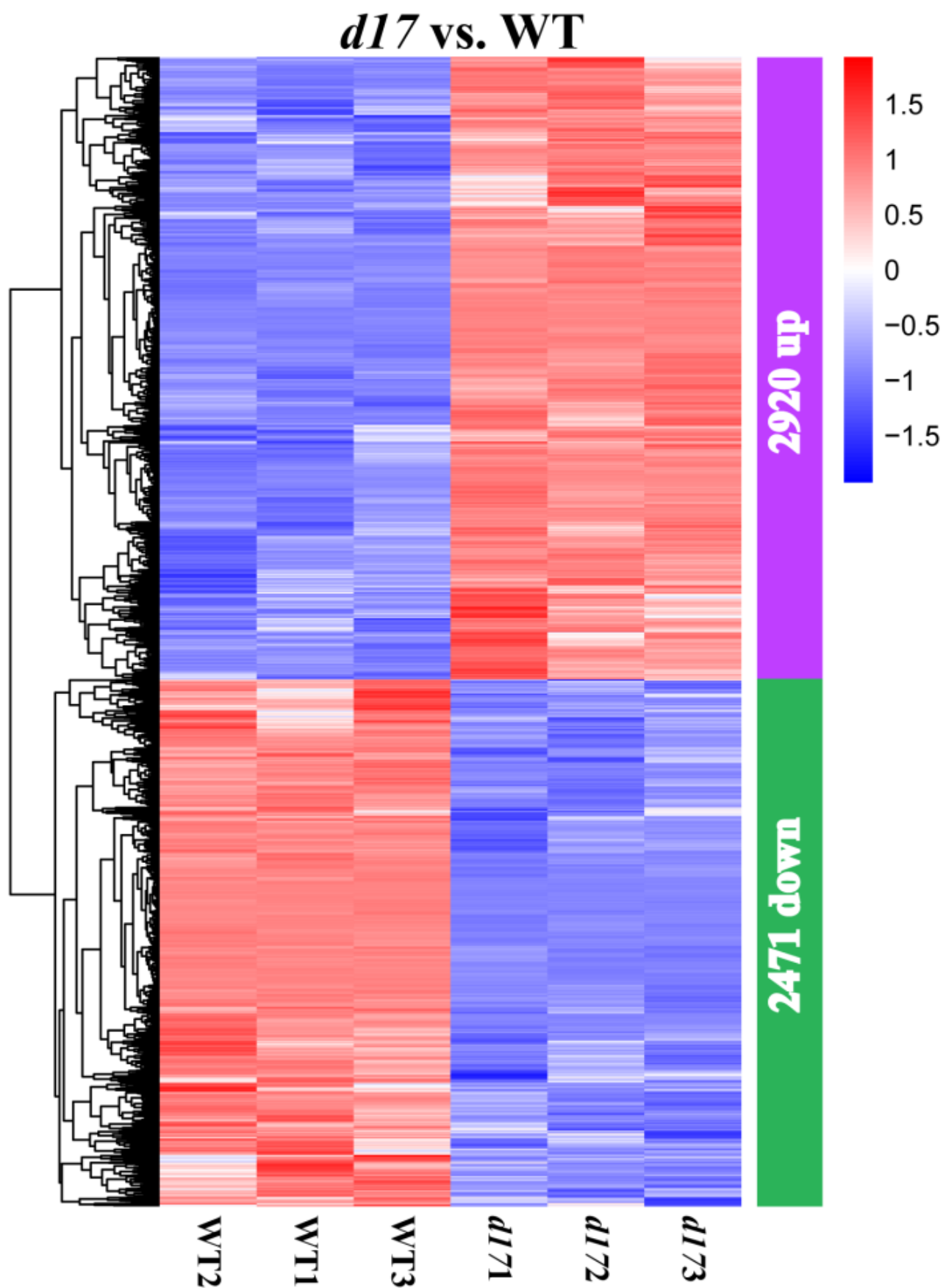
Fig. 1. Hierarchical clustering and heat-map based on Fragments Per Kilobase Million (FPKM) values in *d17* and WT, showing significantly up- and down-regulated genes in among *d17* vs. WT comparison. WT, wild-type rice; *d17*, strigolactone-biosynthetic mutant. *The RNA-seq data were retrieved from the GenBank (http://www.ncbi.nlm.nih.gov) using the accession number PRJNA545916 (Nasir *et al*., 2019a).
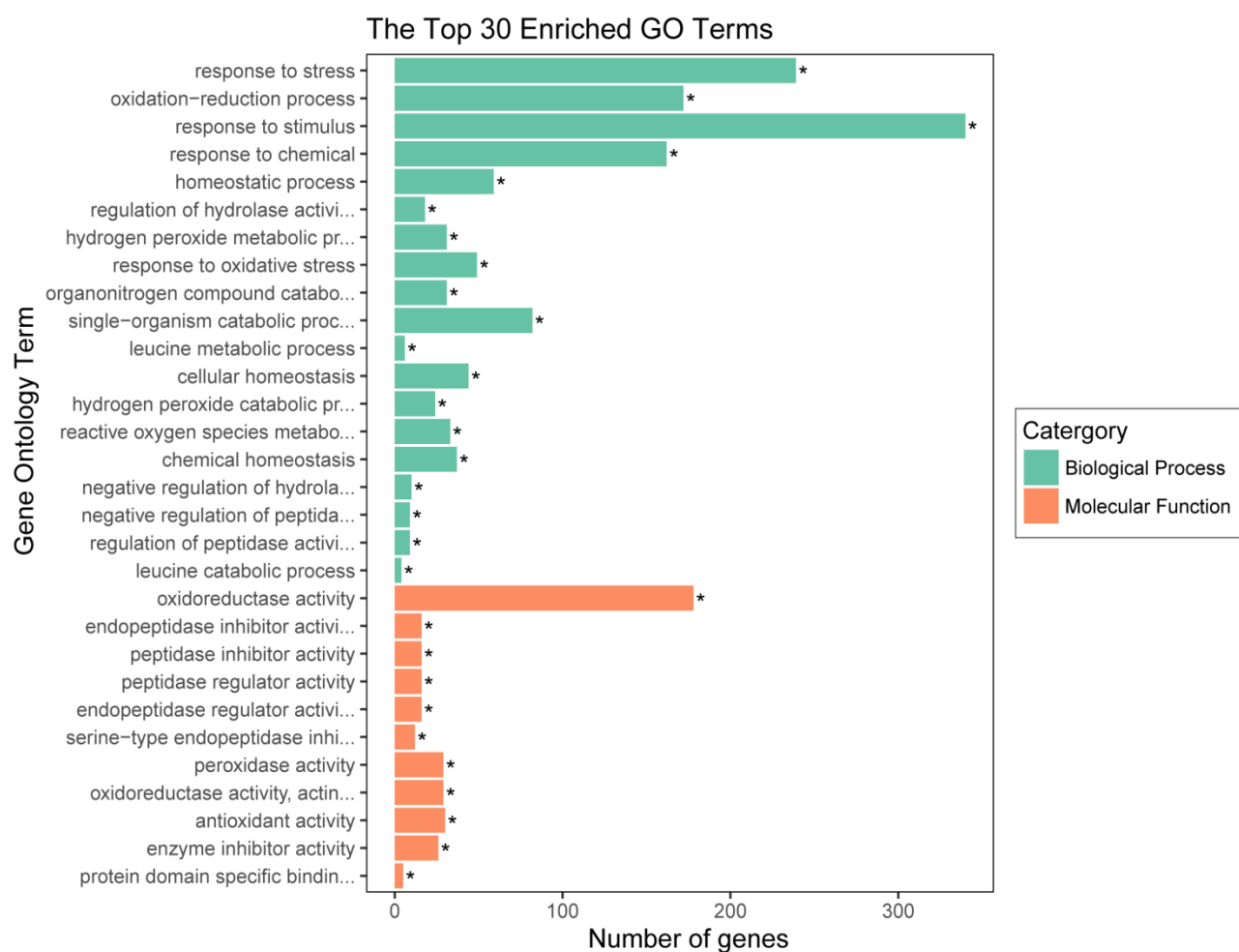
Fig. 2. Top 30 highly enriched Gene Ontology (GO) terms of down-regulated genes obtained from the comparison of *d17* vs. WT. WT, wild-type rice; *d17*, strigolactone-deficient mutant. *The RNA-seq data were retrieved from the GenBank (http://www.ncbi.nlm.nih.gov) using the accession number PRJNA545916 (Nasir *et al.*, 2019a).

Previously, it has been shown that *A. thaliana* SL-deficient and signaling mutants were less resistant to drought stress and salt stress compared to that of WT (Ha *et al.*, 2014). Furthermore, the defensive role of SLs has also been confirmed in various plants species such as tomato, mosses and *A. thaliana* (Vera *et al.*, 2014; Piisilä *et al.*, 2015; Marzec, 2016). In support of these results, here, down-regulation of a significant number of stress-associated transcripts/pathways in *d17* compared to that of WT, hinting towards the possible role of strigolactones in environmental stress resilience in rice.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis: KEGG pathway database was used in order to investigate significantly induced metabolic pathways in WT genotypes with respect to the *d17* mutant genotypes. Results showed that KEGG metabolic pathways, including 'glyoxylate and dicarboxylate metabolism', 'peroxisome', 'phytohormone signal transduction', 'galactose metabolism', 'valine, leucine and isoleucine degradation', 'fatty acid degradation' and 'phenylalanine metabolism' were significantly enriched in the WT relative to the *d17* mutant lines (Fig. 3). The detailed information are shown in Fig. 4. Here, the

metabolic pathway analysis (which is based on transcriptome datasets) indicates that SLs act upstream of various stress-related metabolic pathways in rice.

Plants are sessile organisms, thus to protect themselves from environmental stresses they evolved a remarkable resistance mechanisms at biochemical, molecular and morphological levels (Mostofa *et al.*, 2018; Nasir *et al.*, 2018; Osakabe *et al.*, 2014). Here the down-regulation of genes which enriched in various stress-associated metabolic pathways further indicate the possibility that SLs are likely function in the mediation of environmental stress tolerance via positive regulation of these metabolic pathways in rice. The responsiveness of SL-biosynthesis and –signaling genes has been reported in different environmental stresses in *A. thaliana* (Marzec & Muszynska, 2015), further hinting at a supportive role of SLs in plant adaptation to environmental stresses. Previous reports have shown that phytohormone signaling is important for regulation of environmental stress resistance in plants (Osakabe *et al.*, 2014; David *et al.*, 2013). Similarly, the involvement of phenylalanine metabolic pathway in mediation of various environmental stress has also been proved in different plant species (Caretto *et al.*, 2015; Oh *et al.*,

2009). Consistent with this, it has also been confirmed that carbohydrate-associated metabolic pathways, which include glyoxylate and dicarboxylate metabolic pathways and galactose metabolic pathways are not only involved in energy production but also participate in environmental stress tolerance in plants. Accordingly, reports have revealed that fatty acid metabolism finally leads to the production of linolenic acid, which as a result provide protection against biotic stress (Tian *et al*., 2018b). As mentioned above, in our study the expression levels of the genes involved in various stress–related metabolic pathways, including

'phytohormone signal transduction', 'phenylalanine metabolism', 'glyoxylate and dicarboxylate metabolic', and 'fatty acid degradation' pathways were significantly suppressed in the *d17* mutants with respect to the WT genotypes (Figs. 3 and 4). As is evident that these metabolic pathways are involved in the regulation of environmental stress tolerance, thus our finding shed light on the regulatory role of SL-biosynthesis in mediation of environmental stress tolerance in rice. Nevertheless, the pathways which function downstream of SLs (identified here) need to be confirmed biochemically in future.
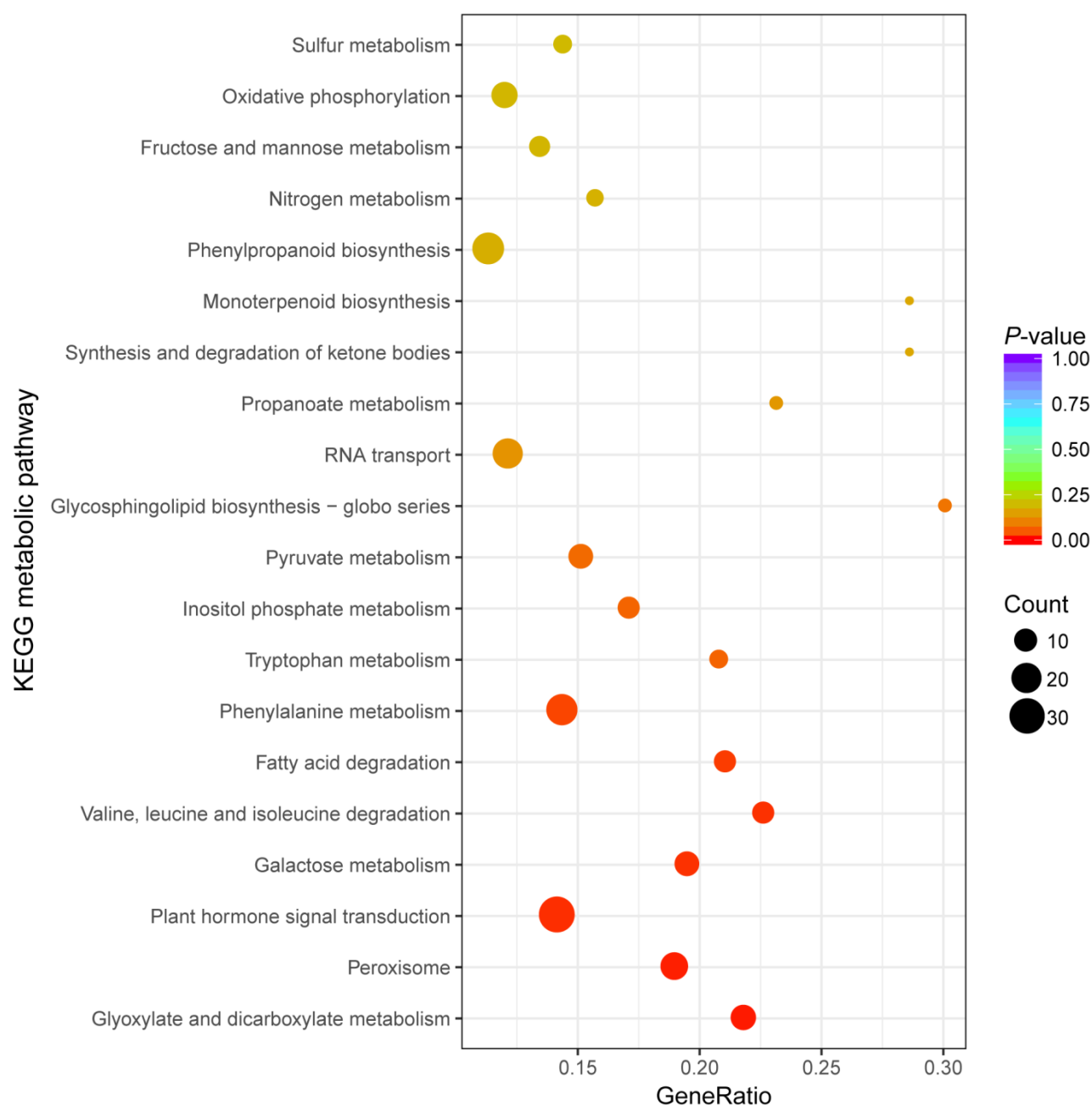

Fig. 3. Significantly enriched Kyoto Encyclopedia of Genes and Genomes pathway (KEGG) metabolic pathways of down-regulated genes obtained from the comparison of *d17* vs. WT. WT, wild-type rice; *d17*, strigolactone-deficient mutant. *The RNA-seq data were retrieved from the GenBank (http://www.ncbi.nlm.nih.gov) using the accession number PRJNA545916 (Nasir *et al*., 2019a).
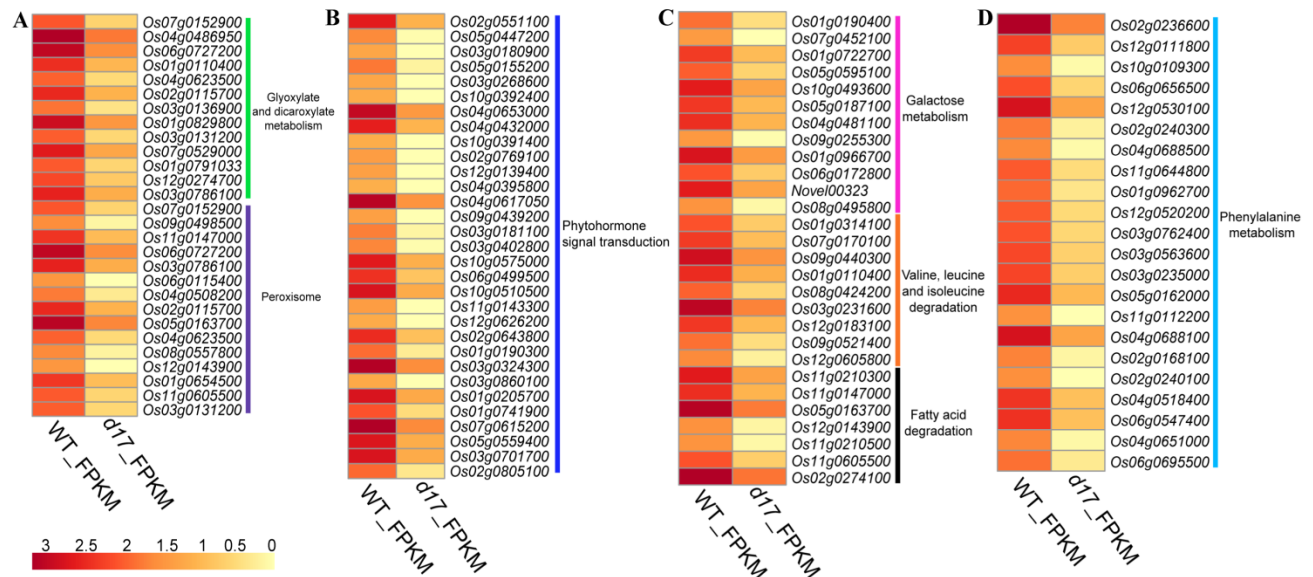
Fig. 4. Heat map of the down-regulated genes in *d17* that are involved in (A) 'Glyoxylate and dicarboxylate metabolism', 'Peroxisome', (B) 'Phytohormone signal transduction', (C) 'Galactose metabolism', 'Valine, leucine and isoleucine degradation', 'Fatty acid degradation' and (D) 'Phenylalanine metabolism'. The colors indicate the average abundance of transcripts based on transformed Fragments Per Kilobase Million (FPKM) values in strigolactone-deficient (*d17*) and the wild-type (WT) plants. *The RNA-seq data were retrieved from the GenBank (http://www.ncbi.nlm.nih.gov) using the accession number PRJNA545916 (Nasir *et al*., 2019a).

Conclusion

The *in-silico* study, presented here, improved our understanding of the molecular pathways function downstream of SL biosynthetic pathway in rice. In addition, the results of the study pointing towards the contribution of SLs in environmental tolerance via regulation of various downstream stress-associated genes/pathways.

Acknowledgements

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