Genome-Wide Identification and Expression Analysis of OsbZIP09 Target Genes in Rice Reveal Its Mechanism of Controlling Seed Germination

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Abstract: Seed dormancy and germination are key events in plant development and are critical for crop production, and defects in seed germination or the inappropriate release of seed dormancy cause substantial losses in crop yields. Rice is the staple food for more than half of the world’s population, and preharvest sprouting (PHS) is one of the most severe problems in rice production, due to a low level of seed dormancy, especially under warm and damp conditions. Therefore, PHS leads to yield loss and a decrease in rice quality and vitality. We reveal that mutation of OsbZIP09 inhibited rice PHS. Analysis of the expression of OsbZIP09 and its encoded protein sequence and structure indicated that OsbZIP09 is a typical bZIP transcription factor that contains conserved bZIP domains, and its expression is induced by ABA. Moreover, RNA sequencing (RNA-seq) and DNA affinity purification sequencing (DAP-seq) analyses were performed and 52 key direct targets of OsbZIP09 were identified, including OsLOX2 and Late Embryogenesis Abundant (LEA) family genes, which are involved in controlling seed germination. Most of these key targets showed consistent changes in expression in response to abscisic acid (ABA) treatment and OsbZIP09 mutation. The data characterize a number of key target genes that are directly regulated by OsbZIP09 and contribute to revealing the molecular mechanism that underlies how OsbZIP09 controls rice seed germination.

Keywords: OsbZIP09; preharvest sprouting; ABA; RNA-seq; DAP-seq; seed germination; rice

1. Introduction

Seeds comprise an extremely important proportion of the world’s diet. Seed dormancy and germination, two distinct physiological processes of seed-bearing plants, are key events in plant development, and are also critical for crop production. Most crops, including rice, were domesticated from wild species that possess a high level of seed dormancy. Cultivated crops germinate easily, which thus ensures high emergence rates after sowing [1]. However, a certain degree of dormancy is critical for seed development and to prevent preharvest sprouting (PHS), which is prevalent in modern crop cultivation and leads to serious agricultural losses. Seed dormancy and germination are precisely controlled by various environmental cues and endogenous stimuli, especially phytohormones [1,2]. Nevertheless, defects in seed germination or the inappropriate release of seed dormancy cause substantial losses in crop yield. Rice is the staple food of more than half of the world’s population.
Warm and damp conditions are the major external cause of PHS, which is often encountered during the rice growth season, especially during the maturity stage [3]. Therefore, PHS is one of the most severe problems in rice production, which leads to yield loss and to a decrease in rice quality and vitality [4,5]. Therefore, inhibiting PHS is important in rice breeding programs, as is dissecting its regulatory molecular network and identifying the major genes involved in PHS.

The phytohormones, abscisic acid (ABA) and gibberellins (GAs), are principal regulators that play antagonistic roles in regulating seed dormancy and germination [1,2,6]. GA promotes seed germination, whereas ABA suppresses seed germination, and the balance between ABA and GA determines whether seeds germinate. During seed development, ABA accumulates, which prevents seed germination on the parent plant [7]. Therefore, ABA-deficiency will lead to a high degree of PHS, which extensively reduces crop yield and grain quality [8]. By contrast, enhanced ABA biosynthesis or its reduced catabolism promotes the over-accumulation of ABA, which suppresses seed germination [9,10]. In addition to ABA content, ABA signaling also regulates seed germination. In general, ABA signal transduction is mediated by the core ABA-signaling cascade, which includes ABA receptors, protein phosphatases 2C (PP2Cs), sucrose nonfermenting 1-related protein kinase 2 (SnRK2), and downstream transcription factors [11]. In the presence of ABA, SnRK2s are autophosphorylated and subsequently activate basic leucine zipper (bZIP) transcription factors, which are critical downstream signaling components. These include ABA-responsive element (ABRE)-binding proteins (AREBs), ABRE-binding factors (ABFs), and ABA-INSENSITIVE 5 (ABI5), which further induce the downstream ABA-responsive transcriptional network and plant responses, such as the inhibition of seed germination and enhancement of stress responses [12,13].

Transcription factors, such as the above-mentioned bZIP proteins, play key roles in mediating and integrating upstream signals and downstream transcriptional networks, which contribute to the adaptation of plants to the changing environment. bZIP proteins are evolutionarily conserved transcription factors and have been identified in almost all eukaryotes [14]. It is proposed that plant bZIP genes originate from four founder genes and that the gene family expanded during evolution [15]. For example, Arabidopsis contains more than 78 bZIP genes [16], and 89 are present in rice [17], 96 in Brachypodium distachyon [18], 131 in soybean [19], and 247 in rapeseed [20].

Despite research on bZIP proteins in rice, most of them have not been cloned or studied. In particular, only several OsbZIPs have been functionally implicated in the regulation of seed germination or PHS. Therefore, the identification and characterization of novel Os-bZIPs involved in the modulation of PHS is important. Moreover, further characterization of the genome-wide target gene profiles of OsbZIP proteins will reveal the molecular regulatory network that underlies rice PHS, and is essential to identify key downstream target genes that could specifically regulate rice PHS and be potentially applied in rice breeding programs. Chromatin immunoprecipitation sequencing (ChIP-Seq) is a promising method for determining transcription factor (TF) directly regulated genes in vivo [21]. However, the limitation of this method is the need for high-quality gene-specific antibodies or tagged transgenic lines, which is technically challenging, expensive, and time-consuming. The recently reported DNA affinity purification sequencing (DAP-seq) technique overcomes these limitations by using an in vitro-expressed affinity-tagged TF accompanied by high-throughput sequencing of a genomic DNA library, thereby generating genome-wide target gene maps that reflect both local sequence context and DNA methylation status [22]. Here, we have identified a novel and unique OsbZIP transcription factor, OsbZIP09, mutants of which exhibited inhibited PHS. We first verified the germination properties of the osbzip09 mutant, and analyzed the OSbZIP09 expression pattern and the sequence and structure of its encoded protein. We performed RNA-seq analysis to investigate OsbZIP09-regulated transcriptome during seed germination. We also identified the direct genome-wide target genes of OsbZIP09 using DAP-seq. Finally, the data of DAP-seq were analyzed in combina-
tion with RNA-seq results to screen key direct target genes of OsbZIP09 that are involved in regulating rice seed germination.

2. Results

2.1. Mutation of OsbZIP09 Inhibits PHS in Rice and Enhances Rice Sensitivity to ABA during Seed Germination

The PHS phenomenon in rice was severe in Yangzhou in 2018, due to several consecutive rainy days accompanied by high temperatures before the rice harvest. The evaluation of PHS in different rice materials in the field led to the observation that several lines originating from the gene editing of the same target gene, OsbZIP09, exhibited less severe PHS than the wild-type control. To analyze the effect of OsbZIP09 mutation on rice PHS in more detail, we used a growth chamber to mimic high temperature and humidity conditions and evaluated the PHS phenotype of osbzip09 mutants. The germination rates of all test panicles from the osbzip09 mutants were notably lower than those of the wild-type control (Figure 1A,B), consistent with the previously observed PHS phenotype in the field. Under normal growth conditions, the germination of osbzip09 seeds was only slightly delayed (Figure 1C, Figure S1). Because ABA is a key inhibitor of seed germination and bZIP transcription factors play essential roles in ABA signaling, we further evaluated the germination of osbzip09 mutants in response to ABA. In general, ABA treatment delayed the germination of osbzip09 and the wild type. However, ABA suppressed the germination of osbzip09 mutant more severely than that of the controls (Figure 1C, Figure S1), suggesting that osbzip09 mutant was more sensitive to ABA during seed germination. Analysis of the shoot length of the germinated seeds after 96 h and 120 h indicated that the difference between osbzip09 and wild type was slight (Figure 1D,E), implying that OsbZIP09 is mainly involved in the regulation of dormancy break and the promotion of seed germination. Based on the result of PHS and seed germination analyses, osbzip09-2, which contained a 1-bp insertion in the target site (Figure S2), was selected as a potential line for subsequent RNA-seq and qRT-PCR expression analyses. Further off-target tests indicated that there was no mutation in the potential off-target sites in osbzip09-2 (Figure S3), confirming that osbzip09-2 was suitable to be a representative line for subsequent expression analyses.

Figure 1. Knock-out of OsbZIP09 inhibits rice pre-harvest sprouting (PHS). Phenotype (A) and germination rate (B) of mature rice panicles after 6 d imbibition in water. Scale bar, 2 cm. (C) Time-course analysis of seed germination under normal conditions. (D) Morphology of germinated seeds 96 h after imbibition (HAI). Scale bar, 0.5 cm. (E) Shoot length of germinated seeds 96 and 120 HAI. * p < 0.05, ** p < 0.01 (t-test); NS: not significant.
2.2. Analysis of the Phylogenetic Relationships, Protein Sequence and Structure, and Gene Expression of OsbZIP09

Because OsbZIP09 is an uncharacterized bZIP transcription factor, we analyzed its amino-acid sequence, protein structure, and gene expression in detail. First, full-length amino-acid sequences of bZIP09 from 23 different plant species were obtained and used to generate a phylogenetic tree. These bZIP09 proteins were classified into four subfamilies, which included 15, 3, 4, and 1 members in groups I, II, III, and IV, respectively (Figure 2A). The bZIP09 amino-acid sequences from eight representative species in group I, including rice, were then aligned, which showed conservation of several regions of the bZIP09 proteins, especially the C terminus where the bZIP domain is located (Figure 2B). Further analyses of the conserved domain at the C terminus, corresponding to 236 to 311 aa in rice, revealed a typical bZIP structure (NX7RX9LX6LX6L), which included a basic DNA-binding region and an adjacent ZIP domain (Figure 2C, Figure S4). Three-dimensional homology modeling of the conserved C terminal region revealed a continuous α-helical structure (Figure 2D). Because osbzip09 was affected in PHS and was also more sensitive to ABA, the OsbZIP09 promoter (2000 bp upstream of the translation initiation codon ATG) was analyzed for the presence of potential cis-acting motifs related to ABA using the PLACE database (http://www.dna.affrc.go.jp/PLACE/, accessed on 18 November 2020). Three ABRE motifs and three DRE motifs (drought-responsive element) were identified (Figure 2E). Expression analysis showed that only 15 min ABA treatment could upregulate the expression of OsbZIP09 (Figure 2F).

2.3. Identification of Genes Co-Regulated by ABA and OsbZIP09 Via RNA-Seq

Because the expression of OsbZIP09 is induced by ABA and osbzip09 is more sensitive to ABA during seed germination, OsbZIP09 potentially regulates components of the ABA pathway to coordinate seed germination. To verify this hypothesis, an RNA-seq experiment was performed using germinated seeds (36 h after imbibition (HAI)) from an ABA-treated wild-type and osbzip09-2 mutant. More than 6.1 Gb of clean bases were generated and the Q30 value exceeded 89.13% for each sample (Figure S5A). More than 87.94% of reads could be uniquely mapped to the rice genome (Figure S5B). Furthermore, more than 91.89% of the reads mapped to exonic regions (Figure S5C). In total, 4267 differentially expressed genes (DEGs) were identified in response to ABA treatment (fold change >1.5, p < 0.05), including 2647 downregulated genes and 1620 upregulated genes (Figure 3A). In osbzip09-2, the expression of 1352 genes was altered compared to the wild-type control (fold change > 1.5, p < 0.05), 474 of which were downregulated and 878 were upregulated (Figure 3A). Comparison of the genes regulated by ABA and OsbZIP09 indicated that 438 genes were regulated in common (Figure S6), which was almost one-third of all the DEGs in the osbzip09-2. Further analysis of these 438 genes showed that 385 showed the same change in expression pattern, with 197 genes being downregulated and 188 genes upregulated in both cases (Figure 3A). This suggests that OsbZIP09 and ABA probably coordinate rice seed germination by coregulating the same set of downstream genes. Gene ontology (GO) analysis of the 438 common targets revealed that genes involved in cell cycle, nuclear decisions, organelle fission, movement of cell, or other components were enriched (Figure 3B). In addition, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis indicated that the common targets were enriched in pathways of phenylpropanoid biosynthesis, cysteine and methionine metabolism, photosynthesis, and galactose metabolism (Figure 3C). The number of genes enriched in the phenylpropanoid biosynthesis pathway was the largest, and this category is closely related to plant growth and defense [23].
Figure 2. Analysis of the phylogenetic relationships, protein sequence, and structure of bZIP09, and the expression pattern of bZIP09. (A) Phylogenetic tree of bZIP09 from different plant species. (B) Amino-acid sequence alignment of eight bZIP09 homologous proteins. Different homology levels were highlighted in different colors. Navy blue, 100%; pink, between 75% and 100%; light blue, between 50% and 75%. (C) Conserved amino-acids in the bZIP09 C terminal region. (D) Protein secondary structure analysis of the C terminal region of bZIP09. The conserved domain includes a continuous α-helical structure. (E) Analysis of the abscisic acid (ABA)-related cis-elements in the OsbZIP09 promoter. (F) Expression of OsbZIP09 in the wild-type (WT) in response to ABA (5 µM) treatment. ** p < 0.01. ACTIN served as the internal reference gene for normalization. Values were obtained from three independent experiments. Data are shown as means ± SD.
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**Figure 3.** RNA-seq analyses of osbzip09-2 and ABA-treated wild-type rice. (A) Venn diagrams showing the overlap between genes up- or downregulated by ABA and in osbzip09-2. ABA up—ABA-upregulated genes; ABA down—ABA-downregulated genes; osbzip09 up—upregulated genes in osbzip09-2; osbzip09 down—downregulated genes in osbzip09-2. (B) Distribution of the top 10 biological process gene ontology (GO) terms for the common differentially expressed genes (DEGs) of ABA treatment and osbzip09-2. (C) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that were enriched among the common DEGs of ABA treatment and OsbZIP09 mutation.
2.4. DAP-Seq Identification of the Genes Directly Targeted by OsbZIP09

Because OsbZIP09 is a novel transcription factor in the regulation of seed dormancy and germination, and several DEGs were identified from RNA-seq analysis, one appropriate strategy to further investigate the underlying mechanism underlying OsbZIP09 function was to identify its direct target genes. Therefore, DAP-sequencing [22] was performed for this purpose. The OsbZIP09-binding sites were analyzed using MACS2 [24]. Peaks that were located 2 kb upstream from the transcription start site (TSS) or 2 kb downstream from the transcription termination site (TTS) were considered to bind to the OsbZIP09 promoter and terminator regions, respectively. In total 7752 binding sites were identified across the whole rice genome (Figure 4A). Among these identified peaks, approximately 18% (1396) were located within the promoter region (2 kb upstream from the TSS), corresponding to 1354 genes (Figure 4B), and a specific core binding motif (CACGTG/C) for OsbZIP09-binding was also revealed. Further gene ontology (GO) analysis indicated that these genes could be assigned into 15 different groups according to their biological processes (Figure 4C). Among these groups, genes belonging to ‘cellular process’ and ‘metabolic process’ constituted the two largest groups and accounted for 36.5% and 28.8% of the total analyzed genes, respectively.

Figure 4. DAP-seq (DNA affinity purification sequencing) analysis of rice OsbZIP09. (A) Distribution of OsbZIP09-binding sites along the twelve chromosomes of rice. (B) Distribution of OsbZIP09-binding sites in genic and intergenic regions. (C) Biological process categorization of OsbZIP09-regulated target genes using gene ontology (GO) analysis.
2.5. Identification and Analysis of the Core Direct Target Genes of OsbZIP09

On the basis of the results from the RNA-seq analysis, 438 common targets of ABA and OsbZIP09 were identified, and approximately 90% of these showed the same qualitative change in expression. Comparison of RNA-seq and DAP-seq data could aid the identification of the direct targets of OsbZIP09 that are involved in the regulating of rice seed germination. The comparison identified 52 common gene targets (Figure 5A). According to the RNA-seq expression data, these common genes were classified into different cluster groups by hierarchical clustering analysis (Figure 5B). Notably, 88.9% of the targets shared the same pattern of gene expression change, and 73.1% of the genes were upregulated in response to ABA treatment and in osbzip09-2 mutant. Next, we divided all the 52 genes into three groups according to their mode of expression change. All the information relating to their expression, description, and OsbZIP09-binding site in their promoter is listed in Tables 1–3. Table 1 lists the 38 genes whose expression was upregulated in response to ABA and in osbzip09-2. These included two genes that encoded F-box proteins, two OsbZIP genes, two OsPP2C phosphatase-encoding genes, six LEAs, and some other genes. Among these, LEA genes constituted the greatest proportion of the common targets and the expression of all LEA genes increased in response to ABA or in osbzip09-2. Moreover, the change in expression change of LEA genes in osbzip09-2 was about 1.5– to 2.0-fold of that in the wild-type control, whereas they were expressed about 2.3- to 8.3-fold more highly in the ABA-treated wild-type plants than in the mock-treated wild type, suggesting that loss of OsbZIP09 could only partially mimic the effect of ABA treatment, which is consistent with the fact that several other transcription factors in the ABA pathway also modulate the expression of LEAs. The LEA genes were considered to be reliable ABA-responsive genes and they are often involved in various stress responses. Table 2 lists eight genes whose expression decreased in response to ABA and in the osbzip09-2 mutant, including DLT (DWARF AND LOW-TILLERING) and OsLOX2, which are involved in brassinosteroid (BR) signaling and the oxidation of polyunsaturated fatty acids (PUFAs), respectively. In addition, several genes encoding protochlorophyllide oxidoreductase, nucleoside phosphorylase, chromomethyltransferase, calvin cycle protein, and receptor-like protein kinase were also downregulated.

![Figure 5](image-url)  
**Figure 5.** Identification and analysis of OsbZIP09 direct target genes responsive to ABA. (A) Venn diagrams showing the overlapping genes between the RNA-seq and DAP-seq data. (B) Hierarchical clustering analysis of the 52 common target genes.
### Table 1. List of OsbZIP09 target genes upregulated by ABA and in osbzip09-2.

| Gene ID          | Transcriptome Analysis Log₂(Fold Change) ¹ | DAP-Seq Analysis | Description               |
|------------------|--------------------------------------------|------------------|---------------------------|
|                  | osbzip09-2/WT | ABA/Mock (WT) | −Log₁₀ (p Value) | Distance to TSS (bp) ² |                      |
| Os10g0173000     | 2.05          | 3.94          | 14.30                | 1616 | expressed protein |
| Os08g0523000     | 2.13          | 2.52          | 11.59                | 95   | F-box protein 463 |
| Os11g0154900     | 0.95          | 1.30          | 17.17                | 114  | OsZIP-2a, OsbZIP80 |
| Os03g0386000     | 0.82          | 3.04          | 12.68                | 1374 | SWRD5            |
| Os03g0820500     | 1.18          | 1.09          | 9.65                 | 373  | OsADF3           |
| Os02g0716800     | 2.20          | 0.96          | 6.91                 | 1543 | PTR2             |
| Os05g0542500     | 0.68          | 1.97          | 12.53                | 552  | OsLEA3           |
| Os01g0702500     | 0.68          | 1.42          | 10.54                | 3    | OsLEA22          |
| Os05g0563900     | 0.87          | 0.81          | 10.25                | 1498 | OsGRX17          |
| Os09g0325700     | 0.63          | 1.53          | 11.80                | 37   | OsPP108, SIPP2C1 |
| Os02g0740500     | 0.85          | 0.90          | 9.65                 | 1299 | expressed protein |
| Os04g0617050     | 0.79          | 0.84          | 9.20                 | 110  | OsSAUR21         |
| Os12g0478200     | 1.12          | 1.02          | 7.73                 | 1383 | GRAM protein     |
| Os05g0198400     | 0.97          | 0.71          | 11.26                | 52   | OsZIP7a          |
| Os10g0524300     | 1.24          | 1.89          | 7.66                 | 1330 | EMBRYO SAC 1     |
| Os09g0484800     | 0.84          | 0.85          | 9.25                 | 101  | pirin, putative  |
| Os10g0159033     | 2.20          | 1.29          | 12.19                | 135  | expressed protein |
| Os03g0723400     | 0.64          | 2.04          | 9.57                 | 6    | expressed protein |
| Os10g0542100     | 1.13          | 1.02          | 8.48                 | 2    | OsMTI-2-1a       |
| Os08g0110200     | 0.66          | 1.20          | 8.45                 | 62   | OsLEA4           |
| Os04g0385600     | 0.77          | 0.99          | 10.06                | 522  | OsFBQ3           |
| Os09g0127700     | 0.64          | 1.36          | 9.25                 | 18   | expressed protein |
| Os03g0170900     | 0.99          | 0.63          | 10.24                | 1082 | OsSUT1           |
| Os11g0454200     | 0.95          | 3.05          | 8.23                 | 97   | OsLEA28          |
| Os11g0451700     | 0.95          | 2.27          | 8.77                 | 118  | OsLEA25, Rab17   |
| Os06g0112100     | 0.93          | 0.85          | 9.88                 | 881  | OsFbox007        |
| Os05g0572700     | 0.97          | 1.49          | 12.68                | 745  | OsPP2C51         |
| Os05g0524100     | 0.77          | 1.30          | 7.79                 | 234  | expressed protein |
| Os07g0422100     | 0.61          | 2.17          | 6.17                 | 247  | OsPM191L2        |
| Os06g0697200     | 0.74          | 0.68          | 7.53                 | 1321 | OsRH35B          |
| Os10g0177200     | 0.71          | 2.29          | 12.03                | 856  | OsDSR-1          |
| Os09g0110600     | 0.88          | 1.93          | 5.95                 | 12   | OsLEA18          |
| Os01g0357000     | 0.68          | 0.98          | 8.55                 | 155  | OsCML16          |
| Os02g0718600     | 0.88          | 1.55          | 13.89                | 388  | expressed protein |
| Os01g0849600     | 1.58          | 1.73          | 6.90                 | 70   | USP protein      |
| Os09g0502500     | 1.19          | 0.66          | 8.12                 | 162  | alcohol dehydrogenase |
| Os12g0510750     | 0.85          | 1.74          | 8.45                 | 162  | expressed protein |
| Os06g0261300     | 2.05          | 1.22          | 4.79                 | 486  | expressed protein |

¹ Log₂(fold change) indicates the log₂ fold change in gene transcript abundance between osbzip09-2 and WT or ABA-treated WT and mock-treated WT. ² Distance to TSS (transcription start site) indicates the distance of the OsbZIP09 binding peak to the nearest transcription start site.

### Table 2. List of OsbZIP09 target genes downregulated by ABA and in osbzip09-2.

| Gene ID          | Transcriptome Analysis Log₂(Fold Change) ¹ | DAP-Seq Analysis | Description               |
|------------------|--------------------------------------------|------------------|---------------------------|
|                  | osbzip09-2/WT | ABA/Mock (WT) | −Log₁₀ (p Value) | Distance To TSS ² |                      |
| Os06g0127800     | −0.58         | −1.84         | 24.06                | 220  | DLT; OsGRAS-32   |
| Os04g0678700     | −0.63         | −1.47         | 15.45                | 111  | OsPORA          |
| Os06g0112100     | −1.30         | −3.33         | 15.80                | 673  | Nucleoside kinase |
| Os02g0769200     | −0.73         | −1.28         | 7.64                 | 435  | LYP5, Os-LYP5    |
| Os10g0104900     | −0.80         | −2.22         | 7.29                 | 701  | OsCMT3a         |
| Os03g0306800     | −0.93         | −0.72         | 6.91                 | 329  | Calvin-cycle protein |
| Os05g0406800     | −0.61         | −1.78         | 5.47                 | 141  | receptor-like protein |
| Os03g0738600     | −0.64         | −2.49         | 6.91                 | 209  | OsLOX2         |

¹ Log₂(fold change) indicates the log₂ fold change in gene transcript abundance between osbzip09-2 and WT or ABA-treated WT and mock-treated WT. ² Distance to TSS indicates the distance of the OsbZIP09 binding peak to the nearest transcription start site.
Table 3. List of OsbZIP09 target genes inconsistently regulated by ABA and in osbzip09-2.

| Gene ID      | Transcriptome Analysis Log$_2$(Fold Change)$^1$ | DAP-Seq Analysis | Description                              |
|--------------|-----------------------------------------------|------------------|------------------------------------------|
|              | osbzip09-2/WT | ABA/Mock (WT) | $-\log_{10}(p)$ Value | Distance To TSS (bp) $^2$ |                          |
| Os08g0278900 | 0.67            | −0.74          | 12.61                     | 80                      | MIR domain protein       |
| Os03g0835150 | 0.82            | −1.59          | 7.48                      | 568                     | expressed protein        |
| Os02g022s200 | 1.17            | −0.83          | 8.95                      | 260                     | IB hydrolase             |
| Os04g0678400 | 0.86            | −0.94          | 6.91                      | 1566                    | OsDof-17; OsDof18        |
| Os03g0377700 | −0.75           | 0.84           | 8.07                      | 1375                    | CSLA5-cellulose synthase |
| Os02g0115900 | 0.70            | −0.72          | 5.95                      | 22                      | OsBip1; BiP3             |

$^1$ Log$_2$(fold change) indicates the log$_2$ of the fold change in gene transcript abundance between osbzip09-2 and WT or ABA-treated WT and mock-treated WT. $^2$ Distance to TSS indicates the distance of the OsbZIP09 binding peak to the nearest transcription start site.

2.6. Expression Analysis and Validation of the Representative Target Genes of OsbZIP09

To validate further the quality of the RNA-seq data, eight representative genes were selected from the 52 key targets for qRT-PCR, including six genes from Table 1, LOX2 (Table 2), and CSLA5 (Table 3). The expression of seven out of the eight genes was consistent with the RNA-seq data (Figure 6A). i.e., the expression of LEA3, LEA4, LEA25, PP2C51, and USP increased in both osbzip09-2 and ABA-treated wild type, whereas that of LOX2 decreased in both samples, and the expression of CSLA5 decreased in response to ABA treatment and increased in osbzip09-2 mutant. Only the expression of LEA18 differed slightly from the RNA-seq data in osbzip09-2 mutant. Correlation analysis of the gene expression between qRT-PCR and RNA-seq data indicated that the values were consistent (correlation coefficient $R^2 = 0.74$), confirming the accuracy of the RNA-seq data (Figure 6B).

Figure 6. Validation of RNA-seq data for the representative common targets by qRT-PCR. (A) qRT-PCR for selected eight representative common targets. (B) Correlation of gene expression between qRT-PCR and RNA-seq data. Fold-change values were log$_2$ transformed.
2.7. Expression Analysis of the Representative Target Genes Regulated by OsbZIP09 and ABA

Because the expression of up to six LEA genes was upregulated by ABA treatment and in osbzip09-2, LEA25 was selected as a representative gene for further analysis, and was also identified by previous proteomic study of rice seed germination [25]. Moreover, LOX2, which was transcriptionally downregulated in this study and is reported to promote germination [26], was also selected for subsequent assay. Sequence analysis revealed that a conserved CACGTCG motif and two CACGTC motifs were identified in the promoters of LEA25 and LOX2 (Figure 7A). To verify further the transcriptional regulation of LEA25 and LOX2 by OsbZIP09 and ABA, the dual luciferase system was used. The promoters of LEA25 and LOX2 were amplified (1.15 kb and 2.05 kb, respectively) and cloned into the pGreen II0800-LUC vector (Figure 7B). In the absence of OsbZIP09, ABA treatment slightly induced the transcription of LEA25. OsbZIP09 suppressed the expression of LEA25 either with or without ABA (Figure 7C), and OsbZIP09 promoted LOX2 transcription (Figure 7D). Further ABA treatment slightly attenuated the promotive effect of OsbZIP09 on LOX2 transcription (Figure 7D). Therefore, the direct transcriptional modulation of downstream target genes is an important molecular mechanism for the OsbZIP09-mediated regulation of rice germination.

Figure 7. OsbZIP09 controls seed germination by suppressing the expression of Late Embryogenesis Abundant (LEA) family genes and enhancing the expression of OsLOX2. (A) OsbZIP09-binding motif analysis in the promoter of LEA25 and LOX2, respectively. (B) Schematic depiction of the reporter and effector constructs used in the dual-luciferase reporter assay. (C,D) OsbZIP09 suppresses the promoter activity of LEA25 but activates the promoter activity of LOX2. Data are shown as means ± SD (n = 5). Statistically significant differences at p < 0.05 are indicated by different letters.

3. Discussion

Pre-harvest sprouting often causes severe losses in grain yield, quality, and germinability in cereal crops, including rice, wheat, barley, and maize [3,27,28]. Diverse endogenous and environmental factors are involved in the regulation of PHS, including humidity, temperature, light, and phytohormones [1,29]. Among these, ABA and GA are the major endogenous determinants that play antagonistic roles in regulating PHS. ABA is critical in the maintenance of seed dormancy, which involves the delay or prevention of seed germination and PHS. Therefore, most PHS in crops is directly or indirectly related to ABA. Many efforts have been made to inhibit crop PHS, including improving growth and cultivation conditions, spraying chemicals, and producing new varieties that are PHS resistant. In general, breeding PHS-resistant crops is the most efficient and environmentally
friendly strategy, and this relies on the identification and characterization of the key genes involved in seed dormancy or germination. However, most progress in identifying and characterizing genes has been made in the model plant Arabidopsis. Although many quantitative trait loci (QTLs) or genetic loci associated with PHS have been isolated in rice [30–34], the number of cloned genes is limited and the molecular mechanism that underlies PHS remains elusive. Most of the reported genes relate to ABA metabolism, such as OsABA1, OsPLA3, PHS1 to PHS4, and OsCNX6 [5,27,35,36]. Recently, several studies have revealed some regulatory modules involved in PHS. For example, mutation of PHS8/ISA1 enhanced the accumulation of sugars in rice endosperm, thus reducing ABA sensitivity via suppressing the expression of OsABI3 and OsABI5 [37]. This suggests that the endosperm sugar content has dual roles in modulating seed dormancy and germination, both as an energy source and as an inhibitor of ABA signaling. Moreover, PHS9 encodes a CC-type glutaredoxin that directly interacts with OsGAP, an interaction partner of the ABA receptor OsRCAR1. Both PHS9 and OsGAP are negative regulators of ABA signaling and regulate seed germination via integrating signaling between reactive oxygen species (ROS) and ABA [38]. It has also been demonstrated that miR156 is also involved in the regulation of rice PHS. Mutation of mir156 releases its suppression of Ideal Plant Architecture 1 (IPA1), which negatively regulates GA signaling, thus enhancing seed dormancy [39]. Importantly, this study showed that mutation of a specific miR156 could suppress PHS without compromising rice productivity.

The ABA signaling pathway requires transcription factors to modulate the downstream transcriptional network and consequently ABA-triggered plant responses, such as the suppression of seed germination. The ABA signaling pathway mainly relies on OsbZIP transcription factors, including AREBs, ABFs, and ABI5. Among these, ABI5 plays a key role in suppressing ABA-mediated seed germination and post-germination growth in a number of plant species, such as Arabidopsis [13,40,41], rice [42], wheat [43,44], barley [45], and Sorghum [46]. In addition to the roles of these classical bZIP transcriptional factors in the ABA pathway, several other bZIP members are also involved in the ABA-mediated regulation of seed dormancy and germination in rice. For example, mutation of OsABF2/SsbZIP46 decreased the sensitivity of rice to high levels of ABA at germination and post-germination growth stages [47]. OsbZIP23 directly interacts with MOTHER OF FT AND TFL 2 (OsMFT2), a negative regulator of rice seed germination, and OsbZIP23 overexpression restores the PHS phenotype of osmft2 knock-out lines [48]. Moreover, OsbZIP72 is activated by ABA and directly binds to the Allene Oxide Cyclase (AOC) promoter and enhances its transcription. This subsequently increases the content of endogenous JA and represses seed germination [49]. Furthermore, OsbZIP75 directly binds to the promoter of DELAY OF GERMINATION 1 (DOG1) and promotes the accumulation of OsDOG1L-3 and inhibits seed germination [50]. However, all the above-mentioned OsbZIPs negatively regulate rice germination. In this study, we demonstrated that OsbZIP09 positively promotes seed germination and its mutation inhibits rice PHS and slightly delays seed germination. Moreover, RNA-seq analysis showed that many genes were coregulated by ABA and in the oszip09 mutant, indicating that OsbZIP09 modulates seed germination via a common set of downstream target genes that are involved in ABA signaling. Phylogenetic analysis and amino-acid sequence comparison indicated that several key domains of bZIP09 were conserved among different plant species, especially the C-terminus that contains a basic DNA-binding region and the adjacent ZIP domain (Figure 2C, Figure S2). This suggests that OsbZIP09 shares a similar DNA binding activity with other bZIP members. We propose that the other non-conserved domains of OsbZIP09 are responsible for its promotive effect on seed germination, which might be mediated by interactions with other regulatory proteins. This deserves further study to identify the OsbZIP09 interaction proteins and dissect the underlying mechanisms.

Analysis of PHS and seed germination, combined with RNA-seq and DAP-seq data, indicated that OsbZIP09 regulates seed germination via interacting with ABA signaling to coordinate the expression of common downstream target genes (Figures 1 and 5). Ap-
proximately 90% of the 52 direct target genes of OsbZIP09 showed the same change in expression in response to mutation of OsbZIP09 and ABA treatment. Notably, six LEA genes were present among the 52 key OsbZIP09 targets and their expression was upregulated in osbzip09 and ABA-treated rice seeds (Figures 5 and 6, Table 1). LEA proteins accumulate in maturing seeds, and represent a hallmark of seed maturation. The expression of LEA genes is induced by drying, freezing, high salinity, osmotic stress, and exogenous ABA [51–53]. The accumulation of LEA proteins potentially protects cellular structures and thus strengthens plant tolerance to dehydration [54,55]. Rice contains 34 LEA proteins which have been subdivided into seven groups [56]. LEAs are often considered to be ABA-responsive genes that mediate plant adaptation to various stresses, such as drought stress and antioxidant stress [57–59]. Recently, two reports have revealed that LEAs are also involved in the regulation of rice seed germination: the germination of OsLEA5-RNAi transgenic rice seeds was less sensitive to ABA treatment [60], and the mutation of OsLEA33 promoted post-germination growth of rice [25]. These studies suggest that OsLEAs play positive roles in plant stress responses and negative roles in rice seed germination. Here, the expression of six OsLEA genes increased in response to the mutation of OsbZIP09 and ABA treatment, which is consistent with the observed decreased PHS and seed germination phenotype in the osbzip09 mutant and ABA-treated wild type. Most importantly, the identified OsLEA genes here were all direct targets of OsbZIP09, implying that the transcriptional regulation of OsLEA family genes is an important mechanism by which OsbZIP09 modulates seed dormancy and germination. Furthermore, OsLEA25, which was selected in this study for qRT-PCR and dual-luciferase validation, was also identified previously in a GA-responsive proteomic assay, in which GA suppressed the accumulation of OsLEA25 and four other OsLEA proteins during seed germination [25]. Therefore, we propose that OsLEA family genes play essential roles in phytohormone-regulated seed germination in rice. In addition to OsLEA25, an AWPM-19-like family gene and a gene encoding universal stress protein (USP) were also identified in two "omic" studies. Proteomic data indicated that GA-suppressed the expression of AWPM-19 and USP proteins, whereas here, ABA treatment and mutation of OsbZIP09 enhanced their expression. AWPM-19 family proteins have been identified in several major crops, including wheat, barley, and rice [61–63]. Two AWPM-19 family proteins, PM19-A1 and PM19-A2, accumulated more in maturing grains of dormant wheat than in non-dormant genotypes [64]. In rice, knock-down of an AWPM-19 gene, OsPM1, which controls ABA influx, promoted seed germination, whereas OsPM1 overexpression had the opposite effect [65]. These studies in wheat and rice indicated that AWPM-19 family proteins negatively regulate seed germination. Although only 8 out of the 52 OsZIP09 targets were downregulated in osbzip09 and ABA-treated seeds, they included two key genes. One was DLT, which encodes a transcription factor that mediates the regulation of plant architecture by brassinosteroid, especially plant height and leaf angle [66,67]. Although no evidence currently exists that DLT regulates seed germination, it associates OsbZIP09 and BR signaling in modulating seed germination and post-germination growth. A second gene, OsLOX2, encodes a lipoxygenase that degrades storage lipids during rice seed germination. Overexpression of OsLOX2 accelerates rice seed germination under normal conditions and decreases seed viability after accelerated aging [26].

We have shown that mutation of OsbZIP09 leads to a notable reduction in rice PHS. OsbZIP09 is a typical bZIP transcription factor that contains conserved bZIP domains and its expression is induced by ABA. To reveal the mechanism whereby OsbZIP09 regulates seed germination, RNA-seq and DAP-seq results isolated 52 key direct targets of OsbZIP09, most of which showed consistent changes in expression in response to ABA and in the osbzip09 mutant. Some of these genes, such as OsLOX2 and LEA genes are involved in controlling seed germination. Therefore, we have successfully identified a number of key direct target genes of OsbZIP09 to explain how OsbZIP09 controls rice seed germination.
4. Materials and Methods

4.1. Generation of Osbzip09 Mutants Using CRISPR/Cas9

One specific target site within the first exon of OsbZIP09 was selected for gene editing. The target sequence was introduced into vector pC1300-Cas9, and the generated construct was transformed into japonica rice ZH11 via Agrobacterium-mediated transformation. The CRISPR/Cas9 vector system and the detailed procedure have been described previously [68].

4.2. Rice Growth Conditions

All rice plants were grown under the same climatic and management conditions during the summer in a paddy field at Yangzhou University (Yangzhou, China). Three replicate plots were used for the experiments, and the plots were arranged in a randomized block pattern, with 6 rows per plot and 10 plants per row. Superior spikelets or mature seeds from superior spikelets from the middle of each plot were collected for seed germination analysis.

4.3. Seed Germination Analysis

To mimic PHS in the lab, mature panicles from osbzip09 mutants and ZH11 wild-type controls were collected and immersed in water, and the number of germinated seeds was recorded after six days. Traditional germination assay of rice seeds under normal conditions was performed as described previously [69]. In brief, dehulled rice seeds were sterilized with 70% ethanol and washed twice with Milli-Q water for each experiment. Sterilized seeds were subsequently germinated in darkness in an artificial climate incubator at a temperature of 26 °C and a relative humidity of 70%. Seeds with a radicle longer than 1 mm were considered to have successfully germinated [70]. Germination rates were recorded every 12 h until 120 h after imbibition (HAI). Each seed germination assay included at least three independent biological replicates, and each replicate contained 30 seeds.

4.4. Phylogenetic Assay, Sequence Alignment, and Domain Analysis of bZIP09

The BLAST function of the National Center for Biotechnology Information (NCBI) database (Bethesda, MD, USA) was used to identify highly homologous bZIP09 protein sequences from different species. A phylogenetic tree was then constructed based on the amino-acid sequence of the full-length protein using MEGA6 software. The amino-acid sequences of bZIP09 orthologs from eight representative species was aligned using Clustal Omega (Hinxton, Cambridge, UK) [71]. The conserved amino-acid sequence of the bZIP09 protein was analyzed and a graphical representation was produced by WebLogo 3 (Berkeley, CA, USA) [72]. Finally, SWISS-MODEL (Basel, Switzerland) was used to analyze the predicated three-dimensional (3D) structure of the conserved domain of bZIP09 [73].

4.5. RNA Extraction and qRT-PCR Analysis

WT seeds treated with ABA (5 µM) or EtOH (mock treatment) for 15 and 30 min were collected for quantification of OsbZIP09 expression via qRT-PCR. Seeds of WT treated with ABA (5 µM) or mock and osbzip09-2 mutant seeds mock-treated for 36 HAI were collected for RNA-seq and qRT-PCR validation. Three biological replicates were included for each experiment, and ACTIN served as a reference gene for normalization. Total RNA was isolated from the harvested seed samples using an RNAsimple Total RNA kit (TIANGEN, Beijing, China), and genomic DNA was removed by treatment with RNase-free DNase I (Qiagen, Hilden, Germany). First-strand cDNA was synthesized using HiScriptIII RT SuperMix for qPCR (Vazyme, Nanjing, China) with an oligo dT primer.

4.6. RNA-Seq Analysis

High-quality RNA (1 µg) was used for library generation, and high-throughput RNA sequencing was performed on an Illumina Novaseq platform (San Diego, CA, USA), and 150 bp paired-end reads were generated. Raw sequencing reads were trimmed, and the
collected clean data were aligned to the genome of rice japonica cultivar Nipponbare (IRGSP-1.0, http://rapdb.dna.affrc.go.jp/, accessed on 10 September 2020) using TopHat2 software [74]. The DESeq2 R package (version 1.16.1, Heidelberg, Germany) was used for differential expression analysis [75]. Genes identified by DESeq2 with a p-value < 0.05 and fold change >1.5 were assigned as being differentially expressed.

4.7. DNA Affinity Purification Sequencing (DAP-Seq) Sampling

DAP-seq was performed according to Bartlett et al. (2017) [76]. First, genomic DNA (gDNA) was extracted from mature seeds of rice. Then a gDNA DAP-seq library was prepared by attaching a short DNA sequencing adaptor to the purified and fragmented gDNA. The adapter sequences were truncated Illumina TruSeq adapters; the TruSeq Universal and Index adapters corresponded to the DAP-seq Adapter A and Adapter B. The DAP gDNA library was prepared using a kit from NEBNext® DNA Library Prep Master Mix Set for Illumina® (NEB, #E6040S/L, Ipswich, MA, USA). OsbZIP09 was fused to the HaloTag using a kit from pFN19K HaloTag T7 SP6 Flexi Vecto (Promega #G184A). OsbZIP09 fused to HaloTag was expressed using a TnT SP6 High-Yield Wheat Germ Protein Expression System (L3260, Promega, Madison, WI, USA), and was purified using Magne HaloTag Beads (G7281, Promega). The Magne HaloTag Beads and OsbZIP09-HaloTag mixture were incubated with 500 ng DNA library in 40 µL PBS (Phosphate Buffered Saline) buffer with slow rotation in a cold room for 1.5 h. The beads were washed five times with 200 µL PBS + NP40 (0.005%), resuspended in PBS buffer, the supernatant was removed, and 25 µL EB buffer was added and samples were incubated for 10 min at 98 °C to elute the bound DNA from the beads. The correct DAP-seq library concentration to achieve a specific read count was calculated on the basis of on library fragment size. Negative-control mock DAP-seq libraries were prepared as described above, without the addition of protein to the beads.

4.8. DAP-Seq Data Analysis

We defined target genes as those that contained DAP-seq peaks located within the transcribed regions of genes, introns, or 2 kb upstream from the transcription start site (TSS), or 2 kb downstream from the transcription termination site (TTS). DAP-seq reads were aligned to the rice genome using Bowtie 2 (Baltimore, MD, USA) [77], which supports gapped and paired-end alignment modes. We ran Bowtie version 2.2.3 with default parameters and reported only unique alignments. DAP-seq peaks were detected by MACS2 (Boston, MA, USA) [24]. We used MACS version 2.0.10 with default parameters, as duplicates were allowed, and the q-value < 0.05. Core motifs were identified by MEME-ChIP (Brisbane, QLD, Australia) [78].

4.9. Promoter Analysis

To analyze ABA-responsive motifs within the OsbZIP09 promoter, 2-kb DNA sequence upstream of the OsbZIP09 initiation codon ATG was obtained from The Rice Annotation Project Database (Rap-db). The promoter sequence of OsbZIP09 was scanned in PLACE database to identify the presence of putative ABA-responsive cis-acting elements. To identify OsbZIP09-binding motifs within the promoters of its target genes, the 2-kb promoter sequence of each gene was analyzed using VectorNTI9 software (version 9, Invitrogen, Carlsbad, CA, USA) to confirm the location and number of the motifs identified by DAP-seq.

4.10. Dual-Luciferase Reporter Assay

Agrobacterium-mediated transient assays were conducted with tobacco leaves by co-expressing reporter and effector constructs. The promoters of LEA25 (1.15 kb) and LOX2 (2.05 kb) were each cloned into the pGreen II0800-LUC vector to generate reporter constructs. The coding region of OsbZIP09 was cloned into the pGreenII 62-SK vector to generate an effector construct [79]. Each reporter construct was transformed into tobacco
leaves together with the OsbZIP09 effector. After 36 h growth, the relative LUC activity of the transformed tobacco leaves with and without ABA (5 μM) treatment were measured using the Dual-Luciferase Reporter Assay System (Vazyme, Nanjing, China). The detailed method is described previously [80]. The sequences of all the primers used in this study are listed in Table S1.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/1422-0067/22/4/1661/s1.

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**Abbreviations**

- ABA: abscisic acid
- ABI5: ABA-INSENSITIVE 5
- ABFs: ABRE-binding factors
- ABRE: ABA-responsive element
- AOC: Allene Oxide Cyclase
- AREBs: ABA-responsive element binding proteins
- AWPM-19: ABA-induced Wheat Plasma Membrane Polypeptide-19
- ANOVA: analysis of variance
- bZIP: basic leucine zipper
- ChIP-seq: Chromatin immunoprecipitation sequencing
- CNX: cofactor for nitrate reductase and xanthine dehydrogenase
- CSLA5: cellulose synthase-like A5
- DAP-seq: DNA affinity purification sequencing
- DOG1: DELAY OF GERMINATION 1
- DRE: drought-responsive element
- HAI: hours after imbibition
- DEGs: differentially expressed genes
- DLT: DWARF AND LOW-TILLERING
- GA: gibberellin
- GAP: GTPase activating protein
- GO: gene ontology
- IPA1: Ideal Plant Architecture 1
- KEGG: Kyoto Encyclopedia of Genes and Genomes
- LEA: Late Embryogenesis Abundant
- LOX2: lipoxygenase 2
- MFT2: MOTHER OF FT AND TFL 2
- NCBi: National Center for Biotechnology Information
- PHS: preharvest sprouting
- PLA3: PLASTOCHRON3
PP2Cs  protein phosphatases 2C
PUFAs  polyunsaturated fatty acids
qRT-PCR  quantitative real-time polymerase chain reaction
QTLs  quantitative trait loci
RCAR1  regulatory component of ABA receptor 1
ROS  reactive oxygen species
RNA-Seq  RNA sequencing
SD  standard deviation
SnRK2  sucrose nonfermenting 1-related protein kinase 2
TF  transcription factor
TSS  transcription start site
TTS  transcription termination site
USP  universal stress protein

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