Transcriptomic Analysis of Responses to Imbalanced Carbon: Nitrogen Availabilities in Rice Seedlings

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Abstract

The internal C:N balance must be tightly controlled for the normal growth and development of plants. However, the underlying mechanisms, by which plants sense and balance the intracellular C:N status correspondingly to exogenous C:N availabilities remain elusive. In this study, we use comparative gene expression analysis to identify genes that are responsive to imbalanced C:N treatments in the aerial parts of rice seedlings. Transcripts of rice seedlings treated with four C:N availabilities (1:1, 1:60, 60:1 and 60:60) were compared and two groups of genes were classified: high C:low N responsive genes and low C:high N responsive genes. Our analysis identified several functional correlated genes including *chalcone synthase* (*CHS*), *chlorophyll a-b binding protein* (*CAB*) and other genes that are implicated in C:N balancing mechanism, such as *alternative oxidase 1B* (*OsAOX1B*), *malate dehydrogenase* (*OsMDH*) and *lysine and histidine specific transporter 1* (*OsLHT1*). Additionally, six jasmonate synthetic genes and key regulatory genes involved in abiotic and biotic stresses, such as *OsMYB4*, *autoinhibited calcium ATPase 3* (*OsACA3*) and *pleiotropic drug resistance 9* (*OsPDR9*), were differentially expressed under high C:low N treatment. Gene ontology analysis showed that high C:low N up-regulated genes were primarily enriched in fatty acid biosynthesis and defense responses. Coexpression network analysis of these genes identified eight *jasmonate ZIM domain protein* (*OsJAZ*) genes and several defense response related regulators, suggesting that high C:low N status may act as a stress condition, which induces defense responses mediated by jasmonate signaling pathway. Our transcriptome analysis shed new light on the C:N balancing mechanisms and revealed several important regulators of C:N status in rice seedlings.

Introduction

Carbon (C) and nitrogen (N) balance is universal and critical for metabolism, growth and development in all cellular organisms. To cope with changing environment conditions, cellular organisms have to sense the nutrient supply and adjust their metabolism accordingly. In cyanobacteria and *Escherichia coli*, a highly conserved protein, named PII, has been known to act as a key regulator for integrating C and N metabolism by sensing α-ketoglutarate and adenylate energy charge [1, 2]. In yeast and mammals, metabolism of C and N are mainly integrated by regulating processes such as the tricarboxylic acid (TCA) cycle and N assimilation through the crosstalk between pathways involving the target of rapamycin (TOR) and sucrose nonfermenting 1 (SNF1) kinases/AMP-activated kinases (AMPK) [3–5]. Similarly, the interaction between C and N metabolic pathway is indispensible for normal plant growth and development. CO_2 fixation and assimilation into photosynthetic products, mainly sucrose and glucose, is dependent on N uptake and assimilation, particularly because a large number of proteins and enzymes (N products), such as RuBisCo (Ribulose-1,5-bisphosphate carboxylase/oxygenase) and thylakoid proteins, are required in large quantities for the photosynthetic processes. Conversely, photosynthetic products provide C skeletons and energy essential for the incorporation of inorganic N into amino acids and other biologically important molecules. Therefore, C and N metabolisms need to be tightly coordinated to maintain a balance between C and N metabolites in plants [6–8]. For example, when plants are supplied with limited N, carbohydrates accumulate in leaves, which would result in the repression of photosynthetic genes [9–11]. On the other hand, when plants are supplied with sufficient N, most N is allocated into chloroplasts but not mitochondria, and this is reflected in differences of photosynthesis and respiration rates with the final results of the accumulation of carbohydrates [12].

Despite mechanisms for C:N balancing are well studied in unicellular organisms and mammals, they are still elusive in plants. This is in part due to the complexity of C:N balancing in plants, as C and N are up-taken through aerial parts and roots, respectively, and need to be redistributed dynamically among different organs [13, 14]. However, understanding the C:N balancing mechanism in plants is of great significance because of their major impacts on crop yield. One way to maintain a proper C:N balance in plants is to regulate the growth of roots and leaves, which uptake and fix N and C, respectively. For example, high nitrate levels inhibit lateral root growth to limit N uptake in *Arabidopsis* [15, 16]. Similarly, high levels of sucrose or glucose inhibit the shoot growth of plants [17, 18]. Recently, a bZIP transcription factor elongated hypocotyls 5 (HY5), which acts as a shoot-to-root mobile signal that mediates C assimilation in the shoot and light promotion of root growth, is suggested to contribute greatly to the maintenance of homeostatic C and N balance in *Arabidopsis* [19]. Apart from the regulation of C:N balance by N and C status, evidence supports a role for C:N ratio or imbalance in this regulation [13, 20, 21]. For example, *nitrate reductase* (*NR*) could be induced by nitrate [22], which is alleviated, however, when cellular carbohydrate content is limited [23]. *Chalcone synthase* (*CHS*) is up-regulated by sugar treatment [24], but under low N condition only [25]. These initial findings raised an interesting question: Do plants have a mechanism for sensing C:N ratios or imbalance? Alternatively, C and N sensors could cross-talk with each other to regulate C:N balance. Microarray analysis revealed over 300 genes were differentially expressed in *Arabidopsis* seedlings subjected to combined C:N treatments compared to C or N treatments respectively, supporting the hypothesis that plants have a C:N sensing mechanism [7]. Besides, studies using *Arabidopsis* seedlings treated with different C:N availabilities (0:60, 0:0.1, 100:60, and 100:0.1) suggested that C:N ratio but not C and N alone played major roles in regulating plant growth [26]. However, another group, in their searching of global gene expression patterns to a matrix of C:N treatments, failed to identify any C:N ratio responsive genes and

suggested that the C:N ratio was not a predominant regulatory mechanism in plants [6]. The different conclusions of these studies implied the difficulty in revealing the molecular mechanisms underlying the C:N balance in plants.

Several genetic studies support the capability of plants to sense C:N imbalance. *Glutamate receptor homolog 1.1* (*GLR1.1*), encoding a putative glutamate receptor, is necessary in seed germination under the exogenous high C:low N condition [27]. *Oversensitive to sugar 1* (*OSU1*), encoding a putative methyltransferase, is indispensible for the adaptation to imbalanced C:N conditions [28]. *Carbon/nitrogen insensitive 1* (*CNI1*)/*Arabidopsis toxicos en levadura 31* (*ATL31*), a RING-type ubiquitin ligase encoding gene, functions in growth phase transition and leaf senescence under high C:low N condition [29, 30]. Imbalanced C:N is also found to regulate the expression of genes that are involved in C and N metabolism in rice [18]. These findings prompted us to speculate that rice plants may sense imbalanced C:N conditions, either high C:low N or low C:high N, and this sensing may lead to the regulation of C and N metabolism in order to achieve the C:N balance. As a first step to test this notion, we need to identify gene markers for imbalanced C:N.

Rice is an important model for cereals and a staple crop worldwide. Understanding C-N nutrients interaction in rice should also help to compare and contrast the mechanisms for the regulation of C:N balance between plants accumulating high levels of photosynthates such as cereals and those that do not, and importantly may provide useful knowledge for improving crop yields. By analyzing the effects of exogenous C:N availabilities on the growth of rice seedlings, we previously identified the balanced and imbalanced C:N conditions for rice seedlings [18]. Herein, we chose four C:N ratios (1 mM:1 mM, 1 mM:60 mM, 60 mM:1 mM and 60 mM:60 mM). Among them, 1:1 and 60:60 were considered as balanced exogenous C:N while 60:1 and 1:60 were imbalanced C:N. By comparative transcriptome analysis, genes were classified into two modes: high C:low N responsive genes and low C:high N responsive genes. A number of C and N regulated genes were identified as the potential regulators of C:N balance. Additionally, several jasmonate signaling and defense response related genes were revealed in high C:low N condition, suggesting that jasmonate signaling might be involved in C:N balancing mechanism under high C:low N conditions.

Materials and Methods

Plant materials and growth conditions

Rice seeds (*Oryza sativa* L. ssp. *Japonica* var. Nipponbare) were decorticated and sterilized in 75% (v/v) ethanol for 1 min and then in 20% (v/v) sodium hypochlorite for 20 min. After washing by sterile water for five times, seeds were kept at 37°C for 24 h to accelerate the germination. Then seeds were transferred to the greenhouse (28°C, 14 h light and 10 h dark) for an additional 48 h. Germinated seeds were cultured in 1/3B5 liquid culture medium [31] for three weeks with medium renewal of every 2 days. Then rice seedlings were refreshed with culture medium without N nutrients for 48 h before the treatments. Four C:N availabilities (1 mM C:1 mM N, 1 mM C:60 mM N, 60 mM C:1 mM N, 60 mM C:60 mM N) were used for the treatments. Sucrose was used as the C source, and the combination of NO_3^- and NH_4^+ in a molar ratio of 12:1 was used as the N source. All of the media contained similar amount of K^+ by replacing KNO_3 with KCl, if necessary. Aerial parts of rice seedlings were harvested at 0, 1, 2, 3 and 4 h after the treatments and kept at -80°C until further analysis.

Purification of total RNA

Total RNA was extracted by RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, including a DNase digestion step. The yield and RNA quality

were assessed using a spectro-photometer NanoDrop 2000 (NanoDrop Technologies, Wilmington, DE, USA) and a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA).

Real-time quantitative PCR analysis

The first-strand cDNA was synthesized by M-MLV reverse transcriptase (Promega, Madison, WI, USA) from 2 μg total RNA. SYBP Green Premix Ex Taq[TM] II (Takara-Bio, Otsu, Shiga, Japan) and the ABI StepOne Plus[TM] Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) were used for the detection. Running conditions were 95°C for 30 s, 40 cycles of 95°C for 5 s, 60°C for 15 s, and 72°C for 20 s. Then melting curves were performed to detect the specificities of primers. *Actin6* (*Os04g0667700*) was used as the internal reference and its primer sequences were 5'-TCAAGCATGTTTACACCCGTCGAG-3' (forward primer) and 5'-AGCAGCATACTTGCTCCATCCGTA-3' (reverse primer). The gene-specific primers are listed in S1 Table. The relative quantification method (using the formula: $2^{-\Delta\Delta Ct}$) was used to evaluate quantitative variation between the replicates examined.

Microarray hybridization

The microarray hybridization was performed by Kangcheng Bio-tech Inc (Shanghai, China). Briefly, total RNA from each sample was amplified and labeled with Cy3-UTP. 1 μg of each labeled cRNA was fragmented with fragmentation buffer (Agilent Technologies) at 60°C for 30 min. The fragmented labeled cRNA was then pooled and hybridized to the Rice Gene Expression Microarray (G2519F-015241; Agilent Technologies) at 60°C for 17 h. After washing and blow-drying with nitrogen gun, microarrays were scanned by Agilent microarray scanner (Agilent Technologies). Scanned images were analyzed by Feature Extraction software (version 11.0.1.1; Agilent Technologies) and the raw gene expression data were imported into GeneSpring GX software package (version 11.5; Agilent Technologies) for further analysis. The 12 microarray data sets were normalized in GeneSpring GX using the Agilent Feature Extraction one-color scenario (mainly using median normalization). All of the data were interpreted using the log-ratio setting. The normalized expression data are listed in S1 File. Microarray raw data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-5118.

Data analysis

Of the total 43,803 probe sets in Rice Gene Expression Microarray, 33,187 probe sets showed signals in all of the twelve samples (each of four C:N treatments contains three biological replicates). Differentially expressed genes were identified by determining the fold-change (FC) and *p*-value of the *t*-test algorithm between two groups (60:1 compared with 1:1, 1:60 compared with 1:1, 60:60 compared with 1:60, 60:60 compared with 60:1) (S2 File). Genes with an FC of $\geq$ 1.5 and a *p*-value of $\leq$ 0.05 between two groups were identified as differentially expressed genes. Hierarchical cluster analysis was performed by R software (http://www.r-project.org). AgriGO [32] was used to perform the gene ontology (GO) analysis for functional categorization of high C:low N and low C:high N responsive genes compared with the genome-wide background with an adjusted *p*-value (False discovery rate, FDR) cutoff of 0.05. The GO categories were derived from Gene Ontology (www.geneontology.org) and comprised three structured networks (biological process, cellular component and molecular function) of defined terms to describe the gene product attributes. High C:low N up-regulated genes were chosen to perform coexpression analysis by RiceFREND online web tool [33]. Functional analysis of the coexpression genes was performed using the KEGG pathway database (www.genome.jp/kegg/pathway.html).

Results

Experimental design to identify genes responsive to imbalanced C:N

To identify genes responsive to imbalanced C:N, we compared gene expression profiles in rice seedlings treated with four C:N availabilities (1:1, 1:60, 60:1, 60:60). These four C:N conditions were chosen based on our previous studies [18]. In brief, 1:1 (1 mM C:1mM N) and 60:60 represent balanced low C:low N and high C:high N, respectively, whereas 1:60 and 60:1 represent imbalanced low C:high N and high C:low N, respectively. Rice seedlings were grown hydroponically in light/dark cycles (14/10 h) for three weeks with 1/3B5 medium (without exogenous C). After nitrogen starvation with nitrogen-free 1/3B5 medium for 48 h, rice seedlings were treated with four C:N conditions and then shoot tissues were collected for RNA expression analysis (Fig 1A). RNA expression profiling was conducted using the Rice Gene Expression Microarray from Agilent (Agilent Technologies). Genes responsive to imbalanced C:N (1:60 and 60:1) would have higher or lower expressions than that under balanced conditions (1:1 and 60:60) (Fig 1B).

Determination of C:N treatment time for the identification of C:N imbalanced genes

To investigate gene responses of rice seedlings to C:N availabilities, we need to determine an appropriate time duration for the treatments. We investigated time course of responses for several key genes known to be affected by both C and N availabilities including *nitrate reductase* (*NR*), *glutamine oxoglutarate aminotransferase* (*GOGAT*), *glutamine synthase* (*GS*), *phosphoenolpyruvate carboxylase* (*PEPCase*) and *pyruvated kinase* (*PK*) [34]. qRT-PCR analysis showed that treatments with imbalanced C:N (both 1:60 and 60:1) induced transient increases in transcript levels for all genes examined with peaks around 2 h for most of these genes (Fig 2B and 2C). Interestingly, 1:60 treatments tended to cause transcript levels for N assimilatory
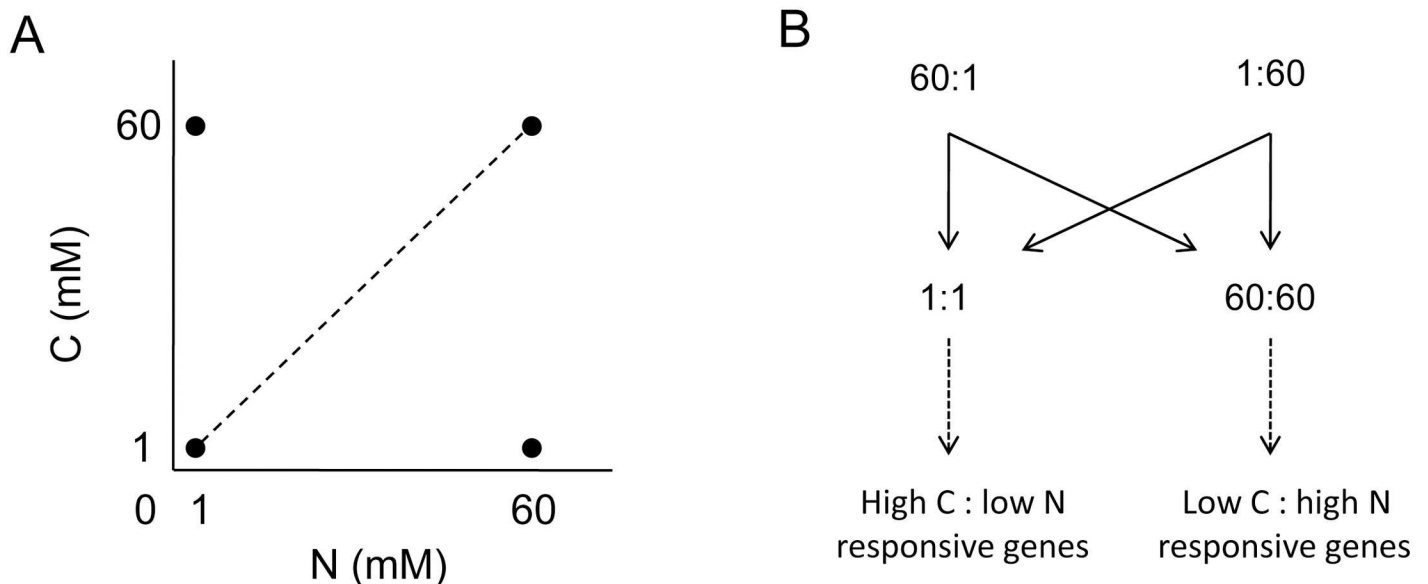

Fig 1. Experimental design to identify genes responsive to imbalanced C:N. (A) N starved rice seedlings were treated with four different C:N conditions: balanced C:N (1:1 and 60:60) or imbalanced C:N (1:60 and 60:1). (B) Hypothetical models of genes responsive to exogenous imbalanced C:N conditions. Genes responsive to imbalanced high C:low N (60:1) or low C:high N (1:60) are proposed to show higher or lower expression levels compared with 1:1 and 60:60 treatments.

doi:10.1371/journal.pone.0165732.g001

genes (*NR*, *GS*, *GOGAT*) to increase again after the first peak, while 60:1 treatments did so for C metabolic genes (*PK* and *PEPCase*). Treatments with balanced C:N also induced changes in transcript levels for the C and N metabolic genes, but the patterns of changes were different (Fig 2A and 2D). 1:1 treatments caused slow but steady increases in transcript levels for all genes except for *NR*, which exhibited a transient increase. 60:60 treatments caused rapid and sustained increases in transcript levels for *PK*, *PEPCase*, and *NR*, but seemed to have little effect on *GS* and *GOGAT*. These expression patterns of the C and N metabolic genes not only confirm the importance of C-N interactions in the regulation of their expression as previously
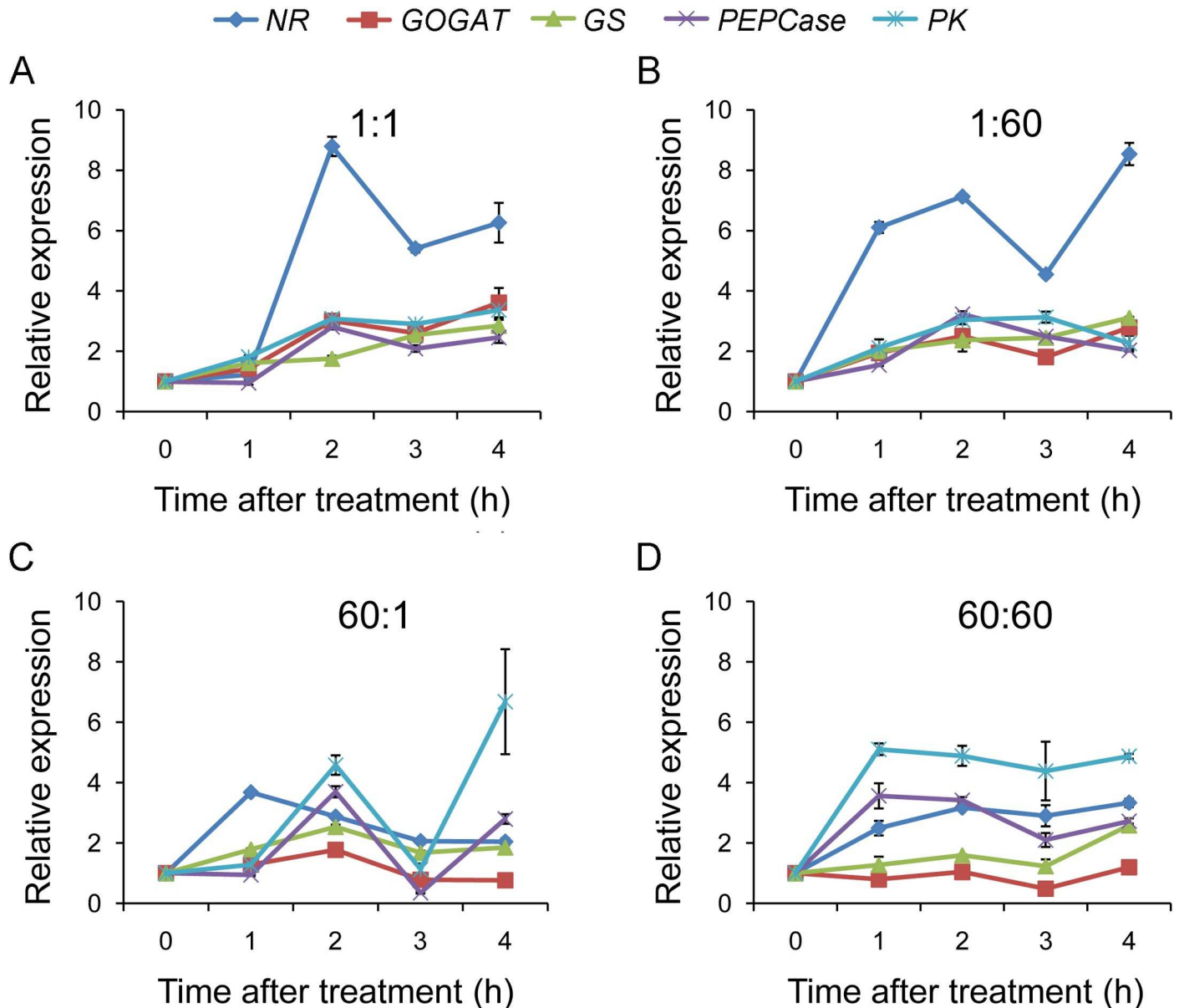

Fig 2. qRT-PCR analysis of CN metabolic genes at different time points after C:N treatments. Expression patterns of *NR*, *GOGAT*, *GS*, *PEPCase* and *PK* were analyzed in rice seedlings treated with four different C:N conditions (A 1:1; B 1:60; C 60:1; D 60:60) for 1, 2, 3 and 4 h. The beginning of the treatment (0 h) was used as the control and *Actin6* served as the internal reference. Values are shown as means ± SDs from three technical replicates. A representative experiment of two biological replicates is shown.

described [18, 35], but also reveal unique gene expression pattern under imbalanced C:N conditions. Importantly, these results suggest that treatments with imbalanced C:N for 2 h will most likely uncover C:N imbalance marker genes.

Identification of high C:low N and low C:high N responsive genes

To identify imbalanced C:N responsive genes, transcriptomic profiles from 60:1 and 1:60 treatments were first compared with those from 1:1 and 60:60 treatments, respectively. Hierarchical clustering was used to evaluate the global differences between treatments and dendrogram representations based on their global genome responses showed the relationships among the treatments (S1 Fig). As the volcano plots shown in Fig 3, large differences were observed in 60:1 vs 1:1 and 60:60 vs 1:60 groups, while relatively small differences were observed in 60:60 vs 60:1 and 1:60 vs 1:1 groups. These results indicated that C, rather than N, has large effect on the expression of genes as previously reported [6, 7]. Besides, more genes were up-regulated than down-regulated by C no matter the concentrations of N source (Fig 3A and 3D). Similarly, there were slightly more genes up-regulated than down-regulated by N under either high C or low C conditions (Fig 3B and 3C).

Based on the above analysis, high C:low N and low C:high N responsive genes could be obtained by identifying the intersections between groups. As the Venn diagram showed (Fig 3E), 63 and 17 genes were categorized as those up- and down-regulated by the high C:low N imbalance, respectively, whereas 10 genes were categorized as those up-regulated by low C: high N imbalance. However, no genes were found to be down-regulated under low C:high N conditions. To gain an insight into the effects of high C:low N and low C:high N status on transcriptomic profiles, we illustrated the expression patterns using heatmaps obtained via hierarchical cluster analysis. Fig 3F and 3G showed the up-regulated and down-regulated genes by high C:low N, respectively. The up-regulated genes by low C:high N were shown in Fig 3H. The annotations of genes listed in the right sides of the heatmaps were described in Table 1.

Several classes of transcription factors including WRKY, C2H2, bHLH, and MYB were found among the 63 high C:low N up-regulated genes. WRKY proteins comprise a large family of transcription factors in plants, which are involved in various plant processes, especially in coping with diverse biotic and abiotic stresses [36, 37]. The *WRKY* gene (*Os01g0584900*) identified here is classified as *OsWRKY77* [38]. C2H2-type zinc finger protein family, which exists in almost all eukaryotes, constitutes one of the largest families of transcriptional regulators in plants [39]. Recent studies revealed that C2H2 zinc finger proteins function as key transcriptional repressors involved in the defense and acclimation response of plants to different environmental stress conditions [40]. The C2H2 zinc finger protein encoding gene (*Os03g0437200*) identified in this study is reported to be induced in the sheaths of rice seedlings under N-deficiency condition [41]. Basic helix-loop-helix (bHLH) protein family is present throughout the three eukaryotic kingdoms and constitutes one of the largest families of transcription factors [42]. Rice genome contains 167 *bHLH* genes [43], among which *OsbHLH6* (*Os04g0301500*) was found here to be up-regulated by high C:low N. The MYB protein family, which comprises one of the richest groups of transcription factors in plants, is involved in plant development, secondary metabolism, hormone signal transduction, disease resistance, and abiotic stress tolerance [44, 45]. There are more than 155 *MYB* genes in rice genome [46] and *OsMYB4* (*Os04g0517100*) was found to be up-regulated here. Overexpression of *OsMYB4* improves the adaptability of several species to abiotic or biotic stresses, for instance, freezing tolerance in *Arabidopsis* and barley [47, 48], disease tolerance in tomato [49], drought and cold tolerance in apple [50]. Laura et al. found that the improved freezing tolerance of *Osteospermum ecklonis* by overexpressing of *OsMYB4* was companied with the accumulation of sugar and proline, suggesting the involvement of *OsMYB4* in CN metabolism regulation [51].
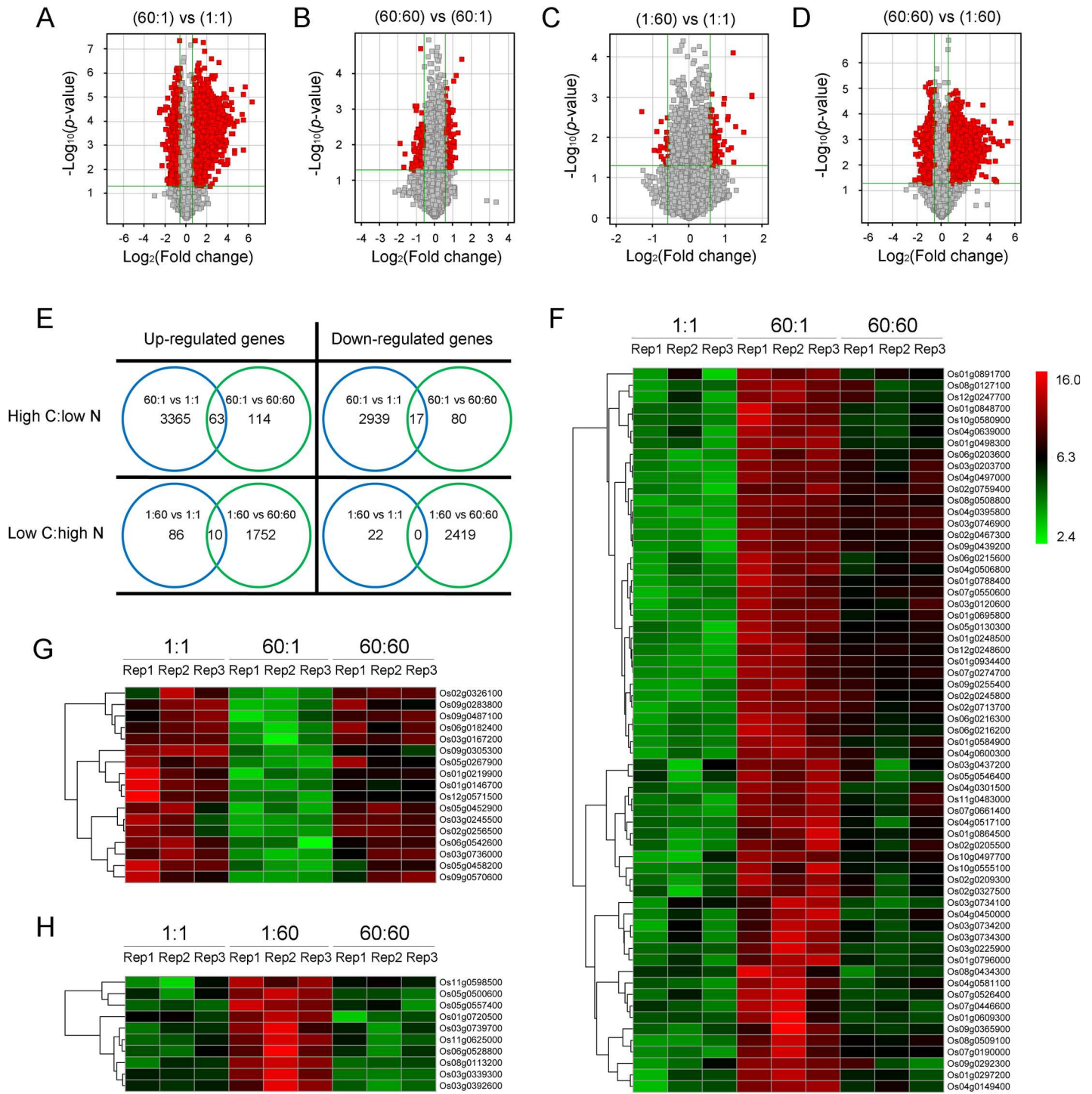
Fig 3. Identification of genes responsive to imbalanced high C:low N and low C:high N. (A-D) The Volcano Plots for differentially expressed genes between treatments. The two vertical lines are the 1.5-fold change boundaries and the horizontal lines are the statistical significance boundaries ($p<0.05$). Genes with fold change>1.5 and statistical significance are marked with red dots. A, 60:1 compared with 1:1; B, 60:60 compared with 60:1; C, 1:60 compared with 1:1; D, 60:60 compared with 1:60. (E) Venn diagram of rice genes (probe sets) responded to C:N treatments. (F-H) Hierarchical cluster analysis of high C:low N and low C:high N responsive genes. The $\log_2$ ratio values of probe sets were used for the analysis with R software. The colored bars represent the value ($\log_2$(fold change)) of the transcripts in each bin after C:N treatments. Green represents down-regulated probe sets, red represents up-regulated probe sets, and dark indicates no significant difference in gene expression. F, High C:low N up-regulated genes; G, High C:low N down-regulated genes; H, Low C:high N up-regulated genes. "vs" represents "compared with".

Table 1. Annotations of genes responsive to imbalanced high C:low N and low C:high N.

	Description	Transcription factor	Name
High C:low N Up			
Os01g0891700	Leucine rich repeat, N-terminal domain containing protein.		
Os08g0127100	Lysine and histidine specific transporter.		OsLHT1
Os12g0247700	Jacalin-like lectin domain containing protein.		OsJAC1
Os01g0848700	Ras-related protein Rab11C.		
Os10g0580900	Conserved hypothetical protein.		
Os04g0639000	Conserved hypothetical protein.		
Os01g0498300	Protein of unknown function DUF563 family protein.		
Os06g0203600	Conserved hypothetical protein.		
Os03g0203700	Plasma membrane Ca^{2+}-ATPase.		OsACA3
Os04g0497000	Allyl alcohol dehydrogenase.		
Os02g0759400	Zn-finger, RING domain containing protein.		
Os08g0508800	Lipoxygenase, chloroplast precursor (EC 1.13.11.12).		OsLOX
Os04g0395800	ZIM domain containing protein.		OsJAZ5
Os03g0746900	Protein of unknown function DUF1677 family protein		
Os02g0467300	Conserved hypothetical protein.		
Os09g0439200	ZIM domain containing protein.		OsJAZ8
Os06g0215600	Oxo-phytodienoic acid reductase.		OsOPR5
Os04g0506800	Sialyltransferase.		
Os01g0788400	Pectinesterase (EC 3.1.1.11) (Pectin methylesterase).		OsPME
Os07g0550600	Transferase family protein.		
Os03g0120600	Conserved hypothetical protein.		
Os01g0695800	Multidrug resistance protein 1 homolog.		
Os05g0130300	Conserved hypothetical protein.		
Os01g0248500	Pathogen-related protein.		
Os12g0248600	Hypothetical protein.		
Os01g0934400	Photosystem II complex PsbP family protein.		
Os07g0274700	B12D family protein.		
Os09g0255400	Indole-3-glycerol phosphate synthase (IGPS).		
Os02g0245800	Inwardly rectifying potassium channel.		
Os02g0713700	Protein of unknown function DUF296 family protein.		
Os06g0216300	Oxo-phytodienoic acid reductase.		OsOPR1
Os06g0216200	Oxo-phytodienoic acid reductase.		OsOPR2
Os01g0584900	WRKY transcription factor.	WRKY	OsWRKY77
Os04g0600300	Alternative oxidase.		OsAOX1B
Os03g0437200	Zn-finger, C2H2 type domain containing protein.	C2H2	
Os05g0546400	Conserved hypothetical protein.		
Os04g0301500	Basic helix-loop-helix region containing protein.	bHLH	OsbHLH6
Os11g0483000	Cytochrome P450 family protein.		
Os07g0661400	Conserved hypothetical protein.		
Os04g0517100	Myb protein.	MYB	OsMYB4
Os01g0864500	Harpin-induced 1 domain containing protein.		
Os02g0205500	Naringenin-chalcone synthase family protein.		OsCHS
Os10g0497700	Phytochelatin synthetase.		
Os10g0555100	Glucosyltransferase like protein.		
Os02g0209300	Non-protein coding transcript, unclassifiable transcript.		
Os02g0327500	Protein of unknown function DUF266 family protein.		

(*Continued*)

Table 1. (*Continued*)

	Description	Transcription factor	Name
Os03g0734100	Conserved hypothetical protein.		
Os04g0450000	FYVE/PHD zinc finger domain containing protein.		
Os03g0734200	Hypothetical protein.		
Os03g0734300	Proteinase inhibitor I20, Pin2 family protein.		
Os03g0225900	Allene oxide synthase (EC 4.2.1.92).		*OsAOS2*
Os01g0796000	(No Hit)		
Os08g0434300	Malate dehydrogenase precursor (EC 1.1.1.37).		*OsMDH*
Os04g0581100	Flavanone 3-hydroxylase.		
Os07g0526400	Chalcone synthase (EC 2.3.1.74).		*OsCHS*
Os07g0446600	Hypothetical protein.		
Os01g0609300	PDR-like ABC transporter.		*OsPDR9*
Os09g0365900	L-ascorbate oxidase precursor (Ascorbase) (ASO).		
Os08g0509100	Lipoxygenase, chloroplast precursor (EC 1.13.11.12).		*OsLOX*
Os07g0190000	1-deoxy-D-xylulose 5-phosphate synthase 2 precursor.		
Os09g0292300	Conserved hypothetical protein.		
Os01g0297200	AAA ATPase, central region domain containing protein.		
Os04g0149400	Hypothetical protein.		
High C:low N Down			
Os02g0326100	Non-protein coding transcript, uncharacterized transcript.		
Os09g0283800	(No Hit)		
Os09g0487100	Hypothetical protein.		
Os06g0182400	Metallophosphoesterase domain containing protein.		
Os03g0167200	IQ calmodulin-binding region domain containing protein.		
Os09g0305300	Plant protein of unknown function family protein.		
Os05g0267900	Hypothetical protein.		
Os01g0219900	Conserved hypothetical protein.		
Os01g0146700	Integrase, catalytic region domain containing protein.		
Os12g0571500	Hypothetical protein.		
Os05g0452900	Conserved hypothetical protein.		
Os03g0245500	Curculin-like lectin domain containing protein.		
Os02g0256500	Conserved hypothetical protein.		
Os06g0542600	(No Hit)		
Os03g0736000	NOT2/NOT3/NOT5 family protein.		
Os05g0458200	(No Hit)		
Os09g0570600	PAP/25A core domain containing protein.		
Low C:high N Up			
Os11g0598500	(No Hit)		
Os05g0500600	GRAS transcription factor domain containing protein.	GRAS	
Os05g0557400	Membrane attack complex component C9 family protein.		
Os01g0720500	Chlorophyll a-b binding protein 2 (LHCII type I CAB-2).		*OsCAB2*
Os03g0739700	Protein of unknown function DUF1334 family protein.		
Os11g0625000	(No Hit)		
Os06g0528800	(No Hit)		
Os08g0113200	RNA-binding region RNP-1 domain containing protein.		
Os03g0339300	Peroxidase (EC 1.11.1.7).		
Os03g0392600	Peptidase S10, serine carboxypeptidase family protein.		

Several C:N signaling and metabolism related genes were also found in the list of high C:low N up-regulated genes. Lysine and histidine specific transporters (LHTs), which are originally described as lysine and histidine selective transporters [52], can transport a broad spectrum of amino acids, especially neutral and acidic amino acids, in plants [53, 54]. They have been suggested to be involved in the import of organic nitrogen into root and mesophyll cells [55] as well as cells of reproductive floral tissues [53]. Here the identified gene (Os08g0127100) encoded one of the six LHTs in rice and was named as OsLHT1 with high transcripts in root, leaf, inflorescence and seeds [56, 57]. Chalcone synthase (CHS) is a key enzyme in flavonoid biosynthesis pathway, and the expression level of CHS is one of the determinants of anthocyanin biosynthesis [58, 59]. CHS is induced by the supplement of sugar [24] and the depletion of nitrogen [25, 60] and is considered as a high C:low N marker gene in Arabidopsis [26]. Two CHS genes (Os07g0526400 and Os02g0205500) were identified to be up-regulated by high C:low N in this study, demonstrating their functional conservation in rice. In addition, a mitochondrial NAD-malate dehydrogenase (MDH) encoding gene (Os08g0434300) was identified in our analysis. Plants contain multiple isoforms of malate dehydrogenases that catalyze the interconversion between malate and oxaloacetate coupled to reduction or oxidation of the NAD(P) pool [61]. It was reported that decreased mitochondrial malate dehydrogenase resulted in enhanced photosynthetic performance in tomato [62].

Furthermore, a number of jasmonate signaling genes were identified as high C:low N up-regulated genes. Lipoxygenase (LOX) catalyzes the conversion of polyunsaturated fatty acids into conjugated hydroperoxides [63]. In plants, LOXs are involved in jasmonic acid biosynthesis [64] and response to biotic and abiotic stresses [65]. Two of the fourteen LOX genes (Os08g0508800 and Os08g0509100) in rice genome [66] were identified in this study. Allene oxide synthase (AOS) is another key enzyme in jasmonic acid biosynthetic pathway in plants [67], which converts 13(S)-hydroperoxylinolenic acid to the corresponding allene oxide, 12,13-epoxyoctadeca-9,11,15-trienoic acid [68]. The rice genome contains at least five AOS genes [68]. The identified one here (Os03g0225900) was designated as OsAOS2 and was reported to be up-regulated by light, heavy metals and pathogen attacks [69, 70]. Oxo-phytodienoic acid reductase (OPR) catalyzes the reduction of oxo-phytodienoic acid to 10,11-dihydro-12-oxophytodienoic acid in jasmonic acid biosynthesis pathway [71]. Three of the ten OPR genes (Os06g0216300, OsOPR1; Os06g0216200, OsOPR2; and Os06g0215600, OsOPR5) in rice genome [72] were identified in this study. Among them, OsOPR1 was reported to be transiently regulated by diverse environmental cues such as wounding, osmotic stress, UV-C irradiation and fungal elicitor [73]. Jasmonate ZIM domain proteins (JAZ) are key regulators of jasmonate hormonal responses by inhibiting DNA-binding transcription factors in the absence of jasmonic acid [74, 75]. ZIM (Zinc-finger protein expressed in inflorescence meristem) domain, also known as TIFY domain, is a plant specific transcription factor containing a core motif TIF[F/Y]XG [76, 77]. The identified two genes here were designated as OsJAZ5 (Os04g0395800) and OsJAZ8 (Os09g0439200) [78]. OsJAZ8 was reported to participate in jasmonate-induced resistance to bacterial blight in rice [79]. Besides, we identified a gene encoding a jacalin-like lectin domain containing protein, OsJAC1, which was reported to be induced by methyl jasmonate and plays important roles in rice defense-related phenomena [80].

Finally we found a series of stress-related genes in the high C:low N list. OsACA3 (autoinhibited calcium ATPase 3, Os03g0203700) encodes one of the fifteen plasma membrane Ca^{2+}-ATPases in rice and is up-regulated under salt and cold stress [81]. Plasma membrane Ca^{2+}-ATPases are transport proteins in the plasma membrane of cells and help in removal of Ca^{2+} from the cell [82]. In most cases, these proteins function in abiotic and biotic stress adaptation by regulating Ca^{2+} level within cells [83]. OsPME (Os01g0788400) encodes one of the 35 pectin methylesterases in rice [84]. Pectin methylesterases are ubiquitous enzymes that modify the

degree of methylesterification of pectins, which are major components of cell walls in bacteria, fungi and plants [85]. In plants, *PME* genes belong to large multi-gene families and are primarily involved in developmental processes and plant-pathogen interactions [86, 87]. *OsAOX1B* (*Os04g0600300*) encodes one of the three alternative oxidases (AOXs) in rice genome [88]. AOX, located in the mitochondrial inner member, is a unique component in plant mitochondrial electron transport chain and catalyses the alternative respiratory pathway [89]. Most biotic and abiotic stresses, including pathogen or virus invasion, drought, high salinity, chilling, high light, high temperature, metal toxicity and nutrient limitation can increase the amounts of AOX, implying its involvement in stress tolerance [90, 91]. Furthermore, the protein content and capacity of AOX are up-regulated at low N condition in spinach (*Spinacia oleracea* L.) [92]. *OsPDR9* (*Os01g0609300*) encodes one of the 23 pleiotropic drug resistance (PDR)-like ATP-binding cassette (ABC) transporters in rice [93]. Plant PDR-like ABC transporters are expressed in response to various biotic and abiotic stresses and transport a diverse array of molecules across membranes [94]. *OsPDR9* was reported to be induced by jasmonic acid, heavy metals, high salt, hypoxic stress and redox perturbations, suggesting its role in stress responses [95] (Table 1).

Compared to the high number of high C:low N up-regulated genes, only 17 genes were found to be down-regulated by high C:low N and 10 up-regulated genes by low C:high N. All of the 17 high C:low N down-regulated genes have not been well studied and their functional information is hardly available. Among the low C:high N up-regulated genes, a GRAS transcription factor encoding gene (*Os05g0500600*) was identified. GRAS transcription factors constitute a large family of plant-specific proteins with at least 32 and 57 members in *Arabidopsis* and rice, respectively [96], and play critical roles in diverse biological processes, such as root development, gibberellic acid signaling and nodule symbiosis [97–99]. In addition, a chlorophyll a-b binding protein 2 (CAB2) encoding gene (*Os01g0720500*) was up-regulated by low C: high N. CAB, an antenna protein in the light harvesting complex, is essential for the uptake of light into photosystem II [100]. There are 17 genomic loci encoding for chlorophyll a-b binding proteins in rice [101]. Sugar has been reported to repress *CAB* gene expression in maize protoplasts [9]. In addition, *CAB* is not only down-regulated by sugar but also up-regulated by nitrogen and is considered as a marker gene of low C:high N status in *Arabidopsis* [26].

Confirmation of microarray results by qRT-PCR analysis

To confirm the reliability of microarray results, we investigated the expression of some genes in rice seedlings with the same C:N treatments by qRT-PCR analysis. Three jasmonate synthetic genes (*OsLOX*, *OsAOS2* and *OsOPR5*), one chalcone synthase encoding gene (*OsCHS*), one chlorophyll a-b binding protein encoding gene (*OsCAB2*) and one peroxidase encoding gene (*OsPERO*) were selected. The three jasmonate synthetic genes as well as *OsCHS* were induced specifically by 60:1 treatment (Fig 4A, 4B, 4C and 4D) while *OsCAB2* and *OsPERO* were induced specifically by 1:60 treatment (Fig 4E and 4F). These expression patterns were consistent to the above analysis, demonstrating that our microarray results are reliable for the investigation of the genome-wide responsive genes to C:N treatments.

Gene ontology (GO)-based functional categorization of imbalanced C:N responsive genes

We employed GO category enrichment analysis to classify the biological functions of genes responsive to imbalanced C:N conditions. We searched the GO terms using AgriGO [32]. Using $p \leq 0.05$ as cut-off, there were no significant GO categories for the genes down-regulated by high C:low N and neither those genes up-regulated by low C:high N. However, significantly
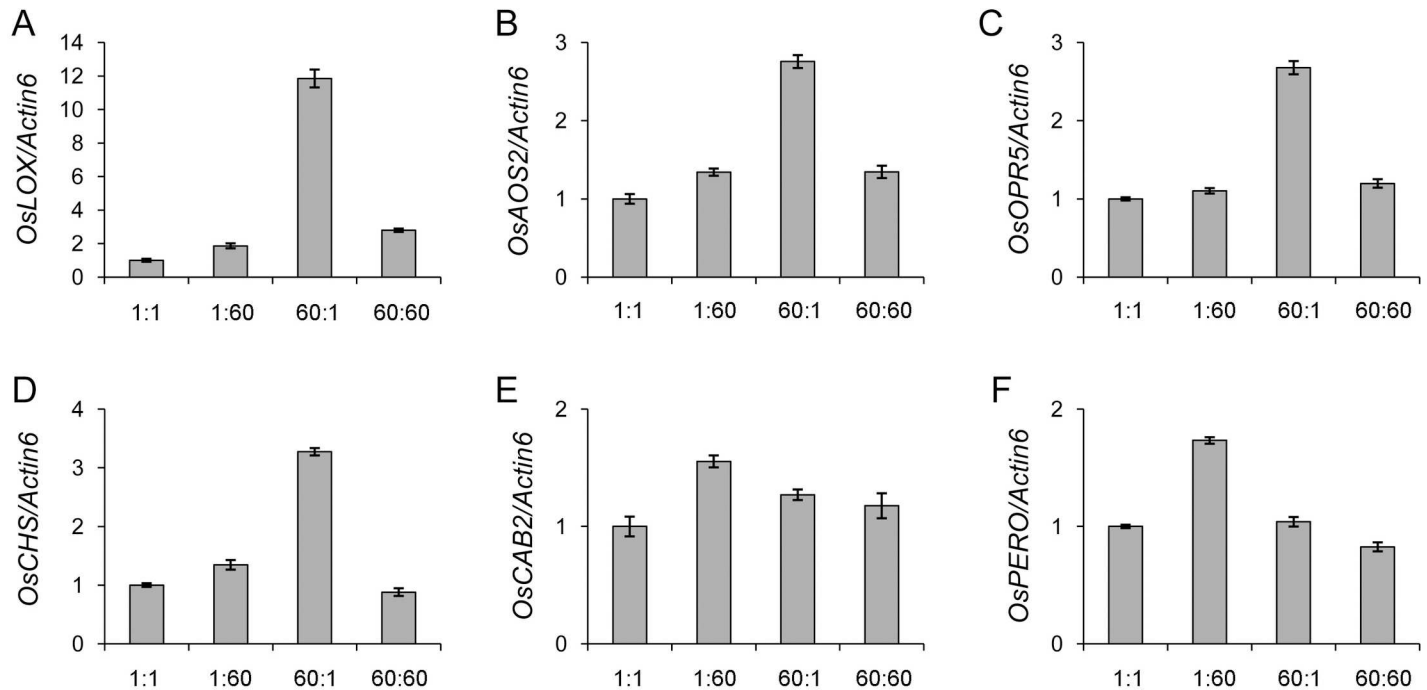
Fig 4. Validation of microarray results by qRT-PCR. (A) *OsLOX*; (B) *OsAOS2*; (C) *OsOPR5*; (D) *OsCHS*; (E) *OsCAB2*; (F) *OsPERO. Actin6* was used as the internal reference. The gray bars indicated the fold change of the genes between treatments (1:60, 60:1 and 60:60) and the control (1:1). Values are shown as means ± SDs from three technical replicates. A representative experiment of two biological replicates is shown.
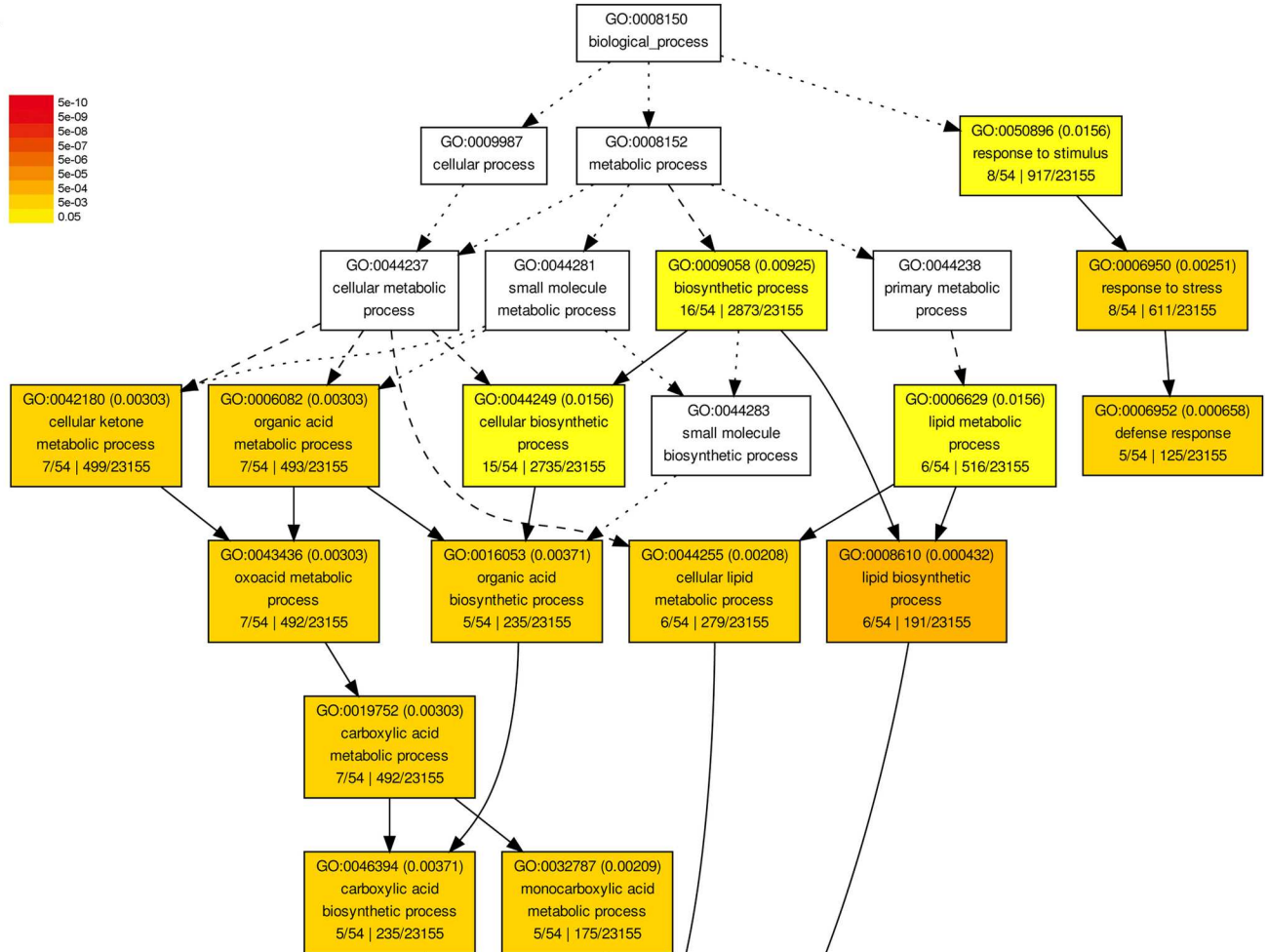
doi:10.1371/journal.pone.0165732.g004

enriched GO terms were identified for the genes up-regulated by high C:low N, which was listed in S3 File. Among the high C:low N up-regulated genes, several GO terms including oxoacid metabolism, organic acid biosynthesis, cellular lipid metabolism and lipid biosynthesis were significantly enriched (Fig 5A). First of all, the intersection of these GO terms was "fatty acid biosynthesis (GO: 0006952)", which included four jasmonate synthesis genes and one chalcone synthesis gene mentioned above (Fig 5C). Second, eight high C:low N genes were enriched in GO term "response to stress", among which two jasmonate synthetic genes and two ZIM domain containing genes as well as one pathogen-related protein encoding gene (*Os01g0248500*) were categorized in "defense response" process (GO: 0006633) (Fig 5A and 5C). The third enriched GO category was "oxidoreductase activity" (GO: 0016491) (Fig 5B). Oxidoreductase activity catalyzes an oxidation-reduction (redox) reaction, in which one substrate acts as electron donor and becomes oxidized while the other acts as electron acceptor and becomes reduced. Six jasmonate synthetic genes (two *OsLOX* genes, one *OsAOS* gene, and three *OsOPR* genes) and a malate dehydrogenase encoding gene were identified among the eleven genes in "oxidoreductase activity" category (Fig 5C).
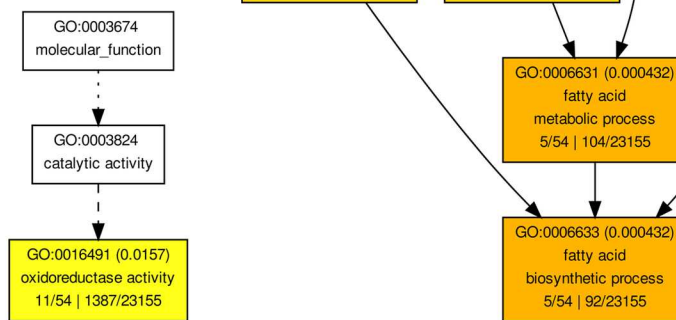
Coexpression analysis of high C:low N up-regulated genes

Coexpression network analysis may provide clues to the functions of genes involved in specific biological processes. To gain better insights into the high C:low N up-regulated genes, coexpression network was developed using RiceFREND online program [33]. Two coexpression networks were formed with 111 and 116 edges in module 1 and module 2, respectively (Fig 6). In module 1, fourteen high C:low N up-regulated genes were the hubs connecting with other 72 genes (S4 File). Among these 86 genes, several ones encode for transcription factors, such as three *MYB* genes (*Os05g0553400*, *Os02g0624300* and *Os04g0517300*), six *WRKY* genes (*Os01g0246700*,

A

Color scale:
5e-10
5e-09
5e-08
5e-07
5e-06
5e-05
5e-04
5e-03
0.05

GO:0008150
biological_process

GO:0009987
cellular process

GO:0008152
metabolic process

GO:0050896 (0.0156)
response to stimulus
8/54 | 917/23155

GO:0044237
cellular metabolic
process

GO:0044281
small molecule
metabolic process

GO:0009058 (0.00925)
biosynthetic process
16/54 | 2873/23155

GO:0044238
primary metabolic
process

GO:0006950 (0.00251)
response to stress
8/54 | 611/23155

GO:0042180 (0.00303)
cellular ketone
metabolic process
7/54 | 499/23155

GO:0006082 (0.00303)
organic acid
metabolic process
7/54 | 493/23155

GO:0044249 (0.0156)
cellular biosynthetic
process
15/54 | 2735/23155

GO:0044283
small molecule
biosynthetic process

GO:0006629 (0.0156)
lipid metabolic
process
6/54 | 516/23155

GO:0006952 (0.000658)
defense response
5/54 | 125/23155

GO:0043436 (0.00303)
oxoacid metabolic
process
7/54 | 492/23155

GO:0016053 (0.00371)
organic acid
biosynthetic process
5/54 | 235/23155

GO:0044255 (0.00208)
cellular lipid
metabolic process
6/54 | 279/23155

GO:0008610 (0.000432)
lipid biosynthetic
process
6/54 | 191/23155

GO:0019752 (0.00303)
carboxylic acid
metabolic process
7/54 | 492/23155

GO:0046394 (0.00371)
carboxylic acid
biosynthetic process
5/54 | 235/23155

GO:0032787 (0.00209)
monocarboxylic acid
metabolic process
5/54 | 175/23155

GO:0006631 (0.000432)
fatty acid
metabolic process
5/54 | 104/23155

B

GO:0003674
molecular_function

GO:0003824
catalytic activity

GO:0016491 (0.0157)
oxidoreductase activity
11/54 | 1387/23155

GO:0006633 (0.000432)
fatty acid
biosynthetic process
5/54 | 92/23155

C

GO term	*p*-value	Genes
Fatty acid biosynthetic process	3.10E-06	*Os06g0216200* (*OsOPR2*), *Os06g0216300* (*OsOPR1*), *Os08g0508800* (*OsLOX*), *Os02g0205500* (*OsCHS*), *Os08g0509100* (*OsLOX*)
Defense response	1.30E-05	*Os09g0439200* (*OsJAZ8*), *Os06g0216200* (*OsOPR2*), *Os06g0216300* (*OsOPR1*), *Os04g0395800* (*OsJAZ5*), *Os01g0248500*
Oxidoreductase activity	0.00032	*Os08g0508800* (*OsLOX*), *Os04g0581100*, *Os04g0497000*, *Os06g0215600* (*OsOPR5*), *Os08g0434300* (*OsMDH*), *Os11g0483000*, *Os03g0225900* (*OsAOS2*), *Os06g0216200* (*OsOPR2*), *Os09g0365900*, *Os08g0509100* (*OsLOX*), *Os06g0216300* (*OsOPR1*)

Fig 5. Gene ontology (GO) enrichment analysis of genes up-regulated by high C:low N. The differentially expressed probe sets were analyzed by SEA (singular enrichment analysis) using AgriGO, and the comparison is displayed in graphical mode. Each box contains GO term number, the false discovery rate (FDR) value, GO term and item number associated with the GO term in the query list and background as well as total number of query list and background. The degree of color saturation of a box is positively correlated to the enrichment level of the term (the yellow-to-red represents the term is up-regulated while non-significant terms are shown as white boxes). Solid, dashed and dotted lines represent two, one and zero enriched terms at both ends connected by the line, respectively. (A) Biological process category analysis of high C: low N up-regulated genes; (B) Molecular function category analysis of high C:low N up-regulated genes; (C) List of screened genes in "fatty acid biosynthesis", "defense response" and "oxidoreductase activity" categories with *p*-values.

Os01g0826400, *Os02g0181300*, *Os05g0343400*, *Os05g0537100* and *Os06g0649000*), two *C2H2* genes (*Os03g0437200* and *Os03g0820400*), two *bHLH* genes (*Os04g0301500* and *Os03g0741100*), two *AP2/EREBP* genes (*Os02g0654700*, *OsERF91*; *Os03g0860100*) and one *HSF* gene (*Heat stress*

A Module 1 (14)

KEGG Pathway	Locus ID	Highlight
Metabolic pathways	Os03g0767000 Os05g0211100 Os08g0448000	●
α-Linolenic acid metabolism	Os03g0767000	●
Phosphatidylinositol signaling system	Os03g0743500	●
Plant-pathogen interaction	Os01g0826400 Os01g0955100 Os03g0743500 Os05g0343400	●
Biosynthesis of secondary metabolites	Os05g0211100 Os08g0448000	●
Ubiquinone and other terpenoid-quinone biosynthesis	Os08g0448000	●
Phenylpropanoid biosynthesis	Os08g0448000	●
Steroid biosynthesis	Os05g0211100	●

1 *Os04g0517100 (MYB)*
2 *Os03g0437200 (C2H2)*
3 *Os04g0301500 (bHLH)*
4 *Os10g0497700*
5 *Os01g0864500*
6 *Os02g0205500 (CHS)*
7 *Os02g0209300*
8 *Os10g0580900*
9 *Os04g0497000*
10 *Os06g0203600*
11 *Os09g0439200 (OsJAZ8)*
12 *Os04g0395800 (OsJAZ5)*
13 *Os11g0483000*
14 *Os04g0639000*

B Module 2 (9)

KEGG Pathway	Locus ID	Highlight
Metabolic pathways	Os03g0235000 Os04g0447700 Os05g0457800 Os06g0215600 Os08g0508800 Os08g0509100	●
α-Linolenic acid metabolism	Os06g0215600 Os08g0508800 Os08g0509100	●
Linoleic acid metabolism	Os04g0447700 Os08g0508800 Os08g0509100	●
Methane metabolism	Os03g0235000	●
Biosynthesis of secondary metabolites	Os03g0235000	●
Phenylalanine metabolism	Os03g0235000	●
Phenylpropanoid biosynthesis	Os03g0235000	●
Arachidonic acid metabolism	Os04g0556300	●
Glutathione metabolism	Os04g0556300	●
Glycerolipid metabolism	Os05g0457800	●
Glycerophospholipid metabolism	Os05g0457800	●
Butanoate metabolism	Os04g0447700	●
Fructose and mannose metabolism	Os04g0447700	●

1 *Os01g0584900 (OsWRKY77)*
2 *Os08g0509100*
3 *Os06g0216200 (OsOPR2)*
4 *Os06g0216300 (OsOPR1)*
5 *Os01g0609300 (OsPDR3)*
6 *Os03g0225900 (OsAOS2)*
7 *Os08g0508800*
8 *Os06g0215600 (OsOPR5)*
9 *Os04g0600300 (OsAOX1B)*

Fig 6. Coexpression network analysis of high C:low N up-regulated genes. (A) Module 1 extracted from coexpression analysis using 14 microarray identified genes. (B) Module 2 extracted from coexpression analysis using 9 microarray identified genes. Red and blue nodes indicate high C:low N up-regulated genes and the red ones are transcription factors. Other genes with known names or encode for transcription factors are marked on the nodes. Genes involved into KEGG pathways are marked with color dots beneath the nodes and the detailed information are listed on the tables.

transcription factor SPL7, Os05g0530400). *OsSPL7* is a rice spotted leaf (lesion-mimic) gene encodes for a heat stress transcription factor [102]. Furthermore, AP2/EREBPs (APETALA2/ethylene-responsive element-binding proteins) belong to large family of transcription factors in plants and play important roles in the cross-talks among different stress signaling pathways [103]. The coexpression of these stress-related transcription factors are consistent with the above mentioned GO analysis category "response to stress" in high C:low N up-regulated genes. In addition, *OsAOS1* (*Os03g0767000*) and, surprisingly, eight of the fifteen *OsJAZ* genes in rice genome (*Os04g0395800, OsJAZ5; Os03g0402800, OsJAZ6; Os09g0439200, OsJAZ8; Os03g0180 800, OsJAZ9; Os03g0181100, OsJAZ10; Os03g0180900, OsJAZ11; Os10g0392400, OsJAZ12; Os10g0391400, OsJAZ13*) were found in module 1 (Fig 6A, S4 File). Similar to *OsAOS2* discussed above, *OsAOS1* encodes for an allene oxide synthase, which serves as a key enzyme for the biosynthesis of jasmonic acid. The transcripts of *OsAOS1* were reported to be up-regulated transiently in response to light and wounding [104]. Among the *OsJAZ* genes in the network, *OsJAZ9* was reported to be a transcriptional regulator fine tuning the expression of JA-responsive genes involved in salt stress tolerance in rice [105]. In order to gain an overview of gene pathway networks, KEGG (Kyoto Encyclopedia of Genes and Genomics) analysis was performed using the online KEGG automatic annotation server. Eight categories were involved in the module 1. Plant-pathogen interaction and metabolic pathways (lipid metabolism and amino acid metabolism) were the most noteworthy ones with four and three genes, respectively (Fig 6A). These results are consistent with the GO categories "defense response" and "fatty acid biosynthetic process", suggesting that high C:low N might serve as an inducer for defense responses, which involve certain fatty acid components.

In module 2, nine high C:low N up-regulated genes were the hubs connecting with other 89 genes (S4 File). Two *WRKY* genes (*Os01g0584900* and *Os11g0490900*), one *HD-Zip* gene (*Homeodomain-leucine zipper, Os09g0379600*), one *GRAS* gene (*Os11g0705200*) and one *bHLH* gene (*Os05g0455400*) were identified (Fig 6B, S4 File). HD-Zip transcription factors, which are unique in the plant kingdom, have diverse functions in plant development and often been implicated in stress adaptation [106]. The identified HD-Zip transcription factor is encoded by *OsHOX25* (*Homebox gene 25*). In rice, two of the 33 *OsHOX* genes have been studied in detail, among which *OsHOX4* was reported to be involved in drought stress [107] and *OsHOX22* functions in drought and salt stress [108]. In addition, the auxin responsive gene *OsSAUR26* (*Small auxin-up RNA 26, Os06g0671600*), the potassium (K^+) transporter gene *OsHAK16* (*High-affinity K^+ transporter 16, Os03g0575200*), the chitinase encoding gene *OsCHT3* (*Chitinase 3, Os06g0726100*) and two pathogenesis-related protein (PR protein) encoding genes *OsPR10A* (*Pathogenesis-related gene 10A, Os12g0555500*) and *OsPR10B* (*Pathogenesis-related gene 10B, Os12g0555200*) were identified in the module 2 coexpression network. Chitinase, a hydrolytic enzyme belonging to the PR proteins, could hydrolyze the cell wall chitin in most fungi and thus in plants chitinase acts in defense against fungal infection [109]. *OsCHT3*, also named as *PR3/OsChia1c*, functions as a defense-related gene in rice [110]. PR10 are small proteins with a molecular weight of 16 kDa. Many biotic or abiotic stresses have been shown to induce the expression of PR10 protein transcriptionally, suggesting their importance in plant defense responses. Three highly homologous *PR10* genes, *OsPR10A, OsPR10B* and *OsPR10C*, are known in rice, but *OsPR10C* appears to be a non-functional pseudogene [111]. These genes together with those in module 1 KEGG analysis (pathogen interaction) suggest enhanced defense responses under high C:low N conditions.

Network genes in module 2 fell into 13 categories of KEGG pathways including α-linolenic acid, linoleic acid metabolism and methane metabolism, etc (Fig 6B). It is known that jasmonate is derived from the unsaturated fatty acid α-linolenic acid (18:3), an octadecanoid abundant in the cellular membranes of higher plants [112]. Thus we propose that genes involved in

α-linolenic acid metabolism in module 1 and module 2 networks might serve as jasmonate precursor synthetic genes. Taken together, the above coexpression network analysis of high C:low N up-regulated genes demonstrated that these genes function in jasmonate-mediated defense responses, suggesting that high C:low N imbalanced C:N nutrient status might serve as a special stress condition in plants.

Discussion

Although it is controversial whether plants are able to sense and respond to C:N ratios [6, 7, 26, 113], it is well known that C and N nutrients interact with each other and that a balanced C:N availability is important for optimal plant growth and development. We speculated that plants would be able to sense and respond to imbalanced C:N availability by regulating genes involved in the C and N nutrient uptake and metabolism as well as genes dealing with the stresses caused by the imbalance of C:N. Our present findings based on global gene expression analysis provide strong evidence for this hypothesis [18, 21, 114]. Our analysis shows that both imbalanced high C:low N and low C:high N conditions induce changes in the expression of genes irrespective of absolute levels of C and N availabilities. In agreement with our hypothesis, these imbalanced C:N responsive genes include those involved C and N metabolism and stress responses.

In this study, we identified 63 up-regulated genes and 17 down-regulated genes by high C:low N. Low C:high N, however, was found to up-regulate the expression of 10 genes (Fig 3E). The larger number of high C:low N responsive genes compared with low C:high N responsive genes might come from the larger number of C regulated genes compared with N regulated genes (Fig 3A, 3B, 3C and 3D). As expected, some of these imbalanced C:N responsive genes are involved in the regulation of C and N assimilation/metabolism in the attempt for rice seedlings to reach C and N homeostasis (balance). Among the 10 up-regulated genes by low C:high N, *OsCAB2* is the homolog of *Arabidopsis CAB*, which has been demonstrated to be a low C:high N marker gene [26]. Several C:N signaling and metabolism related genes are also found to be regulated by high C:low N. Among them, two *OsCHS* genes are the homologs of *Arabidopsis CHS*, which is reported as high C:low N marker gene [26]. Increase in chalcone synthase levels is associated with higher anthocyanin accumulation and reduced chlorophyll contents and photosynthesis. *OsMDH* is another gene identified in high C:low N condition. Decreased MDH activity leads to enhanced photosynthetic performance in transgenic tomato plants [62]. If this is the case in rice, increased MDH levels would decrease the photosynthesis and contribute to the recovery of balanced C:N status under high C:low N condition. Furthermore, *OsLHT1* identified here might also serve as a regulator to maintain balanced C:N status, because overexpression of *LHT* resulted in increased amino acid uptake capacity and thus N use efficiency under limited inorganic N supply in *Arabidopsis* [55]. Last but not the least, one of the three *AOXs* in rice, *OsAOX1B*, is induced under the high C:low N condition. It was reported that under low N stress, the protein content and capacity of AOX would be preferentially increased and would efficiently consume excess carbohydrates, suppressing the rise of C:N ratio to maintain at a moderate level [92, 115]. A recent study also suggests that plants under disrupted C:N reduce the electron transport rate at PSII level and thus the excess of energy must be dissipated through alternative respiratory pathway [116]. In fact, the function of alternative respiratory pathway has long been controversial, for its electron transport from ubiquinone to water does not contribute to a transmembrane potential and wastes energies only [89]. Further studies found that AOX could be taken as a survival protein involved in the adaptation of stress conditions in plants [90, 117]. Despite AOX has been suggested to affect nitrate and carbon assimilation simultaneously [118], it is hard to distinguish the role of *OsAOX1B* identified here: dedicates into the recovery of balanced C:N or serves as a marker gene for stress condition?

We found that some jasmonate signaling components (two *OsLOX* genes, one *OsAOS* gene, three *OsOPR* genes and two *OsJAZ* genes), as well as several stress responsive genes, such as *OsMYB4*, *OsACA3* and *OsPDR9*, were up-regulated by high C:low N. GO category enrichment analysis found that these genes were mainly distributed in "fatty acid biosynthesis" and "defense response" processes. In addition, coexpression network analysis primarily identified two groups of new genes: jasmonate signaling components (e. g. *OsAOS1*, *OsJAZ* genes) and defense related genes (e. g. *OsSPL7*, *OsCHT3*, *OsPR10A* and *OsPR10B*). These results indicated that high C:low N status might result in defense responses regulated by jasmonate signaling in rice seedlings. C and N metabolism have been previously suggested to be involved in the plant defense responses [119, 120]. However, the mechanisms by which C:N contribute to plant immune responses are poorly understood. In spite of this, some preliminary studies are consistent with our observations. For example, sucrose treatment transcriptionally induces pathogenesis related genes [121] while N supply generally decreases defense responses, specifically, the high availability of N sources significantly increase the susceptibility of potato (*Solanum tuberosum*) to the oomycete *Phytophthora infestans* [122]. Moreover, defense related transcription factor genes *MYB51* and *WRKY33* are dramatically induced under high C:low N treatment in *Arabidopsis*, and a regulator of the C:N response, *Arabidopsis* ATL31 ubiquitin ligase, positively regulates the defense response against bacterial pathogens [123].

These findings raise an interesting question: What is the biological significance for the activation of defense responses by high C:low N treatment? Here we propose two possible explanations. First, increased jasmonate content or defense responses under high C:low N condition might be beneficial for the recovery of C:N balance. Given that C and N metabolites are apparently altered by pathogen infection [124] and long-distance transport of resources is one of the strategies in plant defense responses [125–127], the stress/defense responses under high C:low N condition might be related to the reallocation of CN nutrients. On the one hand, N transport and partitioning could be altered by jasmonate treatment [128, 129]. On the other hand, jasmonate mediated defense responses can decrease local photosynthetic rates by reducing photosynthetic electron transport and gas exchange [130–132] as well as accelerate the import of C from source leaves to sink tissues [126, 133]. The second explanation is that the induction of the defense responses might complement the defense ability, which is attenuated by the high growth rate under high C:low N condition. TOR proteins could sense nutrient conditions and control cell growth by regulating relevant transcriptional and translational processes. High C condition would activate TOR signaling pathway and thus the fast growth phenotype [5, 134]. According to the growth-defense hypothesis, fast-growing plant species should suffer more from pathogens than slower-growing species [135, 136]. In this case, the inductive defense responses under high C:low N condition might serve as a protective measure when most resources are invested into growth processes. Nonetheless, further studies are needed to investigate the potential relationship between C:N balancing mechanisms and jasmonate related stress/defense responses.

In addition to genes that can be categorized into functional pathways described above, imbalanced C:N conditions also induced a series of pioneer genes with unknown function and not associated with any genes with known GO categories. Almost all of the genes induced under low C:high N conditions (8 out 10) and 17 genes repressed under high C:low N conditions are in this category. We performed GO analysis to find some clues for the function of these genes, however, no enriched terms were found. Thus, these findings suggest possible novel mechanisms underlying the regulation of imbalanced C:N responses. Further studies should reveal these novel mechanisms and whether they are unique to plants accumulating high levels of carbohydrates such as cereal species.

Supporting Information

S1 Fig. Hierarchical cluster analysis of rice genes (probe sets) between CN treatments (1:1 and 60:1, 1:1 and 1:60, 1:60 and 60:60, 60:1 and 60:60). The $\log_2$ ratio values of probe sets were used for the analysis with R software. The colored bars represent the value ($\log_2$(fold change)) of the transcripts in each bin after CN treatments. Green represents down-regulated probe sets, red represents up-regulated probe sets, and dark indicates no significant difference in gene expression. (A) Hierarchical clustering of rice genes (probe sets) between 1:1 and 60:1; (B) Hierarchical clustering of rice genes (probe sets) between 1:1 and 1:60; (C) Hierarchical clustering of rice genes (probe sets) between 1:60 and 60:60; (D) Hierarchical clustering of rice genes (probe sets) between 60:1 and 60:60.
(TIF)

S1 File. The raw microarray data for the rice expression profiling under four CN treatments.
(XLS)

S2 File. Differentially expressed gene lists between CN treatments.
(XLS)

S3 File. The GO category enrichment analysis results of up-regulated probe sets in high C: low N treatment.
(XLSX)

S4 File. Coexpression analysis results of module 1 and module 2 gene lists.
(XLSX)

S1 Table. List of gene specific primer sequences used in qRT-PCR.
(DOCX)

Acknowledgments

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