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An integrated RNA-Seq and network study reveals a complex regulation process of rice embryo during seed germination

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ABSTRACT

Seed germination is a crucial stage for plant development and agricultural production. To investigate its complex regulation process, the RNA-Seq study of rice embryo was conducted at three time points of 0, 12 and 48 h post imbibition (HPI). Dynamic transcriptional alterations were observed, especially in the early stage (0–12 HPI). Seed related genes, especially those encoding desiccation inducible proteins and storage reserves in embryo, decreased drastically after imbibition. The expression profiles of phytohormone related genes indicated distinct roles of abscisic acid (ABA), gibberellin (GA) and brassinosteroid (BR) in germination. Moreover, network analysis revealed the importance of protein phosphorylation in phytohormone interactions. Network and gene ontology (GO) analyses suggested that transcription factors (TFs) played a regulatory role in functional transitions during germination, and the enriched TF families at 0 HPI implied a regulation of epigenetic modification in dry seeds. In addition, 35 germination-specific TF genes in embryo were identified and seven genes were verified by qRT-PCR. Besides, enriched TF binding sites (TFBSs) supported physiological changes in germination. Overall, this study expands our comprehensive knowledge of multiple regulation factors underlying rice seed germination.

1. Introduction

Seed germination is a rapid transition process from dormancy to seedling growth. Briefly defined, seed germination begins with water uptake and ends with radicle emergence [1]. It can be divided into a rapid imbibition phase and a plateau phase of water uptake [2]. Germination is an efficient process, which is accompanied by dramatic metabolic and physiologic changes, such as mobilization of stored reserves, energy production, signaling transduction and transcription activation.

There are a number of factors controlling seed germination. Historically, germination is considered to be affected by the balance of abscisic acid (ABA) and gibberellin (GA) [1], but all other phytohormones have been increasingly reported to be involved in the process [3–6]. Transcription factors (TFs) dynamically alter the transcriptional activities through recognizing TF binding sites (TFBSs), leading to metabolic changes [7,8]. Interestingly, other mechanisms such as chromatin remodeling and protein phosphorylation have been put forward [9–12]. Recent advances have provided much information for certain regulatory mechanisms of germination separately, but the comprehensive regulation of the efficient process is still unclear.

Rice is an important food crop and its genome has been sequenced as a model cereal. Based on the RNA-Seq study of rice embryo during seed germination, we integrated transcriptional dynamics and gene networks to analyze the combined regulation by multiple factors, including seed genes, phytohormones, TF families and TFBSs. In addition, the roles of phosphorylation and epigenetic modification were investigated. These analyses provide an overall understanding of the regulation process of germination.

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2. Materials and methods

2.1. Seed growth

Dry seeds of rice (*Oryza sativa L* Japonica. cv. Nipponbare) were grown in distilled water and incubated at 26 °C in the dark. Seeds were selected at three time points of 0, 12 and 48 h post imbibition (HPI), representing the dry, imbibed and germinated status, respectively. Embryos were rapidly cut from seeds, snap frozen in liquid nitrogen and stored at −80 °C.

2.2. RNA preparation and sequencing

Total RNA was extracted from embryos using TRIzol® Reagent (Invitrogen) as per the manufacturer's instructions. Oligo (dT) magnetic beads (ABI) were used to get the purified poly(A) mRNA. cDNA library was constructed by the SOLiD™ Whole Transcriptome Analysis Kit (ABI). Transcriptome sequencing was performed using the Applied Biosystems SOLiD System according to the manufacturer's protocol.

2.3. Gene expression

Generated reads were aligned to the reference genome of Japonica (MSU 7.0) in the Rice Genome Annotation Project (RGAP) using TopHat (v.1.4.1) [13]. Only uniquely matched reads were selected and used. Gene expressions were calculated and normalized to the reads per kilobase of transcript per million mapped reads (RPKM) using Cufflinks (v.1.3.0) [13]. The expression cutoff was defined as RPKM $\geq$ 0.1.

Hierarchical clustering of all expressed genes was conducted using R software. In addition, the genes were divided into three classes based on at which time point they showed the highest expression. Differentially expressed genes (DEGs) were determined by DEGseq [14], with the cutoff threshold of P $\leq$ 0.001.

2.4. Functional annotation

To determine statistically enriched (P $\leq$ 0.05) gene ontology (GO) terms of expressed genes, genes expressions were analyzed by parametric analysis of gene set enrichment (PAGE) in AgriGO (v.1.2) [15]. It was based on the background of *Oryza sativa* MSU 7.0 nonTE and default options of Fisher test and FDR adjustment method. To study metabolic pathways in Kyoto Encyclopedia of Genes and Genomes (KEGG) [16], expression profiles of important pathways were clustered based on the averaged gene expressions. Besides, MapMan (v.3.5.1R2) [17] was performed to visualize metabolic overview of transcript changes between adjacent time points, based on fold changes of gene expressions (Log2FC) and the background of Osa_MSU_v7.

2.5. Seed genes

SeedGeneDB (http://sgdb.cbi.pku.edu.cn/) was adopted to observe expression patterns of the genes that are dominantly expressed in rice seeds (defined by microarray data), especially the genes encoding embryo abundant proteins.

2.6. Gene collection, expression profiling and network analysis for phytohormones

Phytohormone related genes in synthesis, degradation and signaling pathways were integrated from three sources: KEGG, the study of Tang et al. and RiceCyc (v.3.3) [16,18,19]. The expression profiles for phytohormones were clustered based on the averaged expressions of genes encoding the same proteins.

To analyze phytohormone interactions, a network of all expressed phytohormone related genes was constructed using gene connections retrieved from RiceNet (v.1) [20], which was then visualized by Cytoscape (v.3.1.0) [21].

2.7. Gene collection, statistical analysis and functional network of TFs

TF genes of rice were collected from three sources: PlantTFDB (v.3.0), PlnTFDB (v.3.0) and the study of Caldana et al. [22–24]. Gene expressions, DEGs and gene classification were analyzed for TFs.

To compare the difference between the percentage of genes in a given family with the percentage of genes in that family in a given class, z-score analysis was applied as the following formula, based on the sample size, frequency, and percentages for each set [7,8]: $z = \frac{\widehat{\pi}1 - \widehat{\pi}2}{\sqrt{\widehat{\pi}(1-\widehat{\pi})\left(\frac{1}{n1}+\frac{1}{n2}\right)}}$. The z-scores were matched to the cumulative standard normal table to calculate P values. Then, TF families were classified into three groups based on z-scores and significantly (P $\leq$ 0.05) enriched families were determined.

To study the functions of TFs in germination, the connected genes (expressed) of expressed TF genes were extracted from RiceNet [20]. For each TF family, the significantly (P $\leq$ 0.05) over-represented GO terms of the family connected genes were statistically determined using the z-score analysis mentioned above. The relationships between TF families and enriched GO terms were constructed and visualized into a network using Cytoscape [21].

2.8. Identification of germination-specific TF genes and qRT-PCR verification

To effectively identify germination-specific TF genes in embryo, our data and several public available datasets were used and selected as following: 1) differentially (P $\leq$ 0.05) and highly (RPKM $\geq$ 50) expressed TF genes at least at one time point in our study; 2) the gene expressions in germinated seeds (both Minghui 63 and Zhenshan 97) ranking top 20 out of 131 development stages in RiceSRTFDB [25]; 3) expressed TF genes (RPKM $\geq$ 1) in embryo (embryo-25 DAP) in the RGAP (RNA-Seq data, http://rice.plantbiology.msu.edu/expression.shtml). As a reference and comparison, gene expressions of the identified specific TF genes in the previous oligo gene chip study of rice seed germination [7] were extracted.

From the germination-specific TF genes peaking at 12 HPI, seven TF genes were randomly selected to be verified by quantitative real-time PCR (qRT-PCR). Rice embryos at three time points were selected and incubated using the same method in “Seed growth” mentioned above. Total RNA was extracted by RNeasy Plant Mini Kit (QIAGEN). cDNA was synthesized from 2 μg of total RNA and oligo (dT) primers, using a Quantscript RT Kit. qRT-PCR was conducted by a CFX96 Real-Time PCR detection system (Bio-Rad). The primers (Table S1) were designed online (http://primer3.ut.ee/). Results were analyzed by CFX manager, and a reference gene of actin (LOC_Os03g50885) was used to normalize gene expressions. Two technical replicates were used for all of the samples.

2.9. TFBS analysis

To analyze TFBSs in germination regulation, the promoter regions (1-kb upstream) of expressed genes in each class were

retrieved and searched for enriched ($P \leq 0.01$) TFBSs using Osiris [26].

3. Results

3.1. Gene expression and transcriptional dynamics during germination

After sequencing, 83,273,725, 71,036,373 and 90,085,278 reads were generated for rice embryo at 0, 12 and 48 HPI, respectively. They were aligned to the reference genome of Japonica (MSU 7.0) (Table 1). Using the uniquely mapped reads, 27,847, 30,173 and 30,934 expressed genes (RPKM ≥ 0.1) were obtained for three time points. In total, 36,063 genes were expressed at least in one stage.

The hierarchical clustering of 36,063 expressed genes during germination revealed a closer relationship between 12 and 48 HPI in comparison with 0 HPI (Fig. 1A). These genes were classified into three classes, comprising 14,502 (40%), 8200 (23%) and 13,361 (37%) genes with the highest expression at 0 (class 1), 12 (class 2) and 48 (class 3) HPI, respectively. DEG analysis showed that 9083 (25%) genes changed significantly ($P <= 0.001$) in germination, including 6911 (2497 up, 4414 down) in the early stage (0–12 HPI) and 3083 (1947 up, 1136 down) in the late stage (12–48 HPI).

3.2. GO enrichment and metabolic analyses

AgriGO analysis assigned 22,797 (63%) expressed genes (7412 DEGs) to 157 GO terms, among which 95, 57 and 77 terms were significantly ($P <= 0.05$) enriched at 0, 12 and 48 HPI (Table S2). The functional categorization revealed dynamic molecular events during germination, such as “epigenetic regulation of gene expression”, “anatomical structure morphogenesis” and “carbohydrate binding” enriched 0, 12 and 48 HPI, respectively.

The expression profiles of metabolic pathways in KEGG were clustered (Fig. 1B). Nucleotide metabolism was relative abundant in dry seeds, carbohydrate and energy metabolism resumed immediately after imbibition and lipid metabolism increased later. In the MapMan analysis, the metabolic overview of transcriptional changes in the first 12 HPI displayed more biological transformations than in the late stage (Fig. S1). Among them, cell wall modification was significantly ($P \leq 0.05$) up-regulated in the early stage for facilitation in water-uptake, while amino acids metabolism and light reaction increased in the late stage in preparation for seeding growth.

3.3. Decreased gene expression patterns of seed abundant proteins

During germination, most (1052) of rice seed genes (1258) in SeedGeneDB were expressed. The numbers of down-regulated DEGs in the early and late stages (207 and 113) were much more than up-regulated (84 and 51). Especially, genes encoding the seed abundant proteins of late embryo abundant proteins (LEA), heat shock protein 20 (HSP20) and seed storage proteins [27] were examined (Fig. 1C). All genes encoding the desiccation inducible LEA and most genes of stress related HSP20 dropped drastically after imbibition. Although half (26) of the genes (52) encoding seed storage proteins increased in the early stage, most (48) of them decreased in the late stage, suggesting a preparation for germination.

3.4. Expression profiling and network analysis of phytohormone related genes

In total, 504 genes in phytohormone synthesis and signaling pathways were collected and 466 genes were expressed (159 DEGs) in germination. Expression profiles of phytohormone related genes were clustered (Fig. S2). For the germination repressor ABA, the key synthesis and signaling enzymes, such as 9-cis-epoxycarotenoid dioxygenases (NCED) and SNF1-related protein kinase 2s (SnRK2), were predominantly abundant in dry seeds, while degradation enzymes increased after imbibition. In comparison, GA increased in water-uptake and its signaling transduction was activated, such as GA INSENSITIVE DWARFs (GID1 and GID2), slender rice 1 (SLR1) and transcriptional activators of GAMYBs. Besides, most genes in brassinosteroid (BR) biosynthesis and signaling pathways including the responsible factor (BZR1) increased evidently after imbibition and accumulated continuously until the germination completion, suggesting a positive role in the whole process. Except the increased tendency of jasmonic acid (JA), related gene expression patterns for other phytohormones were not consistently observed, such as ethylene (ET), cytokinin (CK), auxin and salicylic acid (SA).

To analyze the phytohormone interactions in germination, a network comprising 195 expressed hormone genes (92 DEGs) and 378 gene connections was constructed (Fig. 2). Among the top ten hub genes (Degree ≥ 11) in the network, six genes encoded four types of phosphorylation related proteins: protein phosphatase 2C (PP2C, LOC_Os01g40094), mitogen-activated protein kinases 6 (MPK6, LOC_Os06g06090), GID2 (LOC_Os02g36974) and BR INSENSITIVE 2s (BIN2s; LOC_Os01g10840, LOC_Os 02g14130 and LOC_Os 06g35530) [10,11]. Accordingly, the four enzymes are core signals in ABA, ET, GA and BR pathways. Expressed genes encoding the enzymes and downstream proteins (such as SnRK2, SLR1 and BZR1) showed different transcriptional activities, basically supporting the roles of phytohormones in germination. Furthermore, these enzyme genes, especially the six hub genes, were connected with each other in the network, suggesting an essential role of phosphorylation in phytohormone interactions during germination. Additionally, the other four hub genes in top ten encoding S-adenosylmethionine synthetases (SAMs; LOC_Os01g22010, LOC_Os05g04510 and LOC_Os01g18860) in ET biosynthesis and 12-oxo-phytodienoic acid reductase (OPR, LOC_Os0611290) in JA signaling might play a potential role in rice germination.

3.5. Enriched TF families and their functional network in germination

Among 2954 TF genes integrated from public datasets, 2601 genes were expressed in germination and 802 genes changed significantly. Based on the statistical analysis of gene expressions, TF families were divided into three groups (Table S3), including 9, 8

Table 1
Summary of sequence data and read alignment statistics.

Time points	Reads	Mapped reads	Uniquely mapped	Expressed genes
0 HPI	83,273,725	58,003,818 (70%)	53,641,468 (64%)	27,847
12 HPI	71,036,373	37,710,560 (53%)	31,605,550 (44%)	30,173
48 HPI	90,085,278	63,798,646 (71%)	51,857,601 (58%)	30,934

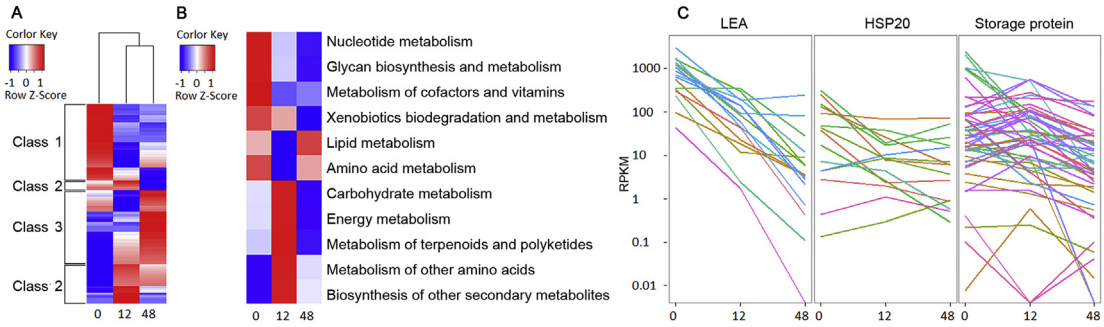

Fig. 1. Dynamic changes in gene expressions and functions. (A) Hierarchical clustering of 36,063 expressed genes. Three classes represent genes peaking at 0, 12 and 48 HPI, respectively. (B) Expression profiles of important metabolic pathways in KEGG. (C) Expression patterns of seed genes encoding embryo abundant proteins of LEA, HSP20 and storage proteins.

and 6 significantly ($P \leq 0.05$) overrepresented families at 0, 12 and 48 HPI, respectively (Fig. 3A).

To analyze the functional roles of TFs in germination, significantly ($P \leq 0.05$) enriched GO terms of the connected genes were determined for TF families, and thus the functional network of TF families were generated and constructed (Fig. S3). Three groups of stage-enriched TF families and their functional connections were extracted (Fig. 3B), which showed a basic consistence with molecular events in different stages. For example, PHD, SET, SNF2 and CAMTA families in group 1 were closely related with "regulation of gene expression, epigenetic" and "cell cycle", which occurred in dry seeds. On the other hand, ERF, bHLH and HD-ZIP families in group 2 were connected with functional alterations in the early stage, such as "anatomical structure morphogenesis", "generation of precursor metabolites and energy" and "carbohydrate metabolic process". In comparison, WRKY, AUX/IAA, TCP, Tify and NAC families in group 3 were involved with "lipid metabolic process", "response to stress" and "photosynthesis" that occurred in the late stage.

3.6. Identification and verification of rice germination-specific TF genes

By integrating public datasets, 35 highly and specifically expressed TF genes during germination were identified in rice embryo, containing 14, 18 and 3 genes in three classes (Table S4). Except four genes that were not detected, a similar gene expression pattern during germination was observed in the previous oligo gene chip study [7], which suggested the validity and sensitivity of our RNA-Seq study.

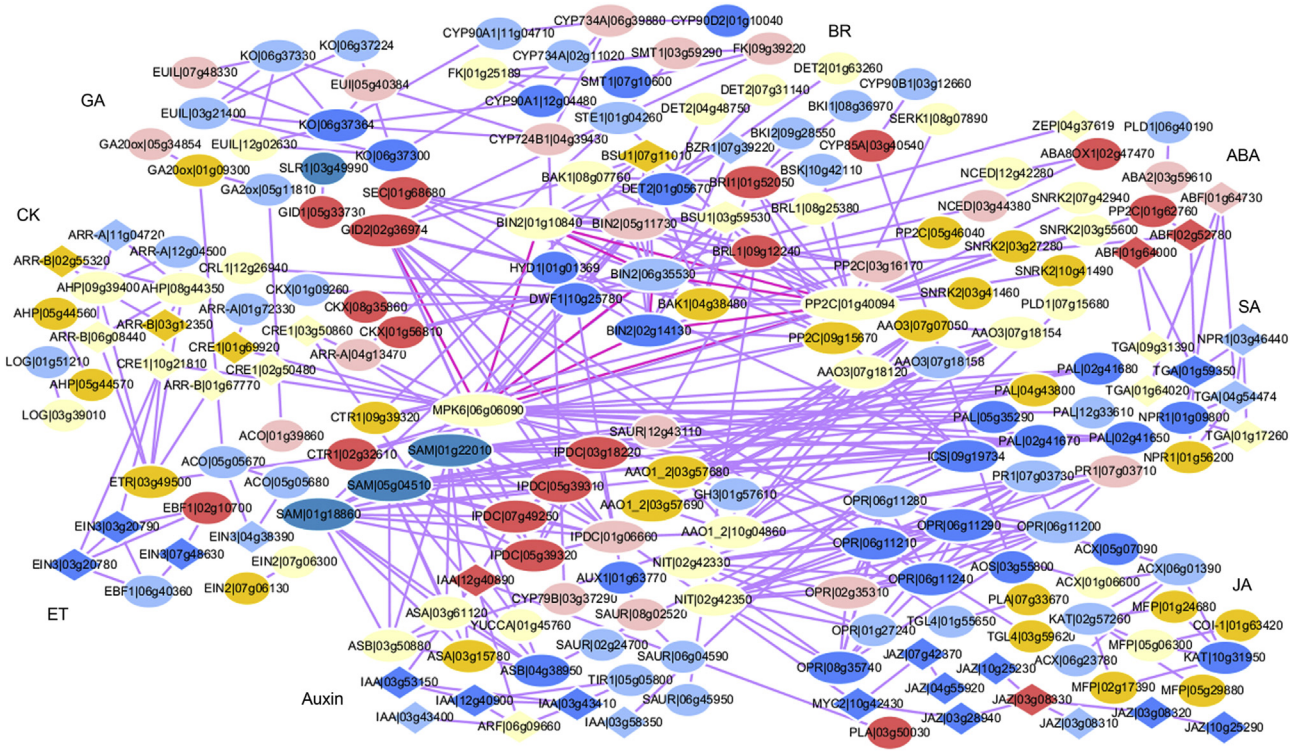

Fig. 2. The network of 195 phytohormone related genes. Gene connections among the six hub genes encoding phosphorylation related PP2C, MPK6, GID2 and BIN2s were highlighted in plum purple. Node label separated by "|" represents enzyme and encoding gene. Yellow, red and blue in ellipse represent three gene classes peaking at 0, 12 and 48 HPI, and deep color means DEG. Triangle represents TF in pathway. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

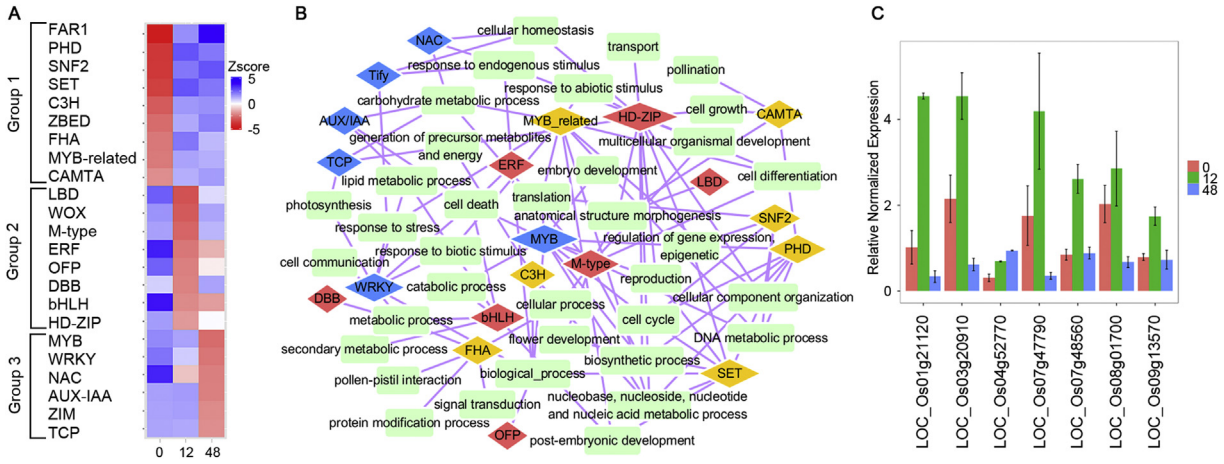

Fig. 3. Stage-enriched TF families and germination-specific TF genes. (A) Three groups of stage-enriched ($P \leq 0.05$) TF families were statistically determined by z-score analysis. (B) The network between significantly enriched TF families and the significantly ($P \leq 0.05$) overrepresented GO terms of the connected genes. Yellow, red and blue in ellipse represent three groups of TF families enriched at 0, 12 and 48 HPI, respectively. Green in round rectangle represents enriched GO term of TF connected genes. (C) The qRT-PCR of seven germination-specific TF genes selected from class 2. They are LOC_Os01g21120 (OsAP2-3), LOC_Os03g20910 (WOX), LOC_Os04g52770 (OsbHLH101), LOC_Os07g47790 (OsAP2-76), LOC_Os07g48560 (OsWOX11), LOC_Os08g01700 (OsbHLH124) and LOC_Os09g13570 (OsbZIP71). The y-axis represents the relative gene expression compared with the marker (LOC_Os03g50885). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

With a transient high expression at 12 HPI, class 2 among the specific genes may represent an important regulatory switch that drives the transcriptomic dynamics in germination. Thus, seven genes were randomly selected from class 2 and validated by qRT-PCR (Fig. 3C). The experimental results basically consisted with gene expression patterns in RNA-Seq. Besides, the *Arabidopsis* homologs of LOC_Os01g21120 (At2g47520), LOC_Os03g20910 and LOC_Os07g48560 (At3g03660, WOX11), and LOC_Os04g52770 (At4g36930, SPATULA) display a transient high expression in imbibed seeds [28], implying their potential roles in germination regulation.

3.7. Enriched TFBSs

TFBSs were searched in the promoter regions of three gene classes using Osiris [26] and statistically enriched ($P \leq 0.01$) elements were obtained (Table S5). The enriched ABRE and GARE at 0 and 12 HPI showed a consistence with the expression patterns of ABA and GA signaling genes. Besides, the light harvesting elements (CAATmotifI and TATAboxII) overrepresented at 48 HPI might be a preparation for seedling growth.

4. Discussion

In this RNA-Seq study, we analyzed the dynamic transcriptional and functional alterations during rice seed germination. Especially, drastic changes occurred in the important stage of the first 12 HPI [7,10]. During the transition from dormancy to germination, we speculated that phytohormones, TFs together with seed genes and TFBSs played a regulatory role in the efficient process (Fig. 4).

We found that the expression patterns of phytohormone related genes coincided with the regulatory roles of ABA and GA in germination. Besides, most BR biosynthesis and signaling genes showed a consistent increase in expression, suggesting a positive

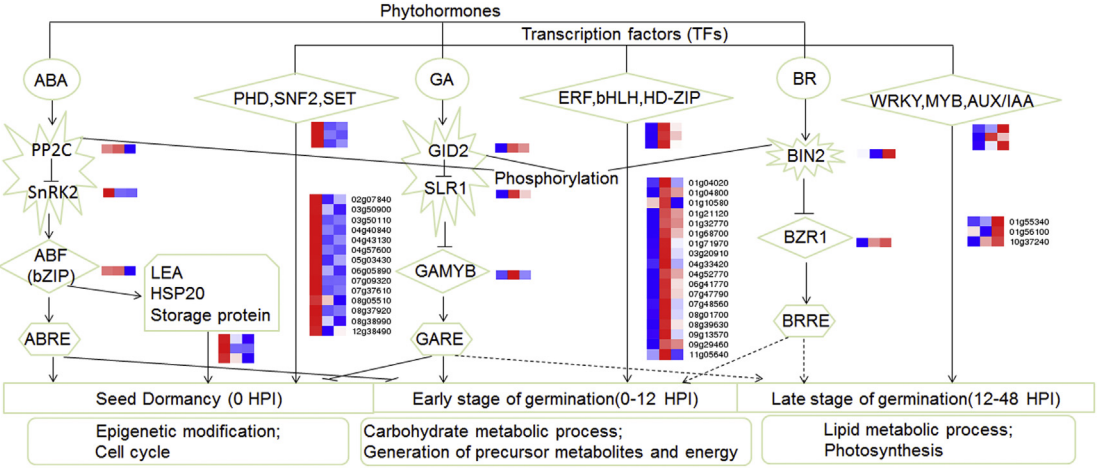

Fig. 4. The complex regulation of rice seed germination. Phytohormones, TFs in combination with seed genes and TFBSs regulated molecular events in embryo during germination. Phytohormones of ABA, GA and BR affected dormancy and germination through the binding of downstream TFs and TFBSs in target genes (such as seed genes encoding LEA) to mediate further responses. The signaling genes encoding phosphorylation related proteins (such as PP2C and BIN2) played a critical role in phytohormone interactions during germination. Three groups of stage-enriched TF families were closely related with molecular events in different germination stages. For example, PHD could play a part in epigenetic regulation in dry seeds. The expression profiles of these factors were clustered using averaged gene expressions. Besides, three classes of 35 germination-specific genes were shown.

effect on germination and subsequent growth. Although BR plays a similar role with GA, its regulatory mechanism seems different from GA. In the network analysis, the hub genes encoding phosphorylation related enzymes in BR (BIN2s) and ABA (PP2Cs) signaling were found closely connected with each other. Coincidently, the antagonism of BR and ABA mediated by BIN2 has been recently evidenced in *Arabidopsis* seed germination [29]. Taken together, we speculated that BR could promote seed germination partly through the phosphorylation-mediated counteraction to ABA in both dicots and monocots.

Network and functional analyses suggested that three groups of stage-enriched TF families might play a role in molecular events in germination. Compared with group 2 and 3, enriched TF families in group 1 were rarely referred in previous reports of seed germination. But our analyses implied that PHD, SNF2 and SET in group 1 could participate in epigenetic regulation, which functional term was overrepresented in dry seeds. Coincidently, a group of PHD-domain proteins have been recently reported as associated with the transition of chromatin activities during germination in *Arabidopsis* [30]. Besides, SNF2 and SET families are also involved in histone methylation and other epigenetic regulations [31,32]. Therefore, epigenetic modification could be a regulatory mechanism controlling the switch between dormancy and germination.

Altogether, we mainly investigated the complex regulation process including phytohormones and TFs in germination and suggested that protein phosphorylation and epigenetic modification worth further study in future.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.110.

Transparency document

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