Stress signaling convergence and nutrient crosstalk determine zinc-mediated amelioration against cadmium toxicity in rice

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ABSTRACT

Consumption of rice (*Oryza sativa* L.) is one of the major pathways for heavy metal bioaccumulation in humans over time. Understanding the molecular responses of rice to heavy metal contamination in agriculture is useful for eco-toxicological assessment of cadmium (Cd) and its interaction with zinc (Zn). In certain crops, the impacts of Cd stress or Zn nutrition on the biophysical chemistry and gene expression have been widely investigated, but their molecular interactions at transcriptomic level, particularly in rice roots, are still elusive. Here, hydroponic investigations were carried out with two rice genotypes (Yinni-801 and Heizhan-43), varying in Cd contents in plant tissues to determine their transcriptomic responses upon Cd_{15} (15 μM) and $Cd_{15}+Zn_{50}$ (50 μM) treatments. High throughput RNA-sequencing analysis confirmed that 496 and 2407 DEGs were significantly affected by Cd_{15} and $Cd_{15}+Zn_{50}$, respectively, among which 1016 DEGs were commonly induced in both genotypes. Multitude of DEGs fell under the category of protein kinases, such as calmodulin (CaM) and calcineurin B-like protein-interacting protein kinases (CBL), indicating a dynamic shift in hormonal signal transduction and Ca^{2+} involvement with the onset of treatments. Both genotypes expressed a mutual regulation of transcription factors (TFs) such as WRKY, MYB, NAM, AP2, bHLH and ZFP families under both treatments, whereas genes enconding ABC transporters (ABCs), high affinity K^+ transporters (HAKs) and Glutathione-S-transferases (GSTs), were highly up-regulated under $Cd_{15}+Zn_{50}$ in both genotypes. Zinc addition triggered more signaling cascades and detoxification related genes in regulation of immunity along with the suppression of Cd-induced DEGs and restriction of Cd uptake. Conclusively, the effective integration of breeding techniques with candidate genes identified in this study as well as economically and technologically viable methods, such as Zn nutrient management, could pave the way for selecting cultivars with promising agronomic qualities and reduced Cd for sustainable rice production.

1. Introduction

With a surge in human population, expected to reach 10 milliard people till 2050, a tandem increase in global food demand is anticipated (Tian et al., 2019). Concurrent with 21st century's accelerated industrialization and administration of contemporary agronomic practices, acute heavy metal and metalloid contamination of soil has been recorded all around the globe (Ashraf et al., 2019; Feng et al., 2020; Ahmad et al., 2021; Deng et al., 2021). Consequent food contamination has caused major environmental, economic and social issues for food safety worldwide, which has resulted in serious implications with respect to human health (Cao et al., 2019). In the priority list of hazardous substances devised by US Agency for Toxic Substances and Disease Registry (ATSDR, 2021), Cd is ranked 7th owing to its perilous carcinogenic propensities, and multifarious studies have been conducted over the last three decades to ascertain its toxicity to humans, animals, plants and other living organisms (Ashraf et al., 2019). In plants, Cd causes severe impairment of the photosynthetic apparatus (Sagonda et al., 2021), and

affects respiration and nitrogen metabolism (Jia et al., 2016), leading to an imbalance of water and nutrient uptake (Yamaguchi et al., 2012).

Amongst the staple food crops, rice is cultivated extensively with around 754.6 million tons' annual production, feeding half of the world's populace and forming a dynamic constituent of world's agricultural economy (FAO, 2017). In view of the proportional booming demand, augmenting rice yield against the odds of abiotic stress factors, such as soil Cd contamination, has become exigent. In contrast with other cereals, rice can cumulate higher Cd contents in its tissues (Uraguchi and Fujiwara, 2013), resulting in a significant contribution to the dietary Cd intake. The chemical resemblance of Cd to zinc (Zn) leads to an active uptake, translocation and bioaccumulation of Cd in plants *via* non-exclusive regulation pathways of Zn, necessitating the formulation of Cd mitigation strategies (Zare et al., 2018; Mapodzeke et al., 2021; Zeshan et al., 2021). Previous studies indicate that Cd-tolerance is commonly accredited to the instigation of genes associated with enzymes that scavenge ROS, metal transporters and chelators during Cd exposure (Sun et al., 2019; Deng et al., 2021).

In regard to nutrient management, zinc is indispensable for sufficient growth of not only plants but also humans (Cakmak and Kutman, 2018). The essentiality of Zn originates from its contribution as an irreplaceable structural co-factor for numerous protein functions in several metabolic processes (Bashir et al., 2019). It has been extensively exploited to counter-balance Cd toxicity, although genotype, dosage and period of Cd and Zn exposure result in varying outcomes (Rizwan et al., 2016; Adil et al., 2020b). As stated by Tkalec et al. (2014), simultaneous presence of Cd and Zn in soil may interact synergistically or antagonistically depending upon the species, metal concentrations in the medium, tissues and the stage of plant development. In tomato (*Lycopersicon esculentum* L.) (Cherif et al., 2011) and Polish wheat (*Triticum polonicum* L.) (Wang et al., 2017), supplementation of zinc hindered cadmium uptake and facilitated Zn translocation to the aerial parts. Therfore, it could be an interesting breakthrough to address the problem of predicted Cd increment and to design strategies to reduce its toxicity.

Transcriptomic analysis offers an outstanding prospective to decipher the regulation of crop's complex molecular machinery in response to abiotic stresses (Wang et al., 2018; Chen et al., 2019). RNA-Sequencing (RNA-seq) has an edge over other such techniques owing to its in-depth coverage of genome, global expression of transcripts, as well as providing comprehensive information about allele-specific expressions and alternative splicing (Oono et al., 2014). In fact, various transcriptomic profiles of cereal crops acquired *via* RNA-Seq during a multitude of abiotic stresses have produced enormous data (Sun et al., 2019; Chen et al., 2019; Quan et al., 2019; Liu et al., 2020). In a previous study, transcriptome responses in roots of dwarf polish wheat were investigated under Cd and Zn alone and combined application to identify the underlying molecular mechanisms (Wang et al., 2017). Presently, there are many reports on the sole effects of Cd-Zn absorption and translocation in plants and on the physio-biochemical aspects worldwide (Cherif et al., 2011), however there is a scarcity of studies on the molecular aspects, particularly RNA-sequencing in rice plants, encompassing Cd-Zn combined effects. Presented here is a transcriptome study of rice roots carried out with an aim to understand the interactive transcriptional expression shifts in two rice genotypes differing in Cd tissue contents, and to determine pathways involved in the resultant molecular re-shuffling at root level leading to increased Cd tolerance with Zn supplementation. It was hypothesized that optimized exogenous application of zinc is advantageous to rice plants against cadmium toxicity which could be detected through the molecular regulation at transcriptomic level. Data mining of this sort could serve as a high value resource for the identification of candidate genes and help establish the relationships among specific signaling modules to manage heavy metal stresses, predominantly Cd stress.

2. Materials and methods

2.1. Plant materials, growth conditions and stress treatment

Two rice genotypes, *viz.*, Yinni-801 and Heizhan-43, were investigated under hydroponic experiments. After surface sterilization with 2% H_2O_2 solution for 15 min, seeds were rinsed several times and soaked with ddH_2O then kept in dark for 2 d at 25 °C. Subsequently, the germinating seeds were placed in a controlled growth chamber with 30/ 22 °C (day/night) temperature, 75–80% relative humidity, light intensity of 225 ± 25 μmol m^{-2} s^{-1} and a photoperiod of 16 h/ 8 h (light/ dark) for 15 d. Afterwards, seedlings were transplanted into 5 L plastic containers (5 seedlings pot^{-1}), acclimatized in a greenhouse with controlled temperature and relative humidity. Under half strength nutrient solution for 5 d, the seedlings were later on transferred into full strength nutrient solution. Composition of nutrient solution was as follows: 2.9 mM NH_4NO_3, 1.7 mM $MgSO_4$, 1.0 mM $CaCl_2$, 1.0 mM K_2SO_4, 0.32 mM NaH_2PO_4, 36 μM EDTAFeNa, 18 μM H_3BO_3, 9.1 μM $MnCl_2$, 0.52 μM $(NH_4)_6Mo_7O_{24}$, 0.16 μM $CuSO_4$, and 0.15 μM $ZnSO_4$ with pH adjusted to 6.5 ± 0.1 using NaOH or HCl solution (Zeng et al., 2008). Cadmium as $CdCl_2$ and zinc as $ZnSO_4.7 H_2O$ were individually added in the solution. Treatments included Cd 15 μM and Cd 15 μmol L^{-1} + Zn 50 μM. Furthermore, plants devoid of Cd were used as control and the experiment was triplicated following completely randomized design. Root samples (2 genotypes × 3 treatments × 3 biological replicates) were rapidly frozen in liquid nitrogen at the time of harvest (15 DAT), then refrigerated at −80 °C.

2.2. RNA extraction, library preparation, sequencing, quality control and mapping

Total RNA (1 μg per sample) was extracted for 18 frozen samples with TRIzol (Invitrogen, USA) following the manufacturer's recommendations and the method described by He et al. (2016). Sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) according to the guidelines provided by manufacturer and were indexed using codes to asssign sequences to each sample (Novogene, China). Purification of poly-T oligo-attached magnetic beads was the first step involved in library construction. Under elevated temperature, fragmentation was carried out in NEBNext First Strand Synthesis Reaction Buffer (5 ×) using divalent cations. M-MuLV Reverse Transcriptase (RNase H) and random hexamer primer were used to synthesize first strand cDNA. Subsequently, the synthesis of second strand cDNA was accomplished using DNA Polymerase I and RNase H. Exonuclease/polymerase activities assisted in conversion of remaining overhangs into blunt ends. After adenylation of 3′ ends of DNA fragments, the preparation for hybridization necessitated the ligation of NEBNext Adaptor with hairpin loop structure. Moreover, the library fragments were filtered using AMPure XP system (Beckman Coulter, Beverly, USA) to preferentially select 250–300 bp long cDNA fragments. Resultant cDNA was then added with 3 μL USER Enzyme (NEB, USA) and incubated for 15 min at 37 °C, then at 95 °C for 5 min. Next, Phusion High-Fidelity DNA polymerase, Index (X) Primer and Universal PCR primers were employed to perform PCR. The obtained PCR products were purified (AMPure XP system) and Agilent Bioanalyzer 2100 system was opted for library quality assessment. After sequencing, clean data were acquired from raw data by removing reads containing adapters and poly-N as well as ones with inferior-quality. Concomitantly, Q20, Q30 and GC content of the clean data were quantified and all downstream analyses were contingent on the clean data with high-quality reads.

2.3. Quantitative and differential analysis

Feature counts v1.5.0-p3 served the purpose of counting the reads numbers mapped to specific gene. Afterwards, FPKM (Fragments Per Kilo base of transcript sequence per Millions base pairs sequenced) of

each gene was determined contingent on the reads count mapped to the gene and length of that gene (Li and Dewey, 2011). After downloading reference genome and gene model annotation files, the index was constructed using Hisat2 v2.0.5, where paired-end clean reads were aligned to the reference genome (Kim et al., 2015a). Although, DESeq2 R package (1.16.1) was used to perform the analysis of differential expression (two conditions/groups; two biological replicates per condition), the resulting *P*-values were adjusted using the Benjamini and Hochberg's approach to control the false discovery rate, rendering genes with *P*-value < 0.05 (after adjustment) as differentially expressed.

2.4. Differential gene expression analysis via GO and KEGG enrichment

Gene Ontology (GO) enrichment analysis of DEGs was implemented by the cluster Profiler R package with corrected gene length bias. Terms with corrected P value below 0.05 were evaluated as significantly enriched. Association between differentially expressed transcripts and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was also performed (http://www.genome.jp/kegg/). Enrichment of DEGs in KEGG pathways was tested by employing the same statistical software as for GO enrichment analysis.

2.5. Quantitative real-time polymerase chain reaction based validation of DEGs

Validation of DEGs was conducted using qRT-PCR with at least two independent biological replicates and three technical replicates (Fig. S1), with a Roche Light Cycler 480 system (Roche, Basel, Switzerland) as defined by Wang et al. (2017). The *OsActin* gene (accession number XM_015774830) was used as the internal reference gene with the primer pair 5TCCATCTTGGCATCTCTCAG3′ and 5GTACCCGCATCAGGCATCTG3′ for the relative amount of RNA. By using the $2^{(-\Delta\Delta Ct)}$ method given by Schmittgen and Livak (2008), the relative gene expression levels were calculated. All the primer sequences used in this study are provided in Table S1.

2.6. Statistical analysis

Statistical analyses were performed using the statistical software graphpad prism (Graphpad Software, San Diego, CA, USA). Data were expressed as mean ± standard error. Duncan's multiple comparison test (DMRT) was used to assess the statistical significance of data at $P < 0.05$ level.

3. Results

In order to elucidate the transcriptomic profile under Cd_{15} and Cd_{15} $+Zn_{50}$ treatments, paired-end reads of the constructed cDNA were subjected to sequencing by employing Novogene platform. About 6.9 Gb reads sample^{-1} were generated with a GC content of 49.16–55.43%, Q30 higher than 93.3%, and an alignment efficiency of 55.47–77.39% to the reference genome, of which more than 54% were uniquely mapped reads (Table S2). Collectively, 46,304,265 clean reads were produced from each library and the sequencing quality was sufficiently high to allow further analysis.

3.1. Analysis of gene expression and DEGs identification

Novel coding transcripts were combined with the reference genome sequence of *Oryza sativa* to obtain complete references, then consequent clean reads were mapped and gene expression levels of each sample under two treatments and control were determined with RNA-Seq by Expectation-Maximization (RSEM). Coherently, 31.4% novel genes encoding proteins with unknown functions were also found, including some that matched the description of protein kinase domain, S-adenosyl-L-methionine (SAM)-dependent carboxyl methyltransferase, zinc-

binding in reverse transcriptase, zinc-finger of the Fin Control System (FCS)-type, Isocitrate lyase family and kinesin motor domain. The number of DEGs was found highest in Heizhan-43 control against its Cd+Zn treated counterparts, Yinni-801 Cd+Zn against its alone Cd treated counterparts, Yinni-801 control against its Cd+Zn treated counterparts, Heizhan-43 against Yinni-801 both under Cd+Zn followed by Yinni-801 plants under alone Cd stress against its respective control (Fig. 1).

Interestingly, the comparison between Cd *vs* CK in both genotypes resulted in a high number of up-regulated DEGs, i.e., 70.6% and 61.52% in Hz_Cd *vs* Hz_CK and Yi_Cd *vs* Yi_Ck, respectively. In addition, 1016 genes were commonly expressed, while 496 and 2407 expressed uniquely under Cd_{15} and $Cd_{15}+Zn_{50}$ treatments, respectively, indicating a high number of Zn-induced gene expression. In Heizhan-43 genotype, 211, 2544 and 322 unigenes expressed differentially when comparisons were made for Hz_Cd_Zn *vs* Hz_Cd, Hz_CK *vs* Hz_Cd_Zn and Hz_Cd *vs* Hz_CK, respectively, while 56 genes expressed mutually. Yinni-801 displayed 688, 1501 and 1900 DEGs when compared for Yi_Cd_Zn *vs* Yi_Cd, Yi_CK *vs* Yi_Cd_Zn and Yi_Cd *vs* Yi_CK, respectively with 189 co-expressing genes (Fig. 1B-D). Principle component analysis (PCA) revealed that RNA-seq samples of two genotypes are clearly separated by PC1 and PC2, explaining for 44.54% of the total variation (Fig. S2). Moreover, the RNA-seq samples of control were clearly separated from Cd and Cd_Zn treatment, indicating Cd toxicity had a significant influence on the transcript levels. The volcano plots depict a 6-fold higher number of expressed DEGs under Yi_Cd_Zn *vs* Yi_Cd (3541) compared to Hz_Cd_Zn *vs* Hz_Cd (579) with 1359 (38.4%) and 187 (32.3%) up-regulated genes; 2182 (61.6%) and 392 (67.7%) down-regulated genes, respectively. DEG count in Hz_Cd_Zn *vs* Yi_Cd_Zn was higher (3423) than Hz_Cd *vs* Yi_Cd (1512) with 1408 (41.1%) and 723 (47.8%) up-regulated; 2015 (58.9%) and 789 (52.2%) down-regulated genes, respectively (Fig. S3A-D). On the basis of their expression levels, all genes were placed into 4 sub clusters (Fig. S4) and 28 module colors with two subsets; one having 11 and the other having 17 modules (Fig. S5).

3.2. KEGG pathway analysis and functional enrichment of DEGs

Out of 117 genes set, more DEGs were up-regulated in Yi_Cd_Zn (29.4% higher) when compared to Hz_Cd_Zn; Yi_Cd (34% higher) against Hz_Cd; Hz_Cd (92.5% higher) against Hz_Cd_Zn; and Yi_Cd (116.2% higher) against Yi_Cd_Zn. Among the top 20 enriched KEGG pathways, the common for all pairwise comparisons were cyano amino acid metabolism and starch and sucrose metabolism. Among Hz_Cd_Zn *vs* Yi_Cd_Zn, Hz_Cd_Zn *vs* Hz_Cd and Yi_Cd_Zn *vs* Yi_Cd, the mutually enriched KEGG pathway was glutathione metabolism, while tyrosine metabolism was enriched for Hz_Cd *vs* Yi_Cd, Hz_Cd_Zn *vs* Yi_Cd_Zn and Hz_Cd_Zn *vs* Hz_Cd (Figs. 2 and 3). Phenylalanine, tyrosine and tryptophan and phenylalanine metabolism enriched for Hz_Cd *vs* Yi_Cd and Hz_Cd_Zn *vs* Hz_Cd. In addition, mismatch repair, brassinosteroid biosynthesis, linoleic acid metabolism and isoquinoline alkaloid biosynthesis were mutually enriched for Hz_Cd *vs* Yi_Cd and Hz_Cd_Zn *vs* Yi_Cd_Zn (Fig. 2). Furthermore, diterpenoid biosynthesis pathway was enriched for Hz_Cd *vs* Yi_Cd and Hz_Cd_Zn *vs* Hz_Cd, whereas cysteine and methionine metabolism, oxidative phosphorylation, glyoxylate and dicarboxylate metabolism, carbon metabolism, phenylpropanoid biosynthesis, gluconeogenesis and carbon fixation in photosynthetic organisms was enriched mutually for Hz_Cd_Zn *vs* Hz_Cd and Yi_Cd_Zn *vs* Yi_Cd (Fig. S6).

The uniquely enriched pathways for Hz_Cd *vs* Yi_Cd were alanine, aspartate and glutamate metabolism, tryptophan metabolism, pantothenate and CoA biosynthesis, proteasome, protein processing in endoplasmic reticulum, arachidonic acid, tropane, piperidine and pyridine metabolism and ABC transporters. However, for Hz_Cd_Zn *vs* Yi_Cd_Zn comparison, sulfur metabolism, plant-pathogen interaction, fatty acid metabolism, other type of O-glycan biosynthesis, nicotinate and
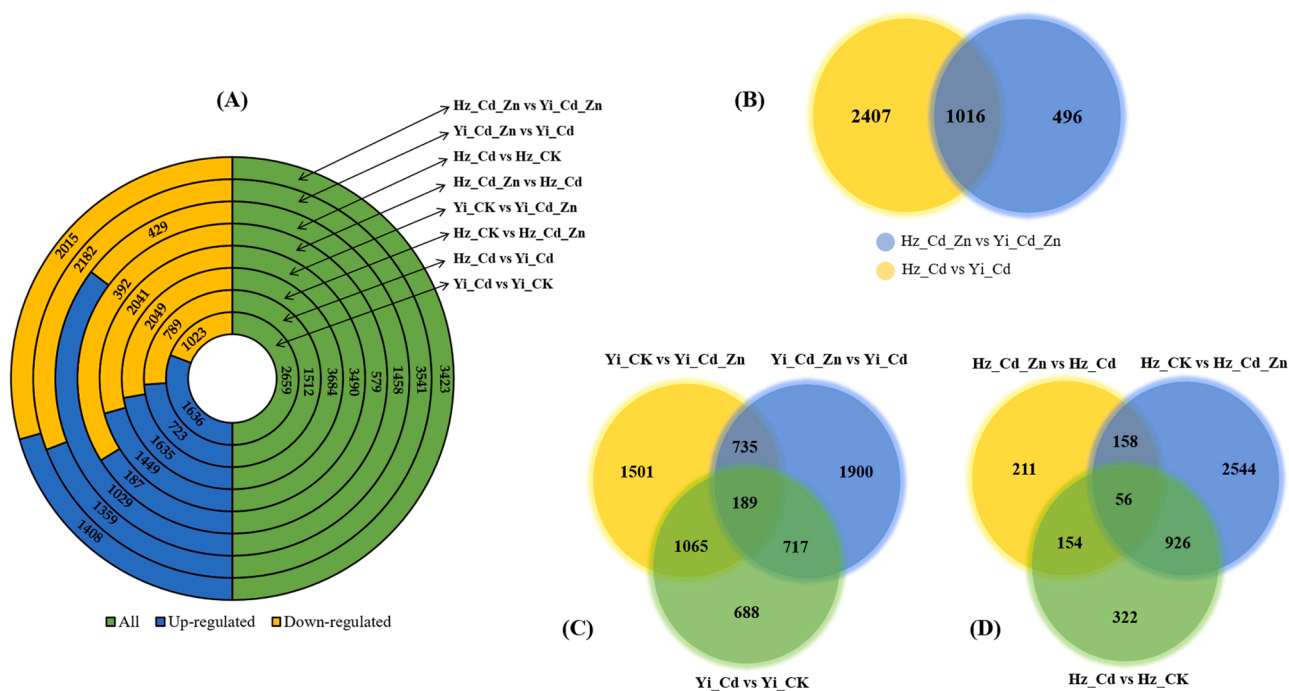
Fig. 1. Comparative gene counts of DEGs between two rice genotypes under Cd and Cd+Zn treatments. (A) differential gene counts between Hz (Heizhan-43) and Yi (Yinni-801) under Control (CK), Cd (15 μM) and Cd (15 μM)+Zn (50 μM) treatments; (B) Venn diagram displaying overlaps among DEGs in Hz-43 *vs* Yi-801 (Cd_Zn *vs* Cd); (C) Yinni-801 (Yi_CK *vs* Yi_Cd_Zn, Yi_Cd *vs* Yi_CK and Yi_Cd_Zn *vs* Yi_Cd); and D, Heizhan 43 (Hz_CK *vs* Hz_Cd_Zn, Hz_Cd *vs* Hz_CK and Hz_Cd_Zn *vs* Hz_Cd).
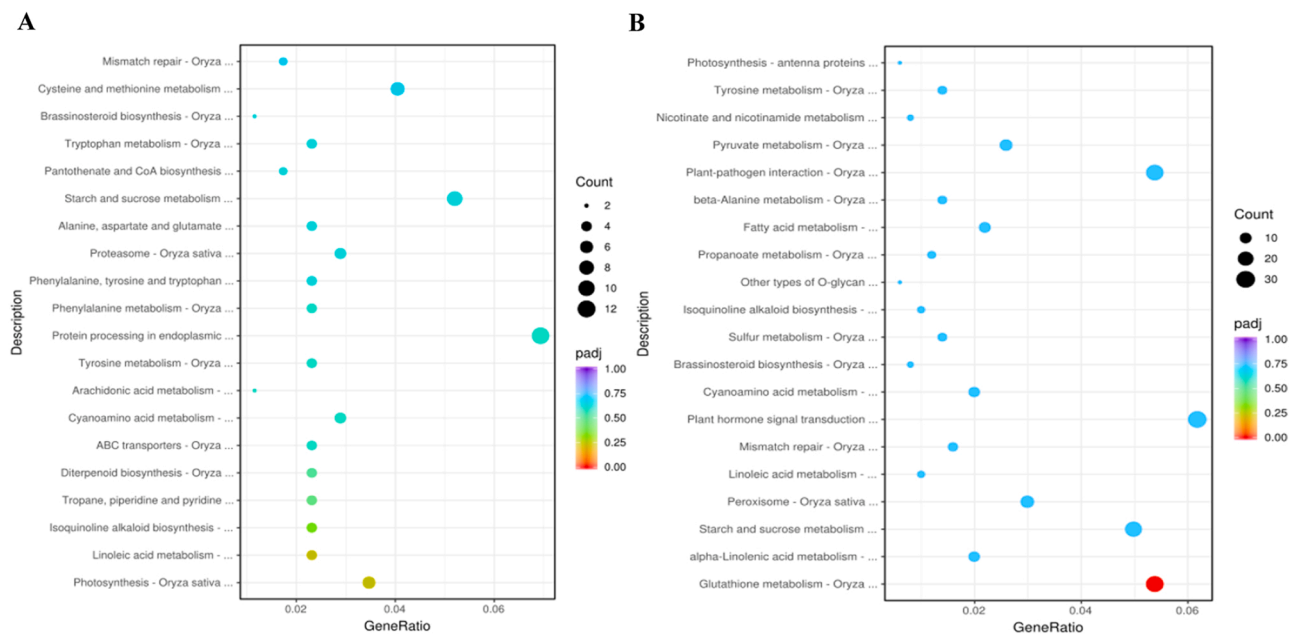

Fig. 2. Scatter plot of enriched KEGG analyses of DEGs. The most significant enriched 20 pathways were revealed in A-B. The DEGs sets "Hz_Cd *vs* Yi_Cd" and "Hz_Cd_Zn *vs* Yi_Cd_Zn", respectively. Each circle represents a KEGG pathway, the ordinates represent the pathway name, and the abscissa represent the rich factor. The color of the circle represents the *q* value; the size of the circle represents the number of genes enriched in the pathway.

nicotinamide metabolism, peroxisome, α-linoleic acid metabolism, β-alanine metabolism, propanoate metabolism, plant hormonal signal transduction and pyruvate metabolism pathways were uniquely enriched (Fig. 2). In case of Hz_Cd_Zn *vs* Hz_Cd, riboflavin metabolism, stilbenoid, diarylheptanoid and gingerol biosynthesis, ascorbate and aldarate metabolism, glycosaminoglycan degradation, fatty acid degradation and flavonoid biosynthesis were enriched specifically. Biosynthesis of amino acids, DNA replication, steroid biosynthesis,

citrate cycle, amino- and nucleotide-sugar metabolism, photosynthesis antenna proteins, ribosome and phagosome were enriched uniquely for Yi_Cd_Zn *vs* Yi_Cd (Fig. S6).

3.3. Gene ontological categorization of DEGs

To investigate the DEGs further, functional annotation was performed based on gene ontology (GO) categories (Fig. 3), and the
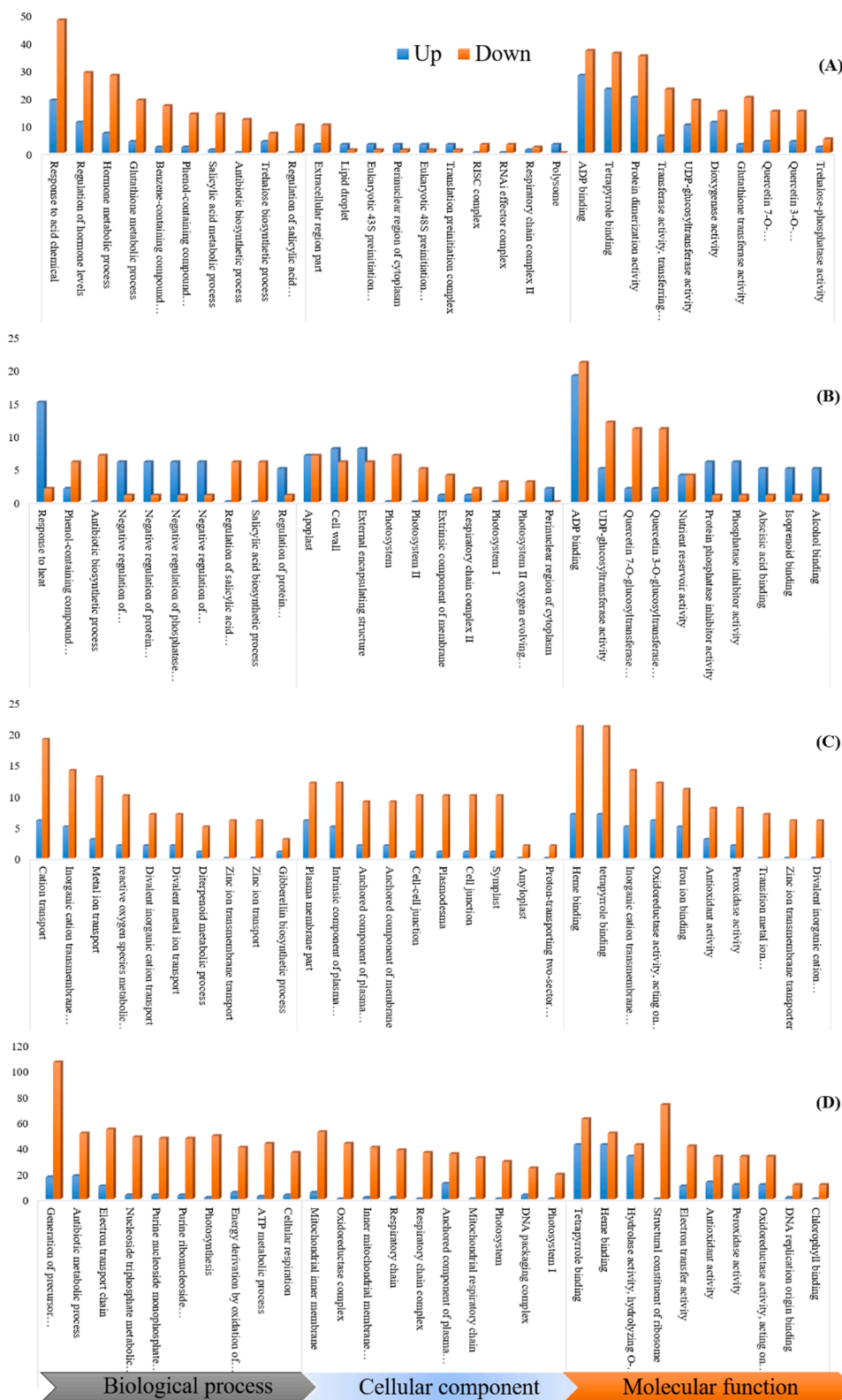
Fig. 3. Relative comparative gene ontology (GO) in Heizhan-43 under $Cd_{15}+Zn_{50}$ in comparison to Yinni-801 under $Cd_{15}+Zn_{50}$ (A), Heizhan-43 under Cd_{15} in comparison to Yinni-801 under Cd_{15} (B), Heizhan-43 under $Cd_{15}+Zn_{50}$ in comparison to its Cd treated counterparts (C), and Yinni-801 under $Cd_{15}+Zn_{50}$ in comparison to its Cd treated counterparts (D). GO terms were organized contingent on q-values < 0.05.

enrichment results suggested that out of 2705 gene sets, Hz_Cd *vs* Yi_Cd comparison showed a higher number of up-regulated DEGs, i.e., 1843 (113.8% higher) in Yi_Cd against 862 in Hz_Cd. In case of Yi_Cd_Zn *vs* Yi_Cd, again the number of DEGs were found higher in Yi_Cd (1980;

173% higher) against Yi_Cd_Zn (725). When compared, Hz_Cd_Zn *vs* Yi_Cd_Zn displayed 1401 (8.2% higher) up-regulated genes in Yi_Cd_Zn against Hz_Cd_Zn (1295). The final comparison between Hz_Cd_Zn *vs* Hz_Cd revealed 54.5% more (1642) up-regulated DEGs in Hz_Cd against

Hz_Cd_Zn (1063). The domains included in the GO analysis were: i) biological process (BP), ii) cellular component (CC), and iii) molecular function (MF). The functions brought forth by comparing Hz_Cd_Zn and Hz_Cd were ROS metabolic process, gibberellin biosynthetic process, diterpenoid metabolic process and ionic transport such as cation transport, divalent inorganic cation transport, metal ion transport, divalent metal ion transport, inorganic cation transmembrane transport (up/down-regulated genes) as well as zinc ion transport and zinc transmembrane transport (strictly down-regulated) (Fig. 3C). For Hz_Cd *vs* Yi_Cd, response to heat was the most enriched among related biological process terms but the genes governing antibiotic biosynthetic process, regulation of salicylic acid biosynthetic process and salicylic acid biosynthetic process were down-regulated (Fig. 3B). Additionally, there was a surge in the up-regulation of negatively regulating genes of phosphoprotein phosphatase, protein dephosphorylation, phosphatase activity and dephosphorylation. In case of Hz_Cd_Zn *vs* Yi_Cd_Zn, response to acid chemical expressed a highest number of DEGs (up/down-regulated) and a highest gene ratio among the related biological terms (Fig. 3A). Moreover, the regulation of hormone levels and hormone metabolic process also stood prominent (both up- and down-regulated genes). Most of the cellular component-related genes were involved in mitochondrial inner membrane and anchored component of plasma membrane (up/down-regulation) in Yi_Cd_Zn *vs* Yi_Cd; whereas, intrinsic component of plasma membrane and plasma membrane part were highlighted for Hz_Cd_Zn *vs* Hz_Cd. Pairwise comparison of Hz_Cd_Zn *vs* Yi_Cd_Zn revealed statistically non-significant gene enrichment for cellular components with 20 genes for extracellular region part (up/down-regulated), and almost the same pattern was exhibited by the genes expressed for Hz_Cd *vs* Yi_Cd, where apoplast, external encapsulating structure and cell wall reached a count of 20 (up/down-regulated) genes of the molecular function-related genes. A higher gene count was observed for Yi_Cd_Zn *vs* Yi_Cd, mainly involved in structural constituents of ribosome, hydrolase activity, tetra pyrrole- and heme-binding (up/down-regulated) (Fig. 3D).

3.4. Stress responsive DEGs

In total, 84 stress related DEGs were found in rice roots, among which 29 were encoding heat shock proteins, 11 were heat stress TFs, 6 were zinc finger stress associated proteins and 6 were ABA stress ripening protein encoding genes. Stress related DEGs regulation took place in a genotype and treatment dependent manner. All treatments included, 13 stress related DEGs were up-regulated, while 16 were down-regulated (Table 1). A universal stress-induced DEG (*Os05g0355400*) was expressed exclusively under Yinni-801 under $Cd_{15}+Zn_{50}$ application, indicating the unique role of Zn in attenuating Cd toxicity in this genotype. Correspondingly, a 50.2 KDA Class I Heat Shock Protein (*Os09g0534600*) was exclusively expressed in Heizhan-43 under $Cd_{15}+Zn_{50}$, which clearly depicts the genotypic difference in response to Cd and Zn supply. Upon alone Cd and Zn addition, expressions of 11 known metallothioneins were altered noticeably. Interestingly, one novel TF transcripts was identified which belonged to PF01439: metallothionein transcription family (novel.1068) with an elevated expression under both treatments but more prominently under Cd stress in both genotypes. In addition, 69% DEGs were up-regulated in Yinni-801 under $Cd_{15}+Zn_{50}$ reaching more than 6 $\log_2$-fold-change values specifically for TAU class GST5 (*Os09g0367700*), GST38 (*Os06g0227500*) and GST19 (*Os10g0527400*). Catalase (CAT) A was up-regulated reciprocally under all treatments in both genotypes, while catalase (CAT) C specifically up-regulated in Yinni-801 as presented in Table S3.

3.5. Memberane transport related DEGs

In this study, a number of transport related DEGs were regulated, including ATP-binding cassette transporter related DEGs that belonged

Table 1
List of stress responsive DEGs in Heizhan-43 and Yinni-801 under Cd and Cd+Zn treatments.

Gene ID	Cd		Cd+Zn		Description
	$\log_2$ fold change		$\log_2$ fold change		
	Heizhan-43	Yinni-801	Heizhan-43	Yinni-801	
Os03g0143400	-0.24	1.17	-0.21	1.19	Heat-Shock protein 60
Os03g0218500	1.29	-0.15	0.73	0.96	HSP 70
Os04g0107900	1.19	0.03	1.18	0.51	Heat-shock protein 90.1
Os01g0136100	4.30	0.42	3.94	1.24	Class I Heat-Shock Protein 1 (16.9 KDA)
Os01g0136200	1.66	-0.39	1.90	0.09	Class I Heat-Shock Protein 2 (16.9 KDA)
Os03g0266900	4.25	2.79	4.31	4.48	Class I Heat-Shock Protein (17.3 KDA)
Os01g0135900	1.04	-0.06	1.76	1.25	Heat-Shock Protein 2 (17.9 KDA)
Os03g0267000	0.00	-0.31	0.18	1.18	Class I Heat-Shock Protein (18 KDA)
Os02g0217900	0.56	0.45	1.13	0.13	HSP19.0
Os11g0244200	1.93	0.00	2.36	0.14	HSP21.9
Os02g0758000	0.50	1.56	0.60	2.49	Class I Heat-Shock Protein (24.1 KDA)
Os03g0245800	3.43	0.04	3.47	0.92	Heat-Shock Protein (26 KDA)
Os09g0456800	1.73	1.90	1.22	1.34	Heat Stress TF B1
Os02g0232000	0.48	0.52	0.96	1.57	Heat Stress TF C2A
Os06g0553100	-0.14	1.28	-0.14	1.78	Heat Stress TF C2B
Os09g0385700	2.24	2.33	2.14	2.78	Stress associated protein gene 17, zf-AN1
Os01g0959200	4.28	3.91	4.28	4.04	ABA Stress Ripening-Inducible 4 Protein
Os01g0959100	3.97	2.64	4.03	3.60	ABA Stress Ripening-Inducible 3 Protein
Os01g0963600	2.68	3.65	1.78	0.95	ABA Stress Ripening-Inducible 2 Protein
Os02g0543000	1.81	2.53	2.14	2.75	ABA Stress Ripening-Inducible 1 Protein
Os07g0687900	0.95	2.18	3.24	4.56	Water Stress Inducible Protein 76
Os03g0348900	0.86	1.08	0.77	1.73	Stress-related RING Finger Protein 1
Os05g0355400	-0.70	0.07	0.52	1.13	universal stress-induced protein A
Os02g0134200	2.38	1.98	2.15	3.11	Stress-Tolerance and Grain-Length
Os10g0569800	5.60	3.87	5.23	4.00	Defense-related gene/RIR1b, Rapid alkalization factor 30
Os03g0195100	-0.04	2.09	0.41	1.62	Aberrant growth and death 2 [AGD2]-like defense response protein 1
Os01g0864500	3.03	2.27	2.86	3.56	Salt and drought sensitive gene 1
Os09g0490200	0.44	0.28	0.72	0.84	Ethylene Insensitive-like gene 3
Os08g0508700	0.95	1.21	1.32	1.89	

Table 1 (*continued*)

Gene ID	Cd		Cd+Zn		Description
	log$_2$ fold change	log$_2$ fold change			
	Heizhan-43	Yinni-801	Heizhan-43	Yinni-801	
Os04g0618700	0.29	0.27	-0.02	0.81	Ethylene Insensitive-like gene 4
Os03g0790500	4.81	1.35	3.37	1.59	Flagellin sensitive2
Os11g0211800	1.85	1.64	0.47	-0.64	GA insensitive dwarf1
novel.1068	5.17	3.47	2.90	2.44	Drought Tolerance 11
Os11g0704500	-0.07	-1.57	0.48	0.20	PF01439: Metallothionein
Os03g0288000	3.15	1.98	1.20	0.73	Metallothionein I-1A
Os01g0974200	-0.30	1.14	-2.22	-1.67	Metallothionein I-1B
Os01g0200700	1.81	-0.06	0.16	-1.53	Metallothionein I-2B
Os12g0570700	2.76	3.63	1.97	3.67	Metallothionein I-3A
Os12g0571100	3.76	2.73	2.99	3.05	Metallothionein I-4A
Os12g0567800	3.53	0.67	2.35	0.61	Metallothionein I-4C
Os01g0149800	1.63	0.32	1.77	0.93	Metallothionein 1F
Os05g0111300	1.02	0.77	-1.10	-1.64	Metallothionein 2A
Os01g0149200	0.04	0.52	0.27	0.93	Metallothionein 2B
Os05g0202800	5.52	5.96	5.20	5.05	Metallothionein 2D
Os07g0580900	1.62	1.14	-0.01	0.71	Metallothionein 3B
Os02g0668100	0.83	0.78	0.08	0.31	geranylgeranyl diphosphate synthase 1
Os03g0188100	1.22	1.20	1.51	2.52	geranylgeranyl diphosphate synthase 2
					Multidrug & Toxic compound Extrusion protein 1

Note: Treatments included Cd (15 μM) and Cd + Zn (Cd 15 μM + Zn 50 μM).

to PDR (Pleiotropic Drug Resistance), MRP (Multidrug Resistance) and members of A, B, E, G and I families of ABC transporters (50), phosphate (10), Zn (9), potassium (16), peptide (4), sulfate (7), copper (5), nitrate (4), vacuolar (2), silicon (2), and ammonium (2) transporters (Fig. 4 and Table S4). Divergence in the expression of the DEGs was observed contingent on treatment and genotype. The expression of ABC transporters under Cd$_{15}$+Zn$_{50}$ was considerably higher in both genotypes than under alone Cd stress, the ABC type G transporter (family member 5) had higher expression levels under Cd$_{15}$ stress than in combined treatment. Additionally, ABC type B transporter (family member 12) expressed exclusively in Yinni-801 under both treatments, with a higher expression under combined Cd and Zn. Furthermore, Zn addition raised the expression of the Zn transporters in Yinni-801 than Heizhan-43. Likewise, a distinct amplification in the expression of *Os03g0575200* (high-affinity K$^+$ transporter 16) was observed in Yinni-801 under both treatments, while HAKT8 and HAKT13 expressed in both genotypes under Cd$_{15}$+Zn$_{50}$. Increased expression of potassium transporters in Yinni-801 indicates an approach to attain better growth under stress conditions and strive for Cd dilution effect. A putative scheme of nutrient cross-talk has been devised based on regulation of nutrient transporters and up/down stream DEGs expression under Cd+ Zn treatment (Fig. 5).

3.6. DEGs encoding transcription factors and key proteins regulating hormonal pathways

Many DEGs encoding transcription factors, including WRKY (63), NAM (36), AP2/ EREBP (48), bZIP (47), Myb_DNA-binding (67), were exclusively/ inclusively expressed (Table S5 and Fig. S5). It is worth noticing that a number of bHLH (63) proteins were up-regulated in Yinni-801 only under Cd stress, while WRKY72 (*Os11g0490900*) showed an increased expression only under Heizhan-43 (both Cd$_{15}$ and Cd$_{15}$+Zn$_{50}$ treatments). Moreover, a novel bZIP_2 TF (novel.863; PF07716: Basic region leucine zipper), with comparatively higher expression in Yinni-801 under both treatments than Heizhan-43, was identified. Out of 48 AP2 TF related DEGs, most were suppressed slightly, while only 2 were mutually up-regulated under both treatments in two genotypes (ERF59 and ERF68). Ethylene response factor 6 expressed positively only in Heizhan 43 under both treatments, while ERF66 and ERF72 expressed in both genotypes under Cd. Additionally, *dehydration responsive element binding protein 1 C* (*DREB1C*) and *ERF79* were expressed only under Cd$_{15}$+Zn$_{50}$ in Yinni-801, which suggests the role of AP2 TFs in Cd$_{15}$ and Cd$_{15}$ +Zn$_{50}$ treatment response. A schematic representation of involved phyto-hormones regulating abiotic stress and their interaction with chiefly enhanced KEGG pathways is depicted in Fig. 6. Among the two genotypes, only Yinni-801 up-regulated the plant hormonal signal transduction pathways under Cd$_{15}$+Zn$_{50}$ treatment. There were no DEGs up-regulated in Heizhan-43 for MAPK pathway as well as hormonal signal transduction pathways. It is quite remarkable to see such distinct genotypic difference in response to the supplementation of Zn against Cd in rice plants.

4. Discussion

4.1. Cadmium exposure elicited stress responsive genes

It has been documented that plants rapidly increase the expression of stress responsive genes with the onset of stress. In the current study, a number of heat stress TFs and heat shock proteins were expressed. Zhao et al. (2011) elucidated that cadmium-stress induces oxidative stress resulting in HSPs expression which in turn inhibits Cd-induced damage in rice plant cells. Heavy metal stress disrupts the confirmation of nascent proteins and an intiation of protective protein production in plants, which might overload the folding process in endoplasmic reticulum, leading to ER stress and a large number of misfolded proteins (Hasan et al., 2017). Current results infer that, although, multiple HSPs were generated mutually under Cd$_{15}$ and Cd$_{15}$+Zn$_{50}$ treatments in both genotypes, the presense of Zn increased the number of low molecular weight (*e.g.,* 17.3 (*Os03g0266900*), 17.9 (*Os01g0135900*) and 24.1 (*Os02g0758000*) KDA) HSPs, which help to prevent misfolded protein aggregation. ABA-stress-ripening (ASR) family is another cluster of proteins that are induced in plants under cold, salinity and water deficit conditions to offer protection, and are known to be conserved within kingdom plantae (González and Iusem, 2014). This study found a number of ASR genes reciprocally up-regulated in stressed plants which validates their role in Zn-endowed tolerance against Cd toxicity. Interestingly, salt and drought sensitive gene 1 (*Os01g0864500*) also expressed inclusively. These results are supported by the physiological, biochemical and electrophysiological responses observed in the previous studies (Adil et al., 2020a; 2020b).

4.2. Cd/Zn treatments modulate distinctive DEGs for the transport of macronutrients

Uptake of essential nutrients as well as toxic elements depends upon membrane transport proteins acting as channels, and in current research certain ABC transporter related DEGs have been identified, belonging to PDR, MRP and members of A, B, E, G and I subfamilies of ABC transporters. ABC transporters had previously been designated as
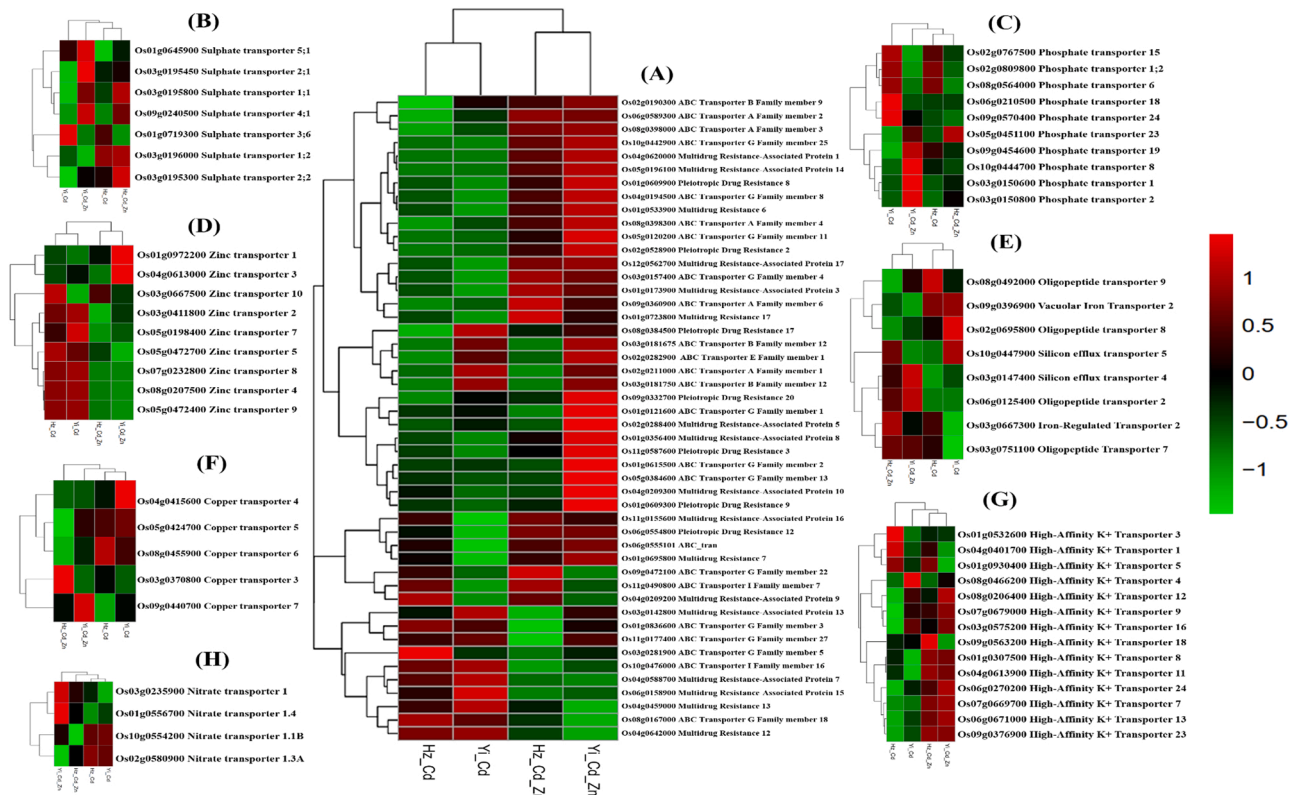
Fig. 4. Hierarchical cluster analysis of identified (A) ABC, (B) sulphate, (C) phosphate, (D) zinc, (E) silicon, peptide and iron, (F) copper, (G) potassium, and (H) nitrate transporter genes expressing differentially (DEGs) in rice root meristems after 40 days of germination. Relative levels of expression are displayed by a color gradient from low (green) to high (red) and are indicated by a scale. Gene counts were represented as *z*-scores in the log2 scale. Data represent means of three independent replicates ± SD.

transporters solely engaged in final detoxification process, *i.e.*, vacuolar deposition, however a study conducted by Kang et al. (2011) has shown their involvement extending far beyond that, for instance, in plant's nutrition and development as well as in response against abiotic stressors. Also, ABC transporter types A, B and G are associated with various metabolic mechanisms encompassing phyto-hormone transport and pathogen defense. Certainly, this might clarify their elevated expressions under Zn supplementation in comparison to the alone Cd treatment in both genotypes. An inclusive expression of MATE protein 1 (*Os03g0188100*) in both genotypes under all treatments, specifically under Cd+Zn in Yinni-80 confirms the role of MATE family transporters and PDR genes in Cd detoxification *via* its elimination out of the cytoplasm, as they have previously been detected to be up-regulated in rice when exposed to Cd stress in a study conducted by Ogawa et al. (2009). ATP-binding function of *MATE* genes facilitates plants against Cd stress through conjugating Cd ions with metal-chelators (metallothionein, phytochelatins and glutathione), turning Cd ions into nontoxic complexes that could easily be extruded from the cells.

As opposed to the Cd treatments, most of the ZIP transporters downregulated under $Cd_{15}+Zn_{50}$ treatments in both genotypes, except for *OsZIP1* and *OsZIP3*, in Yinni-801. Reportedly, zinc exerts antagonistic impact on iron (Fe) and phosphorus (Pi) (Xie et al., 2019). Of note, Naeem et al. (2018) stated that supplementation of potassium (K) with optimized Zn and Pi application to plants fully retrieve Pi-induced loss in grain Zn contents and also increase grain Zn uptake in wheat plants. In this study, multiple high affinity K transporters under combined Cd and Zn treatments were up-regulated, specifically in Yinni-801 genotype indicating a mechanism to revive the osmotic potential and stomatal activity for the maintenance of transpiration/CO_2 intake. As reported by Singh et al. (2020), the deficiency of K in rice instigates the accumulation of jasmonic acid's bioactive form, i.e., JA-isoleucine in rice, which

in turn modulates various K transporters and root architectural modifications. It was quite interesting to observe an increased expression level of jasmonyl-L-isoleucine synthase 2 (*Os01g0221100*) under Cd+Zn treatment, more so in Yinni-801, which explain the recuperative tendencies of this genotype against Cd toxicity with the aid of Zn.

Cadmium toxicity negatively affects nitrate transport and assimilation in plants by regulating many nitrate-related genes, for instance nitrate transporter (NRT) and glutamate dehydrogenase (Shaofen et al., 2019). In the current study, among 4 DEGs regulating nitrate transport, *OsNRT1.1B* (*Os10g0554200*) and *OsNRT1.3A* (*Os02g0580900*) were mutually expressed in tested genotypes under Cd_{15}, while *OsNRT1* (*Os03g0235900*) and *OsNRT1.4* (*Os01g0556700*) were expressed significantly only under $Cd_{15}+Zn_{50}$ in Yinni-801, which indicates the impact of Zn on nitrate transportation within rice roots. Chen et al. (2012) explained that reallocation of nitrate to roots is a typical mechanism that increases Cd endurance in plants. Due to a difference in nitrate-related genes induction, Cd_{15} and $Cd_{15}+Zn_{50}$ plausibly regulate nitrate metabolism differently. Also, a surge in the expression of phosphate transporters was observed, where *OsPHT8* and *OsPHT19* mutually up-regulated, while *OsPHT1* (*Os03g0150600*) and *OsPHT1;2* (*Os02g0809800*) expressed in Yinni-801 under $Cd_{15}+Zn_{50}$ and Cd_{15} treatments, respectively (Fig. 5). Phosphoenolpyruvate/ Phosphate translocator 3 (PEP/PT3) detectably increased in Yinni-801 under Cd stress and in Heizhan-43 under both treatments. Fabiańska et al. (2019) described the contribution of PHTs and PTs in plant metabolism as well as in signaling processes in sensory plastids of the vascular system. Moreover, Lancilli et al. (2014) stated that in *Brassica juncea*, sulfate (SO_4) transporter *Sultr1;1* and *Sultr1;2a/b/c* serve as fully functional high-affinity SO_4 transporters and the differential behavior of *Sultr1;2* variants after Cd exposure suggest the presence of a unique signal transduction pathway governing root SO_4 uptake under stress-induced
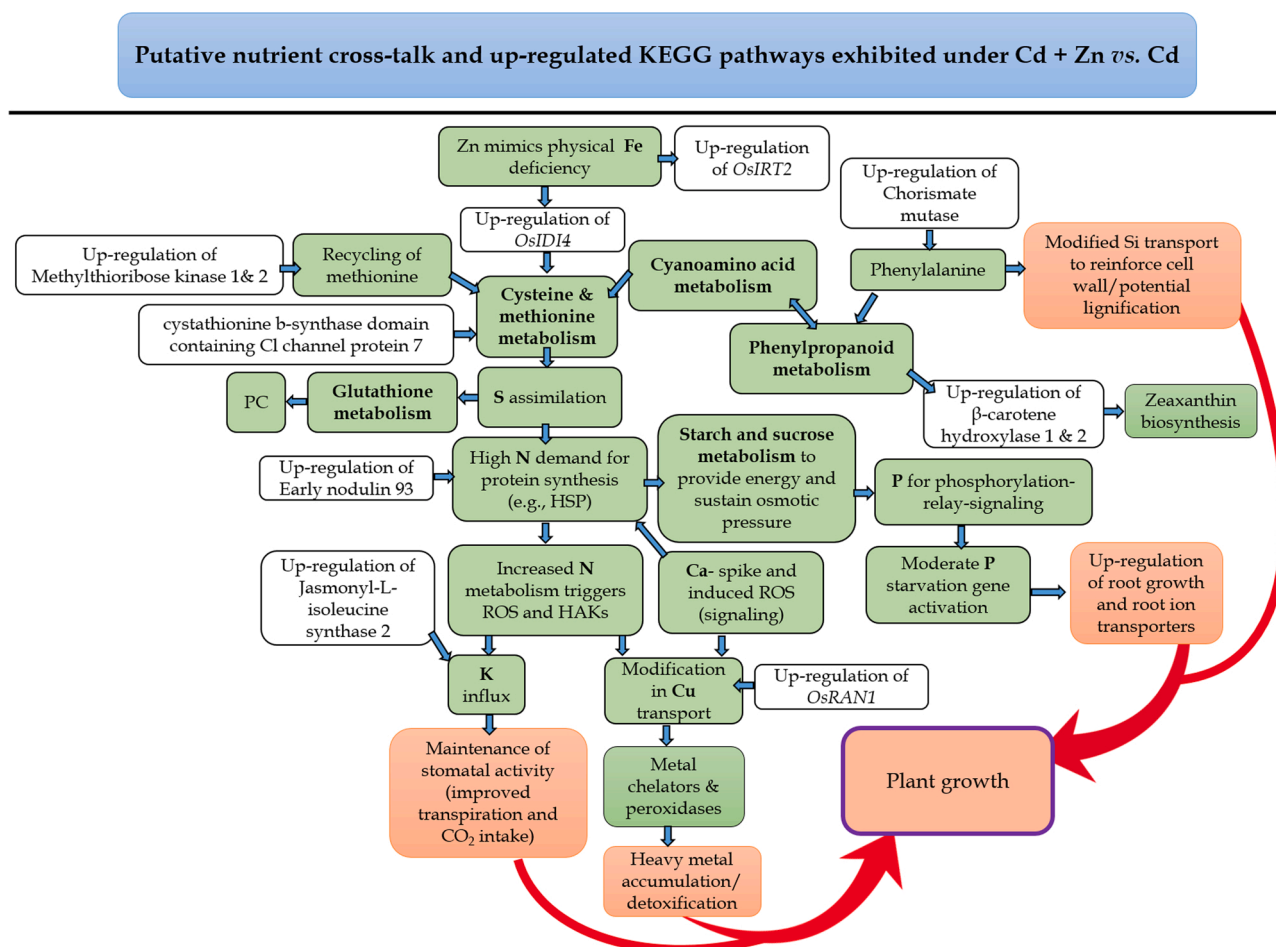
Fig. 5. Putative nutrient cross-talk and up-regulated KEGG pathways exhibited under Cd+Zn *vs.* Cd. Green boxes represent nutrients and their relation with highly enriched pathways, white boxes represent up-regulated genes, and pink boxes represent the successful physiological advantage attained as a result of their cross-talk.

metabolic state. According to current results, *Sultr3;6* was expressed mostly in Yinni-801 under Cd treatment and *Sultr4;1* under $Cd_{15}+Zn_{50}$, while *Sultr*2;1 expressed reciprocally under both treatments in both genotypes. An increased activity of SO_4 transporters under Cd and Zn addition justifies the sulfide incorporation into cysteine (Cys), which acts as an intermediate for methionine (Met), the tripeptide glutathione (g-Glu-Cys-Gly) GSH, and other compounds containing sulfur (Takahashi et al., 2011).

4.3. Cd/Zn treatments regulate distinctive responses of Cd detoxification genes

Phytochelatins are produced from reduced glutathione (GSH), consequently the synthesis of compounds involved in cadmium detoxification and, concurrently, in cadmium tolerance, depends closely on sulfur metabolism (Kulik et al., 2012). Significant up-regulation of a gene encoding putative aminotransferase (*OsIDI4*; *Os09g0453800*) was observed under combined treatment in Yinni-801. This gene catalyzes the transamination step for methionine (Met) regeneration in the Met cycle (Kobayashi et al., 2005). Additionally, expression levels of *OsIDI2* (encoding α-subunit of eukaryotic initiation factor 2B [eIF2Bα]-like protein; *Os11g0216900*) as well as methylthioribose (MTR) kinase 1 (*Os04g0669800*) and 2 (*Os04g0669900*), also increased under similar treatment in both genotypes. Consequently, the results indicate marked enrichment of cysteine and methionine metabolism for Cd+Zn *vs* Cd comparison in genotype Yinni-801 (Fig. 5). Genes encoding metal chelators like metallothioneins (MTs) when overexpress, could enhance Cd tolerance by binding Cd in roots (Sekhar et al., 2011). Remarkably, upon

alone Cd and Zn addition, expression of 11 known metallothioneins was altered noticeably. One novel TF transcripts was identified, which belonged to PF01439: metallothionein transcription family (novel.1068), with an elevated expression under both treatments but more prominently under Cd stress in both genotypes. Glutathione *S*-transferases (GSTs) are multifunctional proteins involved in cellular detoxification by conjugating the GSH to a diverse range of substrates and these S-glutathionylated Cd or Zn complexes are sequestered into the vacuoles, ultimately decreasing the transport and accumulation of heavy metals in rice (Cummins et al., 2011; Jozefczak et al., 2015). Pairwise comparison of Heizhan-43 and Yinni-801 under $Cd_{15}+Zn_{50}$ resulted in a high enrichment of glutathione pathway (ko00480), where most DEGs related to glutathione metabolism were up-regulated in Yinni-801. Strengthening the detoxification mechanism supports the plant under Cd stress, and an increase in Yinni-801 GST activity corroborates the positive impact of Zn in the current study.

Phenylalanine ammonia-lyase (PAL) is ubiquitously found in plants and acts as a catalyst for the first step of phenylpropanoid pathway which involves the deamination of phenylalanine to cinnamate and ammonia aiding in the production of flavonoids/ anthocyanins, coumarins, phytoalexins, lignins and other aromatic compounds (Fraser and Chapple, 2011). It became evident upon analysing ko00940 map in KEGG database, that a variety of DEGs apropos of phenylpropanoids synthesis were regulated in Yinni-801 as compared to Heizhan-43. The regulation of PAL enzymes indicate that Cd stress conditions stimulate defensive mechanism by activating phenylpropanoid metabolism and the resultant lignification leads to secondary cell wall thickening (Tobimatsu et al., 2013). A potential involvement of silicon (Si) in rice
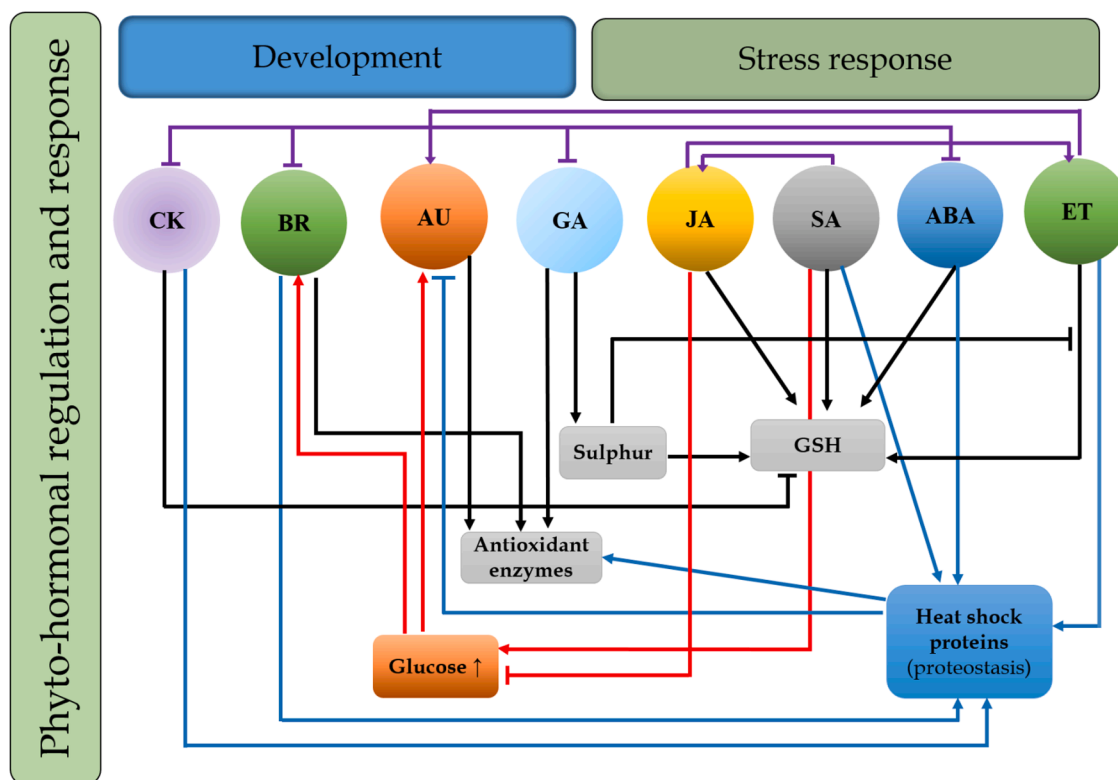
Fig. 6. Schematic representation of phytohormones involved in plant development/ stress response and their plausible association with highly enriched metabolic pathways under $Cd_{15}+Zn_{50}$ treatment. Black lines represent connection with glutathione metabolic pathway and antioxidant enzymes, blue lines indicate involvement with heat shock response, red lines indicate relation with increased glucose contents, and purple lines show hormonal interaction. Arrows and blunt ends exhibit positive and negative correlation, respectively. Abbreviation: CK-Cytokinin; BR-Brassinosteroid; AU-Auxin; GA-Gibberellic acid; JA-Jasmonic acid; SA-Salicylic acid; ABA-Abscisic acid; ET-Ethylene.

root lignification has been documented (Fleck et al., 2011), which is in line with current results as the pattern of silicon efflux transporters changed with the addition of Zn under Cd stress. While working on rapeseed, Ivanova et al. (2010) demonstrated that high Zn contents facilitate copper uptake *via* roots but reduce its transfer to the aerial parts. Similarly, data obtained in this study shows a varying but significant impact of Zn upon copper (Cu) transporters under Cd stress, inferring the efficient use of Cu, as it plausibly impacts phenylalanine ammonia-lyase (PAL), lignin contents and class III plant peroxidases (POXs) (Printz et al., 2016). Several studies have claimed that numerous Cd- or Zn-induced genes imperatively participate in an adaptive response against resultant oxidative stress (Di Baccio et al., 2011; Zeng et al., 2011; Lin et al., 2013); accordingly, the results obtained in this study betoken a reciprocal up-regulation of catalase (CAT-A) under all treatments in both genotypes, whereas for CAT-C, a specific up-regulation (only in Yinni-801) was observed.

4.4. Cd/Zn treatments affect key DEGs for transcription factors and hormone signaling

Protein like messengers, transcription factors (TFs) are attached to cis-regulatory elements and plausibly modulate gene expression by transmitting messages between a stimulus and a response (Borevitz et al., 2000; Zeng et al., 2014). In plants, several TFs have been identified including bHLH (basic helix-loop-helix), WRKY, MYB, bZIP and AP2/EREBP (Apetala ethylene responsive element binding protein) associated with Cd stress (DalCorso et al., 2010). The bHLH TFs regulate many cellular processes, such as hormonal response, metal homeostasis and floral organ development among others, and according to Yang et al. (2018), 167 bHLH proteins have been identified. Estimation of Cd and Fe contents in Arabidopsis exhibited that Cd sequestration improved in

roots and Fe homeostasis enhanced in shoots concomitant with the overexpression of *bHLH39* and *bHLH29* (Wu et al., 2012). Considerably, a number of bHLH proteins were up-regulated in Yinni-801 under Cd stress, which may explain the higher Cd contents in its roots when different element concentrations were analyzed (Adil et al., 2020b). During abiotic stress, WRKY TFs are vital constituents of plant signal transduction feedback. According to Song et al. (2010), the *OsWRKY72* overexpressing lines of *A. thaliana* displayed that this gene interfered with ABA signal and auxin transport pathway regulating multiple physiological processes. Although, *WRKY23* expressed mutually between the genotypes among the treatments but the expression was more pronounced in Yinni-801. The same gene has been demonstrated to furnish Cd tolerance in bent grass (Yuan et al., 2018). Compared to Cd stress, more WRKY genes were up-regulated under $Cd_{15}+Zn_{50}$ treatment in both genotypes (Table S5 and Fig. S7). Comparatively higher expression of a novel bZIP_2 TF (novel.863; PF07716) in Yinni-801 under both treatments signify the participation of bZIP TFs in stress signal transduction in this genotype compared to Heizhan-43. In congruence with prior studies, the TF family MYB expressed highly under Cd stress, more noticeably in Heizhan-43 (Quan et al., 2019; Zeng et al., 2014).

An interactive Cd-rice transcriptomic study exhibited highly expressed AP2/EREBP TFs, with most belonging to DREB and ERF subfamilies (Oono et al., 2014), in consonance with which the present study identified about 48 DEGs encoding AP2 TFs (Table S5 and Fig. S7). This TF family is involved in facilitating plants to acquire resistance against various biotic and abiotic stressesors (DalCorso et al., 2010). Evidently, a novel TF transcript (novel. 466) was identified which belonged to the PF00847: AP2 domain expressing only under Cd stress in both genotypes. Reportedly, ethylene response factor (ERF) subfamily responds to multiple abiotic stressors in plants, including drought and

high salinity; however, out of 48 relevant DEGs identified in this study, only two were mutually up-regulated under both treatments in two genotypes (*ERF59* and *ERF68*). Plant hormones are signaling molecules deemed important for growth regulation as well as for abiotic stress perception and response mechanisms; albeit, the pathways adapted by hormones to achieve these goals are quite intricate (Bücker-Neto et al., 2017). Many components of ABA signaling pathway have been identified in rice, including SnRK2s (Kobayashi et al., 2005), clade A PP2Cs and PYLs (Kim et al., 2015b). Addition of Cd and Zn in both rice genotype differentially activated the *OsPP2Cs* in present study, impeding phosphatase activity and resulting in improved Cd tolerance. In signal transduction pathway of abscisic acid, *Sucrose non-fermenting (Snf1)-related protein kinases of Group2* (*SnRK2s*) act as the precursors of HSPs (Todaka et al., 2015), and Ca^{2+} have been reported to modulate SnRK2-mediated responses directly. The higher expression of *OsSnRK2*-calcium sensor 1 in the roots suggests that Yinni-801 responded differently to Zn addition under Cd stress.

Phyto-hormones, namely salicylic acid (SA), gibberellic acid (GA), jasmonic acid (JA), ABA, and ethylene (ET) have been linked either directly or indirectly with glutathione metabolism (Nguyen et al., 2021), participating in the detoxification of Cd-induced ROS. There is a positive involvement of brassinosteroids (BR), cytokinins (CK) and some aforementioned plant hormones (illustrated in Fig. 6) in Cd-induced heat shock response, whereas auxin is negatively affected by HSPs (Bücker-Neto et al., 2017). Along with typical hormones, genes involved in sugar signaling (*e.g.*, Hexokinase 7; *Os05g0187100*) as well as certain rapid alkalization factors, such as defense-related gene/RIR1b (Rapid alkalization factor 30; *Os10g0569800*) were also found to be highly expressed under combined Cd-Zn treatment in both genotypes, more pronouncedly in Yinni-801 (Sakr et al., 2018). For instance, geranylgeranyl diphosphate (GGPP) is formed by GGPP synthase (GGPPS) in plastids, which functions as an essential for the biosynthesis of gibberellins, chlorophylls and carotenoids (Zhou et al., 2017). A ubiquitous expression of *GPPS1* and *GPPS2* at lower levels is reported in all plant tissues (Beck et al., 2013). Interestingly, the expression levels of *GPPS1* (*Os07g0580900*) and *GPPS2* (*Os02g0668100*) were elevated under Cd treatments than under Cd+Zn in both genotypes, which suggests that rice roots increased the production of above mentioned metabolic terpenoids to survive the oxidative stress induced by Cd, while Zn supplementation had a rescuing effect (Table 1).

5. Conclusion

The avenue of RNA-Seq technique helped to comprehend the complex regulatory gene networks that underlie zinc mediated mellowing of Cd toxicity. The results revealed that there was a distinct variation between the two genotypes at transcriptional level. Genes involved in hormonal signal transduction to activate defensive mechanisms inside the plants were highly expressed. Some genes encoding TFs such as Pkinases, WRKY, bHLH, AP2/EREBP, MYB, NAM and zinc finger proteins were mutually regulated under both treatments, which partake significantly in the homeostasis of Cd and Zn. Interestingly, a number of *protein phosphatase 2 C* (*PP2C*) genes were up-regulated under $Cd_{15}+Zn_{50}$ treatment along with *SnRK2*-Ca sensor 1 gene, which significantly up-regulated under the same treatment only in Yinni-801. These results indicated that Yinni-801, when provided with Zn, adopts the strategy to manage Cd-stress induced hormonal imbalance, manages the production of HSPs, generates enough detoxifying, chelating and excluding agents and keeps the photosynthetic machinery going so as to provide enough energy for the continuation of plant development and growth, which may otherwise, significantly retard. Although addition of Zn boosted Cd-tolerance in Yinni-801, the high dosage did result in certain degree of toxicity, specifically in Heizhan-43. This study explored and identified the novel DEGs and pathways underlying Zn-mediated amelioration of Cd toxicity, pivoting a path towards advanced opportunities for studying Cd and Zn relations, and is also a benchmark for rice breeders to improve Cd tolerance.

Ethical approval

This article does not contain any studies with human participants oranimals performed by any of the authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.113128.

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