Identification of potential Zinc deficiency responsive genes and regulatory pathways in rice genotypes by Weighted Gene Co-expression Network Analysis

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Abstract

Background

Zinc (Zn) malnutrition has been linked to serious health concerns in humans. Targeting genetic biofortification of rice grain Zn can efficiently improve the global Zn nutritional status. To genetically enhance rice grain Zn content, the genetic and molecular mechanisms of Zn deficiency response need to be elucidated. Here, Differential Gene Expression Analysis (DGEA) and Weighted Gene Coexpression Analysis (WGCNA) were established to identify modules of coexpressed genes, the most preserved genes and molecular pathways regulating Zn deficiency response in rice varieties.

Results

Twelve modules of coexpressed genes were obtained by WGCNA using 1649 differentially expressed genes (DEGs) from our DGEA and 2121 Zn genes from earlier studies. Three modules (TTA-M, TRA-M and CA-M) were judged the most relevant for Zn deficiency based on their richness in the well-recognized Zn deficiency responsive (ZDR) genes and molecular pathways. 96 (17%), 188 (47.6%) and 96 (24%) genes from TTA-M, TRA-M and CA-M modules respectively, were significantly expressed in the DGEA. These coexpressed DEGs (CoDEGs) were considered as the most preserved Zn deficiency responsive genes. Of the well-known ZDR genes, only *OsZIP8*, *OsZIP10*, *OsMT1a* and *OsNAAT1* were preserved. Functional annotations for all CoDEGs from the identified ZDR modules showed that glutathione metabolism and biosynthesis of secondary metabolites were the most quickly upregulated molecular pathways. Lastly, a biology-informed gene-gene interaction network analysis (GGIN) indicated that CoDEGs including *OsLSI3*, *OsWOX11*, *OsNRT1.1B*, *OsPSK5*, *OsSWN6* and *OsMID1* strongly interact with the recognized ZDR genes in the ZDR modules. Curiously, these CoDEGs were previously validated for other economic traits in rice.

Conclusion

Findings from this study provide comprehensive insights into the molecular mechanisms of Zn deficiency response in rice and may facilitate gene and pathway prioritization, to enhance Zn use efficiency (ZUE) and Zn biofortification in rice.

1. Background

Zinc malnutrition is affecting about 1.2 billion of people and has been associated with detrimental health concerns including children stunting, weak immune system and brain development (Black, 1998; Dardenne, 2002). Regarding the current pandemic of COVID-19, Zn malnutrition will exacerbate infection rates (Akhtar et al.; Huizar et al., 2020; Van Der Straeten et al., 2020), underlining the need for Zn biofortification.

Fortifying Zn in rice, a staple crop for nearly 3.5 billion of people, can significantly benefit the global Zn nutritional status (Van Der Straeten et al., 2020). Agronomic approaches to enhance grain Zn content

through chemical fertilization for example, are not only uneconomical, but also ineffective, as Zn tends to form insoluble complexes which limit its uptake from plant root tissues (Alloway, 2008). Favorably, natural genetic variations in rice germplasm are sufficient to attain Zn concentration requirements of 28 ppm in the polished rice grain (Van Der Straeten et al., 2020). Zn efficient (ZE) genotypes have relatively higher ZUE and perform better than Zn inefficient (ZI) genotypes under low Zn soils or nutrient solutions (Nanda et al., 2017). Also, ZE genotypes exhibit higher growth of crown roots (Nanda and Wissuwa, 2016a) and greater rates of the exudation of low-molecular weight organic acids (LMWOA) metal-chelators, such as nicotianamine synthase (NAs), mugineic acid (MAs) or 2'-deoxymugenic acid (DMAs) from the phytosiderophore family (Ishimaru et al., 2011; Ptashnyk et al., 2011; Widodo et al., 2010). Such traits have been linked to the loading of both Zn or Fe in rice grains (Banakar et al., 2017; Masuda et al., 2012). In addition to these LMWOAs, Zinc transporter genes mainly *ZIP* (zinc-regulated transporters and iron-regulated transporter proteins), *HMA*s (heavy metal ATPases) and *MTP*s (metal tolerance proteins) are essential for the uptake, distribution, and redistribution of Zn across different plant tissues under low Zn deficiency conditions (Ishimaru et al., 2005; Olsen and Palmgren, 2014). Further, transcription factor (TF) genes such as *OsNAC*, *OsIRO2*, and *OsIRO3* are also known to regulate the expressions of several Zn or Fe responsive genes (Banerjee and Chandel, 2011; Ogo et al., 2006). As to molecular pathways affected by Zn deficiency, previous transcriptomic studies have consistently shown that Zn deficiency responsive (ZDR) genes significantly enrich Zinc transmembrane activity, transmembrane transport activity, phenylpropanoid biosynthesis and glutathione metabolism functional gene sets (Bandyopadhyay et al., 2017; Nanda et al., 2017; Zeng et al., 2019b). Nevertheless, the most conserved ZDR genes and molecular pathways are not known. Also, the interaction networks involving the known (experimentally validated) ZDR genes are yet to be investigated.

Weighted gene co-expression network analysis (Langfelder and Horvath, 2008) is a systems biology approach that leverages on the extent of co-expression between genes to define their connectivity. Subsequently, it allows studying the universal network nature of a transcriptome (Zhao et al., 2010). For example, integrating transcriptomic analysis and WGCNA in rice enabled to find the global Cadmium (Cd)-regulated DEGs (Tan et al., 2017). Also, WGCNA was useful to functionally annotate the genome of rice (Childs et al., 2011) and construct the atlas of maize by integrating different omics data (Walley et al., 2016). In a transcriptome co-expression network, genes are represented by nodes which are linked together by edges based on the weighted co-expression across samples. The most connected genes within a network are assembled as modules, while the highly connected genes within a module are referred to hubs (Zhang and Horvath, 2005). Co-expression network architecture is inherent of cellular organization, with the functional modules built up from several interacting molecules (Hartwell et al., 1999).

Here, DGEA and WGCNA were implemented to identify (1) functional modules of coexpressed genes in Zn-stressed rice genotypes (2) coexpressed DEGs (CoDEGs) and their preserved molecular functions, and (3) genes showing strong connectivity with the experimentally validated (herein referred to well-known or recognized) ZDR genes. Subsequently, modules of coexpressed genes were constructed by combining DEGs from our own samples including ZI (IR26 and IR64) and AUS (WCP22 and KALBOR026) genotypes,

and the other publicly reported ZDR genes. The significance of a module for Zn deficiency response was dictated by the recognized ZDR functional annotations enriched by genes coexpressing in that module. Genes from the identified ZDR modules which are DEGs in our DGEA were judged as the most preserved, and so were their respective functional annotations. These functionally annotated CoDEGs are good candidates to track functional markers which can be incorporated into breeding schemes to improve rice grain Zn content. Lastly, gene-gene interaction network (GGIN) or protein-protein interaction network (PPIN) of the ZDR modules identified genes or proteins with strong connectivity with the well-recognized ZDR genes. We speculate that these identified hub genes could also be involved in the mechanistic of Zn stress response as their linked well-known ZDR genes.

2. Results

Identification of differentially expressed genes.

Gene expression analysis was performed for root and shoot tissues of two ZI (IR26 and IR64) and AUS (UCP122 and KALBOR026) rice genotypes using DESeq2 R package (Love et al., 2014). To obtain sufficient DEGs for the next stage of WGCNA, two approaches were used. A condition-based gene expression analysis (CEA) where gene expression for each ZI or AUS genotype was studied under Zn deficiency condition (Zn supply vs Zn deficiency), and a genotype-based expression analysis (GEA) by setting ZI genotypes as reference level for each of AUS genotype (IR26/IR64 vs UCP122/KALBOR026). Overall, 1649 unique genes (**Table 1 and Additional file 1-10: Table S1-10**) were differentially expressed ($FDR < 0.01$, $\log_2 \text{fold} \geq 2$ or < 2). 1179 of the 1649 DEGs were novel ZDR genes, while 470 DEGs have been reported in previous studies (Fig. 1a **and Additional file 11: Table S11**). Under CEA, the well-known ZDR genes such as *ZIP* (*OsZIP1*, *OsZIP5*, *OsZIP10* and *OsZIP8*), a phytosiderophores family gene (*OsNAAT1*), and a metallothionein gene (*OsMT1a*) were induced by Zn deficiency treatment in ZI or AUS genotypes. Under low Zn supply conditions and GEA, the expressions *ZIP* genes and phytosiderophores family genes (*OsNAAT1* and *OsNAS3*) were significantly higher in the root sample of AUS genotypes than in the same tissue of IR26 genotype (**Additional file 9–10: Table S9-10**).

Functional annotation of differentially expressed genes.

Functional annotations of DEGs (under CEA) by STRING tool version:11 (Mering et al., 2003) indicated that Zinc ion transmembrane transporter activity GO term was significantly enriched by DEGs upregulated by Zn deficiency treatment in both ZI and AUS genotypes. However, significant differences between UCP122 and other genotypes were detected in the enrichment of the DNA-binding transcription factor activity GO term. The latter was significantly upregulated in the root tissue of UCP122 yet downregulated in the same tissue of KALBOR026 and IR26 genotypes. Similarly, Zinc ion transmembrane transporter activity GO term was upregulated in root tissues of UCP122, while for all the other genotypes, it was only upregulated in crown tissues.

Table 1
Number of DEGs obtained under CEA and GEA in AUS and ZI genotypes.

Contrast levels		Contrast categories	Expressed genes (FDR < 0.01)		
			log ₂ fold ≥ 2	log ₂ fold < 2	Total
Zn supply vs Zn Def (CEA)	ZI	IR26_root	93	19	112
		IR26_crown	14	56	70
		IR64_root	20	24	44
		IR64_crown	35	276	311
		Total non-redundant genes	145	312	457
	AUS	UCP122_root	129	11	140
		UCP122_crown	4	10	14
		KALBOR026_root	49	94	143
		KALBOR026_crown	36	8	44
		Total non-redundant genes	203	87	290
ZI vs AUS genotypes (GEA)	Under Zn deficiency				
		IR26 vs UCP122_crown	97	178	275
		IR26 vs UCP122_root	155	387	526
		IR26 vs KALBOR026 root	137	398	535
		IR26 vs KALBOR026 crown	27	55	82
		IR64 vs UCP122_crown	98	83	181
		IR64 vs UCP122_root	46	123	169
		IR64 vs KALBOR026 root	54	205	259
		IR64 vs KALBOR026 crown	115	94	209
		Total non-redundant genes	497	912	1419
	Under Zn supply				
		IR26 vs UCP122_crown	23	37	60
		IR26 vs UCP122_root	58	119	177
		IR26 vs KALBOR026 root	49	80	129
		IR26 vs KALBOR026 crown	11	31	42

Contrast levels	Contrast categories	Expressed genes (FDR < 0.01)		
	IR64 vs UCP122_crown	25	60	85
	IR64 vs UCP122_root	27	157	184
	IR64 vs KALBOR026 root	26	73	99
	IR64 vs KALBOR026 crown	11	51	66
	Total unique genes	129	294	413

Also, cellulose metabolic process was downregulated in crown tissues of all the genotypes but was not enriched by DEGs from the UCP122 genotype. These findings infer that UCP122 cultivar may have unique phenotype under low Zn supply conditions. All the significantly enriched functional gene sets are provided (Table 2 and Additional file 12–15: Table S12-15).

Table 2

Functional annotations of DEGs in ZI and AUS genotypes after Zn deficiency treatment (under CEA).

Samples	Go term/ KEGG pathway_ID	GO term/pathway description	FDR
ROOT_KALBOR026	GO:0015250	Water channel activity	+0.00087
	GO:0015318	Inorganic molecular entity transmembrane transporter activity	+0.00087
	GO:0015075	Ion transmembrane transporter activity	+0.0199
	map00480	Glutathione metabolism	+0.0282
	map00940	Phenylpropanoid biosynthesis	+0.0378
	GO:0140110	Transcription regulator activity	*0.00018
	GO:0003700	DNA-binding transcription factor activity	*0.00065
	GO:0044249	Cellular biosynthetic process	*0.0012
CROWN_KALBOR026	GO:0005385	Zinc ion transmembrane transporter activity	+3.45E-05
	GO:0010333	Terpene synthase activity	+3.45E-05
	GO:0022890	Inorganic cation transmembrane transporter activity	+0.00011
	map00904	Diterpenoid biosynthesis	+0.0036
	GO:0030243	Cellulose metabolic process	*0.0052
	GO:0071555	Cell wall organization	*0.0067
ROOT_UCP122	GO:0005488	Binding	+0.00019
	GO:0003700	DNA-binding transcription factor activity	+0.0045
	GO:0003824	Catalytic activity	+0.0045
	GO:0005385	Zinc ion transmembrane transporter activity	+0.0134
	GO:0030244	Cellulose biosynthetic process	+0.038

+ : GO terms/KEGG pathways significantly (FDR < 0.05) enriched by upregulated genes.

* : GO terms/KEGG pathways significantly (FDR < 0.05) enriched by downregulated genes.

Samples	Go term/ KEGG pathway_ID	GO term/pathway description	FDR
CROWN_UCP122	NO significant annotations found		
ROOT_IR26	GO:0050667	Homocysteine metabolic process	+0.0057
	GO:0008652	Cellular amino acid biosynthetic process	+0.031
	GO:0009086	Methionine biosynthetic process	+0.031
	map00940	Phenylpropanoid biosynthesis	+0.0044
	map01110	Biosynthesis of secondary metabolites	+0.0044
	map00130	Ubiquinone and other terpenoid-quinone biosynthesis	+0.0298
	map00360	Phenylalanine metabolism	+0.0298
	GO:0005488	Binding	*0.0278
	GO:0046872	Metal ion binding	*0.0278
	GO:0003700	DNA-binding transcription factor activity	*0.0371
CROWN_IR26	GO:0005385	Zinc ion transmembrane transporter activity	+1.62E-06
	GO:0006073	Cellular glucan metabolic process	*1.70E-08
	GO:0030243	Cellulose metabolic process	*1.70E-08
	GO:0071555	Cell wall organization	*1.26E-07
ROOT_IR64	GO:0046872	Metal ion binding	+0.0142
	map00940	Phenylpropanoid biosynthesis	+0.006
	GO:0034219	Carbohydrate transmembrane transport	*0.003
	GO:0051119	Sugar transmembrane transporter activity	*0.0026
CROWN_IR64	GO:0005385	Zinc ion transmembrane transporter activity	+1.62E-06
+ : GO terms/KEGG pathways significantly (FDR < 0.05) enriched by upregulated genes.			
* : GO terms/KEGG pathways significantly (FDR < 0.05) enriched by downregulated genes.			

Samples	Go term/ KEGG pathway_ID	GO term/pathway description	FDR
	GO:0006073	Cellular glucan metabolic process	*1.70E-08
	GO:0030243	Cellulose metabolic process	*1.70E-08
	GO:0071555	Cell wall organization	*1.26E-07
+ : GO terms/KEGG pathways significantly (FDR < 0.05) enriched by upregulated genes.			
* : GO terms/KEGG pathways significantly (FDR < 0.05) enriched by downregulated genes.			

Construction of the global network of coexpressed modules of genes.

To construct the global network of coexpressed genes, raw counts from nine Zn-deprived samples were obtained from <https://www.ebi.ac.uk/ena>, then processed and merged with eight Zn-deprived samples from our own study (Fig. 1b and **Additional file 17: Table S17**). Samples from public datasets included ZE (Nipponbare, IR55179 and RIL46) and ZI (KP, IR64, IR74) genotypes. The selected genes for WGCNA were 1649 DEGs from this project and 2121 Zn responsive genes reported from seven previous studies (Fig. 1a and **Additional file 11: Table S11**).

We set the module minimum size and the threshold to merge correlated modules at 16 and 0.6, respectively. The merged raw counts from different projects were corrected for batch effects, tissue types and plant growth stage-mediated covariances. The expression variance was also stabilized as indicated in WGCNA tutorial

<https://horvath.genetics.ucla.edu/html/CoexpressionNetwork/Rpackages/WGCNA/faq.html>. These parameters resulted into 2076 genes which coexpressed across 13 different modules (Table 3), including a module of unassigned genes. All the modules were relabeled with short abbreviations for reference.

Functional annotations of the identified modules.

To associate modules to Zn deficiency responsiveness, GO terms and KEGG pathways enrichment by genes within each module were studied using STRING version:11 (<http://string-db.org/>) (Mering et al., 2003). Apart from genes from P-M module, all the other modular genes significantly enriched at a minimum one GO term or KEGG pathway (Table 3). Intriguingly, molecular functions and genes recognized for Zn uptake, distribution, and grain Zn/Fe content functions in rice, preferably coexpressed in TTA-M, TRA-M and CA-M modules (Fig. 2a). Therefore, all the subsequent characterizations were implemented on these putative ZDR modules.

Table 3
Functional annotations of coexpressed modules of genes.

Module's colour Assigned label Number of genes in a module	Module preserved GO terms preserved KEGG pathways (Bonferroni p -value < 0.05)
Blue TTA-M 555 *	Transmembrane transport activity ; Zinc ion transmembrane transport ; iron ion homeostasis, etc. Metabolic pathways ; Photosynthesis, etc.
Midnightblue TRA-M 395 *	Transcription regulator activity; Zinc ion transmembrane transporter activity, etc. Biosynthesis of secondary metabolites; alpha-Linolenic acid metabolism.
Greenyellow CA-M 393 *	Catalytic activity; metal ion binding, etc. Metabolic pathways; Phenylpropanoid biosynthesis, Glutathione metabolism, etc.
Grey60 AP-M 145	Apoplast No significant KEGG annotations found.
Royalblue PB-M 217	No significant GO terms phenylpropanoid biosynthesis; fatty acid metabolism, etc.
Lightcyan CG-M 65	Cell growth glyoxylate and dicarboxylate metabolism; cutin, suberine and wax biosynthesis; biosynthesis of unsaturated fatty acids.
Darkturquoise PHEN-M 96	No significant GO annotations Phenylpropanoid biosynthesis; phagosome; stilbenoid, diarylheptanoid and gingerol biosynthesis.
Darkgreen PHOT-M 41	Photosynthesis Cyanoamino acid metabolism. Starch and sucrose metabolism; glycine, serine and threonine metabolism.
Darkgrey CA-M 35	Cold acclimation; photosynthesis; response to abiotic stress porphyrin and chlorophyll metabolism.
Black RES-M 122	Response to external stimulus; response to extracellular stimulus Amino sugar and nucleotide sugar metabolism, etc.
Pink DR-M 395	No significant GO annotation found DNA replication, alpha-Linolenic acid metabolism, plant-pathogen interaction.
Purple P-M 79	No significant GO annotations No significant KEGG annotations found.
Grey FAT-M 94	No significant GO annotation found Fatty acid elongation.
* Zn deficiency responsive modules (ZDR modules).	

Zn deficiency responsive modules are putative regulators of Zn deficiency response in rice.

Many of the well-known ZDR genes and their molecular pathways were found in ZDR modules. Genes coexpressing in the TTA-M module included for example, Zinc ion transmembrane transporters (*OsZIP3*, *OsZIP8*, *OsHMA2* and *OsHMA3*), a MAs transporter (*OsZIFL4/OsTOM1*), transmembrane transporters (*OsYSL16*, *OsYSL18* and *OsMTP4*), metal-NA complexes transporters (*OsYSL4* and *YSL6*), metal binding (*OsMT4IC*, *OsMT1B* and *OsMT1a*) and iron homeostasis genes (*OsSHR1* and *OsNRAMP6*). Equally,

functional annotations including Zinc ion transmembrane transporter activity (*OsMTP1* and *OsZIP2*), organic substance (*OsMCM4*, *OsMPK7*, *OsCIPK9*, *OsCESA6*, *OsCSLF6*, etc.), oxidoreductase activity (*OsCKX5*, *OsDAO*, genes from *CYP* family genes, etc.), transmembrane transporter activity (*OsNRAMP6*, *OsMST6*, *OsABCG44*, etc.) and transcription regulator activity (*OsIRO2*, *OsERF67*, *OsONAC12*, *OsSWN6*, *OsDREB1C*, *OsJAZ TFs* genes, etc.) were detected the TRA module. CA-M was another important module, with catalytic activity as its most enriched GO term. Genes including *OsZIP10* and MAs biosynthesis genes (*OsSAMS1*, *OsSAMS2*, *OsNAAT1* and *OsDMAS1* coexpressed in the CA-M module. Collectively, these findings are suggestive of potential key roles of ZDR modules in the regulating Zn deficiency molecular mechanisms in rice. All the genes coexpressed across ZDR modules and their function annotations are provided (**Additional file 18–23: Table S18-23**).

Preserved Zn deficiency responsive genes and their molecular functions.

To find the most preserved genes, we intersected ZDR module genes with DEGs from the DGEA (under CEA). 96 (17%), 188 (47.6%) and 96 (24%) genes from TTA-M, TRA-M and CA-M modules respectively, were responsive to Zn deficiency in our DGEA (**Fig. 2b and Additional file 24–28: Table S24-28**). Among all the well-known ZDR genes, the most preserved were Zinc transmembrane transport genes *OsZIP8* (TTA-M) and *OsZIP10* (CA-M) which were significantly upregulated in crown tissues of all ZI genotypes and KALBOR026 genotype, a metallothionein gene *OsMT1a* (TTA-M) which was upregulated in the root tissue of UCP122 genotype, and a phyto siderophores family gene *OsNAAT1* (CA-M) which upregulated in root and crown tissues of all AUS genotypes and IR64 genotypes (**Additional file 29–30: Table S29-30**). Enrichment analysis of all coexpressed DEGs (CoDEGs) showed that genes which upregulated in the root tissue of UCP122 genotype affected the highest number of functional sets vs genes from all the other sample genotypes.

Of the 140 genes upregulated in root tissue of UCP122 genotype (Table 1), 88 genes coexpressed in TRA module, and significantly enriched 77 GO terms including DNA-binding transcription factor activity, cellulose biosynthetic processes, catalytic activity, response to stimuli, etc (**Fig. 2c and Additional file 34: Table S34**). Contrary, in the same module, five of the GO terms upregulated in the root sample of UCP122 genotype (including DNA-binding transcription factor activity, binding, sequence specific DNA binding, etc.) were rather downregulated in the root tissue of KALBOR26 genotype. This was consistent with our findings with the DGEA that suggested possible phenotypic differences between UCP122 and other varieties during Zn deficiency conditions. On the other hand, another set of CoDEGs from the TTA-M module upregulated the glutathione metabolism in root tissues of both AUS genotype. The other preserved and upregulated functional gene sets included the secondary metabolites biosynthesis (CA-M module) and transmembrane transport (TTA-M module) which were significantly enriched by CoDEGs from the root sample of IR26 and KALBOR026 genotypes, respectively (**Fig. 2c**).

Identification of hub genes in Zn deficiency responsive modules.

The Maximal Clique Centrality (MCC) algorithm (Chin et al., 2014) was applied to identify hub genes across ZDR modules. The first 30 hub genes as ranked by their scores from the strongest to weakest, are

provided (**Additional file 35–37: Table S35-37**). Among the well-known ZDR genes, only *OsZIP3* and *OsYLS18* (both from TTA-M module) featured in the top 30 hub genes. To identify the most Zn deficiency relevant hub genes, we rather adopted a biology-informed approach where CoDEGs directly connected with at least three recognized ZDR genes, or proteins directly interacting with at least one well-known ZDR protein were considered as ZDR specific hub genes or proteins (**Additional file 38: Fig. S1-2**). The well-known ZDR genes or proteins for which the interactions were searched against were: *OsZIP10*, *OsNAAT1*, *OsSAMS1*, *OsSAM2*, *OsDMAS1* and *OsYSL 10* (CA-M module); *OsIRO2*, *OsZIP2*, and *OsNRAMP6* (TRA-M module); *OsZIP3*, *OsZIP8*, *OsMT1a*, *OsHMA2*, *OsHMA3*, *OsYSL6*, *OsYSL 14*, *OsYSL 18*, *OsNRAMP4*, *OsNRAMP7* and *OsTOM1* for the TTA-M module. Genes meeting the set criteria included *OsHHLH120*, *OsWOX11*, *OsPSK5*, *OsNAC121*, *OsPGIP1*, *OsMID1*, *OsMYB55*, *OsJAmyb* and *OsMPH1* (CA-M module), *OsSWN6*, *ONAC12*, *OsERF67*, *OsHHLH108* (TRA-M module), and *OsHMA1*, *OsLIS3*, *OsGSTU12*, *OsNRT1.1B*, *OsMTI4C*, *OsMT1g*, *OsZIP79* and *OsTRX* for TTA-M module (**Fig. 3a-c and Additional file 18–20: Table S18-20**).

Curiously, the <https://funricegenes.github.io/> search database for functionally validated genes in rice, showed that several of the CoDEGs (**Additional file 29–31: Table S29-31**) linking up the recognized ZDR genes have established agronomic functions such as transport of essential nutrients, biotic and abiotic stress tolerance (**Table 4 and Fig. 3a-c**).

Table 4
Experimentally validated genes which are hub genes for the known ZDR genes.

Module	Gene name	MSU_ID	Gene functions (retrieved from https://funricegenes.github.io/)
TTA-M	<i>OsNRT1.1B*</i>	<i>LOC_Os10g40600</i>	Key regulator of Nitrogen use efficiency in rice. Enhances of Selenium concentration in rice grains.
	<i>OsLSI3*</i>	<i>LOC_Os10g39980</i>	Essential for Silicon distribution in vascular structures of rice.
	<i>OsMT1g*</i>	<i>LOC_Os12g38290</i>	Confers multiple abiotic stress tolerance in rice.
	<i>OsRNS4*</i>	<i>LOC_Os11g05480</i>	Improves salinity stress tolerance.
	<i>OsTRX</i>	<i>LOC_Os12g08730</i>	Enhances carotenoid and chlorophyll content.
CA-M	<i>OsBHL120</i>	<i>LOC_Os09g28210</i>	A QTL for root thickness and lengths in upland rice.
	<i>OsWOX11*</i>	<i>LOC_Os07g48560</i>	Enhances crown root development. Enhances drought stress tolerance. Improves potassium deficiency tolerance. Regulates cytokinin signaling pathway.
	<i>OsMYB55⁺</i>	<i>LOC_Os05g48010</i>	Regulates amino acid metabolism. Enhances heat stress tolerance and immunity. Regulates hormonal signaling.
	<i>OsMPH1⁺</i>	<i>LOC_Os06g45890</i>	Modulates plant height and enhances drought stress tolerance. Enhances Cadmium stress tolerance.
	<i>OsMID1^{*,+}</i>	<i>LOC_Os05g37060</i>	Improves drought stress tolerance Negatively regulates Arsenic stress tolerance
	<i>OsPSK5*</i>	<i>LOC_Os12g05260</i>	Target of osa-miR168a regulating seed vigor
	<i>OsPGIP1*</i>	<i>LOC_Os05g01380</i>	Enhances sheath blight tolerance
	<i>OsSWN6*</i>	<i>LOC_Os04g45340</i>	Positively regulates the secondary wall biosynthetic processes.
* : Coexpressed in ZDR modules and expressed in our DGEA (CoDEGs).			
⁺ : R2R3-type MYB transcription factor gene.			

3. Discussion

This study indicates that integrating WGCNA and prior biological knowledge on ZUE trait is an effective method to add value on previous findings from GWAS, qtl mapping, transgenic, miRNA and gene transcriptomic studies on Zn. This approach enabled to establish modules of coregulated genes, determining conserved molecular functions, and predicting new putative Zn responsive genes which may facilitate the improvement of Zn use efficiency and Zn biofortification in rice.

The significance of Zinc deficiency responsive modules.

Our results indicated that molecular pathways and genes known to play essential roles in Zn homeostasis and Zn loading into rice grain coexpressed in ZDR modules. These genes included for instance, *ZIP* genes (*OsZIP2*, *OsZIP3*, *OsZIP10* and *OsZIP8*), heavy metal ATPase genes (*OsHMA1*, *OsHMA2* and *OsHMA3*), mugeneic acid (MAs) or nictiamine synthase (NAs) genes (*OsNAAT1*, *OsTOM1*, *OsDMAS1*, *OsSAMS1*, and *OsSAM2*), metallothionein (*MT*) protein coding gene (*OsMT1a*) and Zn/Fe transcription regulator genes (*OsIRO2* and *OsIRO3*). *OsZIP8* gene is involved in Zn uptake and transport in rice (Lee et al., 2010). And the expression of *OsZIP10* gene correlated with high iron (Lee et al., 2009) and Zn (Maurya et al., 2018) concentrations in rice seeds. Apart from *ZIP* genes, *OsHMA2* and *OsHMA3* have also been experimentally validated. *OsHMA2* is a long-distance transporter of Zn and Cd (Yamaji et al., 2013), while *OsHMA3* enhances Cd stress tolerance and the expression of other Zn transporter genes (Cai et al., 2019; Sasaki et al., 2014).

MAs and NAs are metal chelators from the phytosiderophores family genes and play essential roles in the accumulation Zn/Fe into grain rice, while mitigating the loading of Cd (Banakar et al., 2017). Earlier studies demonstrated that *OsTOM1*, a DMA efflux transporter gene, enhanced tolerance to both Zn (Ishimaru et al., 2011) and Fe (Nozoye et al., 2011) deficiencies in rice. Further, targeting MAs genes was proposed as a single strategy to improve both Zn/Fe in rice endosperm (Singh et al., 2017). *MTs* genes are also metal chelators that bind transition metals such as Zn (Sinclair and Krämer, 2012). Transgenic rice plants overexpressing *OsMT1a* showed enhanced expressions of Zn-induced CCCH zinc-finger TFs, ROS scavenging capacity, drought stress tolerance and Zn concentrations in rice tissues (Yang et al., 2009).

Coexpression of all these Zn transporters, phytosiderophores family genes and metal chelators genes across TTA-M, TRA-M and CA-M modules suggests relevant functions of ZDR modules in the molecular mechanism Zn deficiency response.

Conserved genes and molecular pathways across Zn deficiency responsive modules.

By intersecting ZDR modules genes and DEGs from our study we obtained the most conserved transcriptionally active genes. The most significantly enriched functional annotations by these CoDEGs were glutathione metabolism, DNA binding transcription factor, transmembrane transport, and biosynthesis of secondary metabolites including phenylpropanoid and phenylalanine metabolism. Interestingly, these conserved molecular pathways were only enriched by genes expressed in root tissues, underscoring the crucial role of root tissue in Zn deficiency regulation (Fig. 2c). Glutathione (GSH; γ -

glutamyl-cysteinyl-glycine) is a low- molecular-weight (LMW) intracellular signalling molecule with indispensable antioxidant and abiotic stress tolerance roles (Noctor et al., 2012). The significant enrichment of glutathione metabolism by Zn stress induced genes was also reported by previous studies in rice (Bandyopadhyay et al., 2017; Zeng et al., 2019a). Zeng et al. (2019a) showed that Zn supply repressed the activity involved in glutathione metabolism genes, suggesting that glutathione has essential roles in Zn stress response. Further, it was shown that the antioxidant roles of GSH enhanced Cd stress tolerance in rice (Chen et al., 2010). In this study, a set of DEGs that upregulated in root tissues of both AUS genotypes and coexpressed in the TTA-M module, significantly enriched the glutathione metabolism pathway. An earlier study indicated that salt stress tolerant rice cultivar Pokkali had significantly greater levels of GSH compared to a sensitive variety, IR64 (El-Shabrawi et al., 2010). Whether the exudation of GSH or the expression of GSH pathway genes is linked to higher ZUE in ZE genotypes is worthy further investigations, for example, through transgenic or crop modelling approaches.

Conversely, secondary metabolites KEGG pathways such as phenylpropanoid biosynthesis was preserved in a group CoDEGs from CA-M module which were upregulated in the root sample of IR26 genotype. The upregulation of phenylpropanoid biosynthesis pathways in rice tissues under low Zn supply was also reported in previous studies (Nanda et al., 2017; Zeng et al., 2019a). Phenylpropanoids constitute the major group of secondary metabolites in plants (Sharma et al., 2019). Phenylpropanoids can chelate metal ions to enhance the mobilization and uptake of essential elements such as Zn, iron, manganese, potassium, calcium and magnesium (Seneviratne and Jayasinghearachchi, 2003). The high conservation of both glutathione and phenylpropanoid biosynthesis pathways in root tissues of AUS and ZI is another evidence for the vital roles of LMW root effluxes in enhancing Zn uptake from low Zn environments.

Closest hub genes of the well-known ZDR genes are known stress responsive genes.

Genes with strong connectivity with the validated ZDR genes were identified. Several of these genes or their homologous were shown to play major roles in different morphogenetic events, biotic and abiotic stress responses by previous investigators. These included for example, a TF gene *OsbHLH120* which interacted with validated ZDR genes in the CA-M module. *OsbHLH120* is a QTL for root growth and thickness, and improves drought tolerance in upland rice (Li et al., 2015). The homologous gene of *OsbHLH120*, *OsiRO2/OsbHLH56*, is a well-known TF gene that positively regulates the expression of several genes involved into Fe uptake in rice (Ogo et al., 2009; Ogo et al., 2007). Both TFs contain an homeodomain Leucine Zipper (HD- Zip 1) element known to mediate of hormonal responses and response to stimuli (Ariel et al., 2007). In contrast, another homologous gene, *OsiRO3*, negatively regulates Fe uptake (Wang et al., 2020; Zheng et al., 2010) but interacted with *OsDMAS1* in the PPIN (**Additional file 38: Fig. S2c**). *OsiRO3* has a similar binding site with *OsiRO2* (5'-CACGTGG-3'). Whether the activity of *OsbHLH120* under Zn/ Fe deficiency is similar to *OsiRO2* or *OsiRO3* requires further studies. Unknown targets of *OsbHLH120/OsiRO3* may contribute to Fe/Zn hypersensitivity.

OsWOX11 was an other important hub gene for known ZDR genes. *OsWOX11* is a regulator of cytokinin signalling pathway, crown and root hair development, and enhances drought stress tolerance (Cheng et al., 2016; Neogy et al., 2019). An earlier study associated crown development with Zn stress tolerance in ZE genotypes (Nanda and Wissuwa, 2016b). In rice, cytokinin inhibits crown root development, while auxin promotes that process (Debi et al., 2005; Kitomi et al., 2011). Here, a cytokining gene (*OsCKX5*) from the ZDR modules was downregulated by Zn deficiency in root sample of KALBOR026 genotype (**Additional file 31: Table S31**), consistent with the significant higher root biomass observed in KALBOR026 genotype relative to ZI genotypes (IR26 and IR64) (Lu et al., 2020). However, *OsWOX11* and its homologous gene *OsWOX6* only upregulated in root samples of a ZI genotype (IR26) (**Additional file 31: Table S31**) with less root biomass relative to AUS genotypes (Lu et al., 2020). *OsWOX11* may have essential roles in Zn stress response, as its neighbour genes. However, more investigations are needed to illuminate the regulation of cytokinin signalling by *OsWOX11* during Zn deficiency conditions.

On the other hand, in the TTA-M, *OsNRT1.1B* gene coexpressed and interacted with known ZDR genes such as *OsZIP3*, *OsHMA2* and *OsYSL18* (Fig. 3). *OsNRT1.1B* is a QTL for nitrate use efficiency divergence in rice cultivars (Hu et al., 2015) and enhances nitrogen uptake under low nitrogen conditions in cultivated rice (Fan et al., 2016). Also, *OsNRT1.1B* improves Selenium levels in rice grains (Zhang et al., 2019). A previous study indicated that *OsNRT1.1B* was induced by Zn stress conditions (Bandyopadhyay et al., 2017). Here, the expression of *OsNRT1.1B* was significantly repressed in root tissues of a Zn sensitive genotype, IR64 (**Additional file 29: Table S29**). As a transporter gene, *OsNRT1.1B* could also have significant functions in the translocation of Zn in different rice tissues.

Still in the TTA-M module, *OsLSI3* linked up *OsHMA3*, *OsHMA2*, *OsYSL14* and *OsYSL18*. *OsLSI3* and its homologue *OsLSI2* involve in the distribution and redistribution of Arsenic across vascular structures in rice (Yamaji et al., 2015). *OsLSI2*, is a silicon efflux transporter responsible for arsenic accumulation in rice grain (Ma et al., 2008). However, exogenous Silicon is known to enhance gas exchange capacity in rice plants under Zn stress (Song et al., 2014), and the uptake of essential micronutrients (Zn and Manganese) and macronutrients (Phosphorous, potassium, Calcium and Magnesium) in rice and other monocots plants under heavy metal stress conditions (Keller et al., 2015; Tripathi et al., 2013). On the other side, Silicon also reduced Zn uptake in root tissues of maize and cotton plants (Bokor et al., 2015). Here, *OsLSI1*, *OsLSI2* and *OsLSI3* were significantly upregulated in root samples of KALBOR026 relative to IR26 in the same tissue under Zn stress conditions (**Additional file 10: Table S10**). *OsLSI2* was also significantly induced by Zn stress relative to Zn supply conditions in root tissues of KALBOR026 genotype. It is likely that *OsLSI2* / *OsLSI3* have similar functions as their linked Zn/cd transporter genes. However, their specific roles in Zn homeostasis remain to be elucidated.

Another gene connecting the well-known ZDR genes was *OsHMA1*. It is presumed that *OsHMA1* involves in Zn stress response or detoxification of Zn excess like its orthologue *AtHMA1* (Takahashi et al., 2012). *OsHMA1* transcripts accumulated by more than 7 folds under Zn-stress condition relative to Zn supply conditions (Suzuki et al., 2012).

In this study, *OsHMA1* was significantly upregulated in crown tissues of AUS (KALBOR026) and ZI (IR64) genotypes (**Additional file 29: Table S29**). This suggests that *OsHMA1* could also have similar functions as its homologous genes *OsHMA2* or *OsHMA3*.

Lastly, many of the validated *R2R3*-type *MYB* TFs genes (Katiyar et al., 2012) linked up several known ZDR genes across the ZDR modules (Fig. 3c and Table 4). These *MYB* TF genes were also reported in previous transcriptomic studies on Zn deficiency in rice, but none of these has been experimentally validated for Zn deficiency functions. Considering that coexpressing and interacting genes are likely to share same functions, the identified hub genes which have been validated for the other economic traits in rice could also have key roles in enhancing Zn deficiency tolerance as their linked well-known ZDR genes. Thus, targeting these putative novel ZDR genes may benefit the concurrent improvement of several agronomic traits in rice.

4. Conclusions

A comprehensive elucidation of functional pathways within the framework of co-expression network enabled to identify 3 key modules of coexpressed genes under Zn deficiency, 380 global conserved genes and their pathways, and key hub genes in the interaction networks of Zn responsive genes. Of the well-recognized Zn deficiency responsive genes, only *OsZIP8*, *OsZIP10*, *OsMT1a* and *OsNAAT1* were expressed in our samples and conserved in the identified key modules. *OsZIP3* and *OsYSL18* were the only recognized Zn responsive genes detected among the top hub genes in the key modules. Several other hub genes with strong interactions with the recognized Zinc deficiency responsive genes were found. Results from this investigation may for instance facilitate genomic selection breeding schemes in targeting relevant molecular pathways and genes to enhance ZUE and Zn biofortification in rice.

5. Materials And Methods

A comprehensive pipeline used from DGEA to hub genes identification is provided (**Additional file 38: Fig. S3**).

Sample description and data acquisition for DEG analysis.

Procedures from sample preparation and Zn deficiency treatment to the generation of the Fragment Per Kilobase Millions (FPKM) data frame were previously performed in our laboratory, and are as described in a published study (Lu et al., 2020). Briefly, samples consisting of one-week old root or crown tissues of two ZI (IR26 and IR64) and two AUS (UCP122 and KALBOR026) genotypes were supplied (control) or deprived of Zn nutrient regime for one month (Zn stress treatment). The FPKM table as generated by our biological collaborators consisted of 36 columns covering the four genotypes, two tissues (root and crown) per each genotype, two replicates per tissue of each genotype and two treatment conditions (Zn supply or Zn deficiency). Two replicates per tissue for each genotype were treated as one independent biological sample under Zn supply or Zn deficiency conditions (**Additional file 16-17: Table S16-17**).

Differential gene expression analysis (DGEA) was only carried out for samples from our laboratory using the Bioconductor R package DESeq2 (Love et al., 2014). Genes were considered differentially expressed if passing the stringent criteria of fold-change ≥ 2 or ≤ -2 , $q\_value \leq 0.01$, and false discovery rate-adjusted p value (FDR) < 0.01 .

Sample description, RNAseq data acquisition and processing for WGCNA

The global gene co-expression network was built using the Weighted Gene Coexpression analysis (Langfelder and Horvath, 2008) with 17 independent biological samples obtained after collapsing related replicates by their averages (**Additional file 17: Table S17**). Eight of the 17 samples were Zn-stressed samples from this study (as described in the section above), six others (IR55179, RIL46, Nipponbare, IR64, IR74 and KP) were from a study by (Nanda et al., 2017), and the remaining were from (Zeng et al., 2019b). Overall, nine genotype samples (IR55179, RIL46, IR64, IR74, IR26, NIPPONBARE, KP, UCP122 and KALBOR026) were used for WGCNA. These samples were representative of different varietal ecotypes including *Japonica* (NIPPONBARE and KP), *Indica* (IR55179, RIL46, IR64, IR74 and IR26) and *AUS* (UCP122 and KALBOR026), and different Zn use efficiency (ZU) levels. Only raw counts from Zn-stressed samples were retrieved from NCBI data repository and fed into RNAseq pipeline.

The RNAseq pipeline first consisted of adapter removal and low-quality reads trimming by Trimmomatic tool (Bolger et al., 2014). Next, clean reads were mapped to the MSU7 rice reference genome using HISAT alignment tool (Anders et al., 2015). The annotation and reference genome files (MSU7) were obtained from Illumina's iGenomes project (support.illumina.com/sequencing/sequencing_software/igenome.html). The poorly aligned reads were filtered out using the filter SAM tool version 0.1.19 (Li et al., 2009). The SAM tool generated a BAM file of quality reads which was fed into the subread tool (Liao et al., 2014) along with gene model annotation file. The resulting gene count matrix was converted to FPKM values and merged with the Zn deficiency FPKM table provided by our biological collaborators. All the used samples were pair-end sequenced libraries.

Data quality processing for WGCNA

The FPKM values from the above steps were rounded to their nearest integers and standardized using the `rlog()` function of DESeq2 R package. Rows with minimum counts less than 10 were also filtered out. As the only variation of interest was genotypic variation, effects from other factors such as tissue type, development stage, Zn stress treatment duration were corrected using `removeBatchEffect()` of limma R package. Samples' PCA and clustering before and after controlling the unwanted variations are as illustrated in (**Additional file 38: Fig. S4a-b**). The resulting normalized FPKM values were finally filtered with their MAD (median and median absolute deviation) to reduce noises by removing low-expressed or non-varying genes (Langfelder and Horvath, 2008).

Gene co-expression analysis and module construction

Only Zn deficiency responsive gene loci comprising of genes reported in 7 previous studies using different methods (e.g., GWAS, qtl mapping, miRNA, transgenic and transcriptomic studies) and DEGs from this study were selected for network construction (**Additional file 11: Table S11**). WGCNA (version 1.49) R package was used to assemble modules of coexpressed genes (Langfelder and Horvath, 2008). The scale free topology was used for choosing the softpower threshold (β) for the computation of adjacency matrix as $a_{ij} = |s_{ij}|^\beta$; where s_{ij} is the correlation between gene i and gene j (Zhang et al., 2005). Soft thresholding refers to reducing low correlation continuously by powering the correlation between genes to that threshold using β parameter. The pickSoftThreshold() function was used to determine the β value. The soft power threshold of 13 was selected as the first power that surpasses the scale-free topology fit index of 0.85 (**Additional file 38: Fig. S5a**) (Ramírez-González et al., 2018). Next, a topological overlap matrix (TOM) was constructed from adjacency matrices using β value of 13 (**Additional file 38: Fig. S5b**). TOM enables to determine the network connectivity of a gene as defined by all its adjacencies with the rest of genes for network generation. Step by step procedure was finally established to construct a consensus network with parameters: minModuleSize = 16; dynamicMods = cutreeDynamic(dendro = geneTree, distM = dissTOM, deepSplit = 2, pamRespectsDendro = FALSE, minClusterSize = minModuleSize). After generation of the dynamic tree modules, modules were labeled to colors and merged with mergeCloseModules() function.

Network visualization and hub genes identification

Gene-gene and protein-protein network structures from ZDR modules were represented graphically using Cytoscape (v3.8) (Cline et al., 2007). Genes or proteins directly connected with at least three well-known ZDR genes or proteins were retained for a finer gene-gene interaction network (GGIN) visualization. The top hub genes in each ZDR modules were based on the Maximal Clique Centrality method implemented via the cytoHubba plugin (Chin et al., 2014) in Cytoscape (v3.8) software.

Identification of transcription regulators, transcription factors, protein kinases genes

Transcription factors were identified using the Plant Transcription Factor Database (PlantTFDB version 5.0 (<http://planttfdb.cbi.pku.edu.cn/index.php>)) (Pérez-Rodríguez et al., 2010). Transcription regulators and protein kinases were predicted using iTAK version 1.6 software

(<http://itak.feilab.net/cgi-bin/itak/index.cgi>) (Zheng et al., 2016).

Functional annotation enrichment analysis

Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways affected by the DEGs from this study, coexpressed genes across all the modules, and the differentially coexpressed genes across ZDR modules were identified using STRING tool version:11 (<http://string-db.org/>) (Mering et al., 2003) at FDR < 0.05.

List Of Abbreviations

ZUE: Zinc Use Efficiency

DGEA: Differential Gene Expression Analysis

ZDR : Zn Deficiency Responsive

ZE : Zn Efficient

ZI : Zn Inefficient

WGCNA : Weighted Gene Coexpression Analysis

DEGs : Differentially Expressed Genes

CoDEGs : Coexpressed Differentially Expressed Genes

MCC: Maximal Clique Centrality

CEA: Condition-based gene Expression Analysis

GEA : Genotype-based gene Expression Analysis

LMWOA : Low-Molecular Weight Organic Acids

GGIN : Gene-Gene Interaction Network

PPIN : Protein-Protein Interaction Network

NAs : Nicotianamine synthase

MAAs : Mugineic Acid

DMAAs : Deoxymugenic Acids

ZIP : Zinc-regulated transporters and Iron-regulated transporter Proteins

HMAAs : Heavy Metal ATPases

MTPs : Metal Tolerance Proteins

ROS : Reactive Oxygen Species

MT : Metallothionein

TF : Transcription factor

TFR : Transcription factor regulator

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All datasets generated for this study are included in the article/supplementary material, further inquiries can be directed to the first author.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

Gouyou Ye conceived the project. Xiang Lu and Blaise Pascal MUVUNYI prepared the samples and collected data. Blaise Pascal MUVUNYI and Sang He analyzed the data. Blaise Pascal MUVUNYI drafted the manuscript. Sang He and Gouyou Ye revised the manuscript. All the authors have read, edited and approved the current version of the manuscript.

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Figures

Fig. 1

Figure 1

Genes and sample genotypes used for WGNA. a A three-level venn diragram illustrating overlapping genes between DEGs from this study, coexpressed genes and reported Zn genes from GWAS, qtl mapping, transgenic, miRNA and gene transcriptomic studies. b Sample dendrogram and classification

of 17 used samples into different ZUE categories. Height represents the average Euclidean distance between samples computed with hclust() function. A heat map of ZUE categories across all genotypes is illustrated underneath the sample dendrogram. Genotypes from the same category of ZUE are represented by red-colored bars. ZUE levels of AUS genotypes are still unknown. Therefore, no specific categories was attributed to AUS genotypes.

Fig. 2

Figure 2

Preserved genes and molecular pathways in ZDR modules. a Hierarchical dendrogram of coexpressed genes/nodes and visualization of ZDR modules. Coexpression distance is equivalent to the topological dissimilarity of nodes (1-TOM). Modules were constructed based on dynamic tree cutting method (see materials and methods for details). b Upset graph representing the number of coexpressed DEGs in ZDR modules. Numbers with larger font size at the top of black bar graphs in the upper pannel represent intersecting genes between DEGs and ZDR modules. c Functional annotations of the preserved DEGs in each ZDR module. A statistical threshold (FDR <0.05) was applied to define the significantly affected GO or KEGG pathways.

Fig. 3

Figure 3

Visualization of gene-gene interaction networks of ZDR modules. a TTA-M module. b TRA-M module. c CA-M module. Edges represent interactions. Edges from the interactions between the well-known ZDR genes (bigger nodes and font size) and other genes are represented with different colored lines (edges). Genes at the center of each module (hub genes) interact with at least three well-known ZDR genes. The

thicker the edge, the stronger is the degree of connections between the nodes it links. Bordered nodes correspond to DEGs from this study. A more detailed network legend is provided at the top of the panel.

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