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Full Length Article

Transcriptome Analysis of Upland Rice in Response to PEG Stress during Seed Germination

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Abstract

Upland rice is an ecotype adapted to dry culture system. In this study, changes in germination characteristics and transcriptome of germinating seeds exposed to 15% polyethylene glycol (PEG) were investigated using upland rice genotype IRAT109. PEG reduced germination potential and inhibited growth of seed radicle and plumule. Under PEG stress, gibberellic acid (GA) content decreased and abscisic acid (ABA) content increased. Transcriptome analysis revealed that 1270 genes were differentially expressed between stressed and non-stressed seeds. Approximately, 56.46% of differentially expressed genes (DEGs) were up-regulated and 43.54% of DEGs were down-regulated under PEG stress. Gene Ontology (GO) analysis categorized 1149 DEGs into 52 functional groups and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis assigned 123 DEGs to 54 pathways. A large number of DEGs related to GA signal transduction, ABA biosynthesis and signal transduction, and defense response pathways were upregulated. Results indicated that genes related with GA signal transduction and ABA play important role in germination under drought stress. Findings of this study are highly useful to understand the molecular mechanism of germination and provide candidate genes for molecular breeding in dry direct-seeded rice.

Keywords: Upland rice; Seed germination; Drought; RNA-seq; Signal transduction

Introduction

Transplanting and direct-seeding are two widely used methods of planting rice (*Oryza sativa* L.). In Asia, nearly 80% of rice is established using the transplanting method (Rao *et al*., 2007; Wang *et al*., 2017). However, due to increase in water scarcity and labor shortage, transplanting rice is facing greater challenges and is not adopted by framers. Meanwhile, dry direct-seeded rice is becoming increasingly popular due to less water, less labor, and easy mechanized planting (Farooq *et al*., 2011; Pathak *et al*., 2011; Mahender *et al*., 2015). However, low germination rate and irregular seedling emergence occur in dry direct-seeded rice due to shortage of water during seed germination, which has adverse effect on the large-scale promotion of dry direct-seeded rice. Therefore, varieties for dry direct-seeding should have rapid and uniform seed germination ability under moderate drought stress (Zheng *et al*., 2016).

Plant seed germination is a series of orderly physiological reaction and morphogenesis processes starting from water absorption and seed expansion. Seeds with high starch contents *i.e*., wheat (*Triticum aestivum* L.) and rice need less water, while seeds with high protein contents like soybean (*Glycine max* L.) and peanut (*Arachis hypogaea* L.) need more water for germination (Bradford and Nonogaki, 2007). In order to meet the water demand during seed germination, farmers usually sow in time when there is adequate soil moisture or irrigate to provide enough water. Meanwhile, researchers are committed to discover genes that regulate seed germination and further improve the germination ability through molecular breeding.

Upland rice is an ecological type that evolved over a long period of domestication under dry conditions. Compared with lowland rice, upland rice has better drought tolerance with high seed vigor, high growth potential, and stable protective enzyme activity under drought stress (Hu and Xiong, 2014). But, there are some shortcomings in upland rice varieties, such as poor yield and grain quality, owing to the insufficiency of breeding work (Bernier *et al*., 2007, 2008). In order to breed high yield, good grain quality and drought tolerance rice adapted to dry direct-seeding, it is necessary to explain drought tolerance mechanism and clone drought tolerance gene from upland rice, then polymerize drought tolerance genes of upland rice with high yield and good grain quality (Arun *et al*., 2018). Although several drought related genes, *ARAG1* (Zhao *et al*., 2010), *TSRF1* (Quan *et al*., 2010), *OsNAC5* (Jeong *et al*., 2013), *SNAC3* (Fang *et al*., 2015), *OsICE1* (Chander *et al*., 2018), *OsLG3* (Xiong *et al*., 2018) have been cloned, there are not enough to explain the drought tolerance mechanism and utilize in breeding. In recent years, the rapid development of

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high-throughput transcriptome sequencing has laid a foundation for functional genomics research in crop species. However, no studies have focused on transcriptome analysis of upland rice in response to drought stress during seed germination. In the present study, the differential transcriptome of germinating seed in upland rice between PEG stress and non-stressed conditions were analyzed using RNA-seq. The aim of the study was to identify drought-responsive genes that regulate seed germination and to provide new insights into the mechanism of germination under drought stress in upland rice. These results will be helpful to molecular breeding in dry direct-seeded rice.

Materials and Methods

Materials and Treatments

A widely used upland tropical *japonica* IRAT109, characterized by strong root system and better osmotic adjustment, was used as the material (Li *et al*., 2015). The seeds of IRAT109 were surface-sterilized with 10% H_2O_2 for 10 min and washed with sterile distilled water. Seeds were then soaked in distilled water at 28°C for 24 h, allowed to germinate in Petri dishes with moistened filter paper. Seeds were germinated and grown in a growth chamber for 5 days irrigated with the H_2O (control, non-stress) or 15% PEG solution (osmotic potential of -0.8 MPa, stress). Growth chamber was set at 10 h light/14 h dark, 28°C day/25°C night temperatures and 75% relative humidity. Each treatment had 3 replicates with 50 seeds per replicate.

Germination Characteristics and Endogenous Phytohormones

Germination vigor is the germination rate after 3 days of treatment (Sun *et al*., 2010). Root length and shoot length were measured after 5 days of treatment. Seeds after one day of treatment were collected to determine the endogenous contents of abscisic acid (ABA) and gibberellic acid (GA3) using enzyme-linked immunosorbent assay kits (China Agricultural University, China), following instruction (Yang *et al*., 2001). Seeds were incubated in 80% (v/v) methanol at 4°C for 48 h. The extracts were collected after centrifugation at 10,000 rpm for 15 min at 4°C, filtered through a Sep-Pak C18 cartridge (Waters, Milford, MA, USA), dried using pure N_2 at 20°C and stored at -80°C. The residues were dissolved in phosphate buffered saline (pH = 7.5) containing 0.1% (w/v) gelatin and 0.1% (v/v) Tween 20 to quantify ABA and GA3 contents.

Total RNA Isolation and Sequencing

Total RNA was isolated from seeds treated with PEG solution or H_2O for one day using TransZol RNA extraction kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Oligo (dT) magnetic beads were used to isolate poly (A) RNA from the total RNA samples. The mRNA was fragmented by heating at 94°C for 5 min. First-strand cDNA was synthesized by random hexamers (six base random primers) for 10 min at 25°C, 50 min at 42°C, and 15 min at 70°C. Second-strand cDNA was synthesized by adding buffer, dNTPs, RNaseH and DNA polymerase I. The double-stranded cDNA was purified by QiaQuick PCR kit (Qiagen, Chatsworth, CA, USA), eluted by EB buffer, and was end repaired using T4 DNA polymerase, Klenow fragment, and T4 polynucleotide kinase. A single "A" base was added to the cDNA products and the fragments were ligated to the Illumina PE adapters. Agarose gel electrophoresis was used to select the 150 bp to 200 bp size of the fragments. Then cDNA was amplified by 15 cycles of PCR for 10 s at 98°C, 30 s at 65°C and 30 s at 72°C. The protocol for library construction was same as previously described by Lou *et al*. (2014). The libraries were sequenced using Illumina HiSeqTM2000 platform at Biomarker Technologies Corporation (Beijing, China). For each sample, the sequencing data were more than 2G and gene identification ratio in rice genome was more than 90%. Three biological replicates, which 6 samples from PEG stressed and non-stressed seeds, were used for RNA-seq.

RNA-seq Data Analysis

Reads with low quality and adaptor contamination were removed by using in-house perl scripts. The clean reads were mapped to the *Oryza sativa* Nipponbare reference genome with TopHat. The sequence alignment files generated by TopHat were provided to Cufflinks (Trapnell *et al*., 2010) and assembled the alignments into transfrags. Cufflinks statistical model probabilistically assigns reads to the assembled isoforms. Using BLASTX, unique reads were aligned to a series of databases including NCBI non-redundant (Nr), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Ontology (GO). Using the DEGseq (2010) R package, differential expression of genes between PEG stressed and non-stressed seeds were analyzed (Anders and Huber, 2010). Differentially expressed genes (DEGs) were ranked based on size and normalized FPKM (fragments per kilo base of exon per million reads). DEGs were analyzed according to the log2 fold change (log2FC) of FPKM ($P < 0.05$ and log2 fold change ≥ 1).

Quantitative Real-time RT-PCR

Total RNA was reverse-transcribed using GoScriptTM reverse transcription system (Promega, Madison, USA). In an applied CFX96 Real-Time PCR System (BIO-RAD), the transcript levels were measured in a total reaction volume of 20 μL containing 5 μL of the reverse-transcribed product, 0.8 μL of forward and reverse primers, 10 μL of GoTaq® qPCR Master Mix (Promega, Madison, USA) and 4.2 μL of ddH_2O. The cycling conditions included an initial denaturation at 95°C for 8 min followed by 38 cycles of amplification (95°C for 15 s and 60°C for 1 min). The transcript levels were normalized using rice ACTIN

(*LOC_Os03g50885*) as the internal reference gene. Three biological replicates were used. The relative expression levels were determined using the $2^{-\triangle\triangle CT}$ method. The details of primers used for quantitative real-time RT-PCR are given in Supplementary Table 1.

Results

IRAT109 is a typical upland rice variety with high seed germination ability. PEG stress affects seed germination compared to non-stress condition (Table 1 and Fig. 1). PEG stress reduced germination vigor by 27.67%. PEG inhibited seed radicle and plumule growth, which resulted in a decrease in root length and shoot length by 69.29% and 30.27%, respectively. Under PEG stress, GA content decreased by 15.81%, ABA content increased by 10.75% and GA/ABA ratio decreased by 23.98%. IRAT109 seeds retained relatively high germination vigor of 72.15% under 15% PEG stress indicating the adaptive mechanism of IRAT109 to cope with osmotic stress during seed germination.

A genome-wide transcriptome analysis was performed using PEG stressed and non-stressed seeds of IRAT109 (Table 2). Approximately 20,150,182 and 28,359,036 reads were generated for stressed and non-stressed seeds, respectively. Out of this, 91.71% and 92.48% of reads were mapped to the reference genome, and 90.33% and 91.32% of reads were uniquely compared with the reference genome. There were 26,512 and 27,425 annotated genes in stressed and non-stressed seeds, respectively. Comparing gene expression profiles between stressed and non-stressed seeds, 1,270 DEGs were identified according to the threshold $P < 0.05$ and $\log2FC \geq 1.00$ (Fig. 2). Consistent expression trends between RNA sequencing and RT-qPCR were observed for 5 genes, which indicated that RNA-seq data were reliable (Supplementary Fig. 1). Approximately 56.46% (717) of DEGs were up-regulated and 43.54% (553) of DEGs were down-regulated under PEG stress. Log_2FC values of 717 up-regulated genes ranged from 1.39 to 9.77. Five genes with the maximum up-regulation were *Os02t0716550*, *Os08t0191100*, *Os07t0170000*, *Os05t0104700* and *Os04t0412350*. *Os02t0716550* participated in biochemical pathways related to lipid metabolism and redox. *Os07t0170000* involved in cell metabolism and biochemical pathways related to redox. *Os08t0191100* and *Os04t0412350* are hydratases, which participated in the metabolic process of polymers. Log_2FC value of 553 down-regulated genes ranged from -7.29 to -1.47. Five genes with the maximum down-regulation were *Os01t0780900*, *Os03t0223301*, *Os12t0511500*, *Os08t0184800*, and *Os11t0701600*. *Os01t0780900* has catalytic activity, which participated in cell growth, regulation of primary metabolic process, cell response to stimulation, and development related to morphogenesis. *Os03t0223301* has catalytic activity that involved in the regulation of macromolecule metabolism. *Os08t0184800* involved in cellular component of plastid. *Os12t0511500* involved in cellular component of cytoplasmic membrane-bounded vesicle, which may be participated in biological process of defense response.

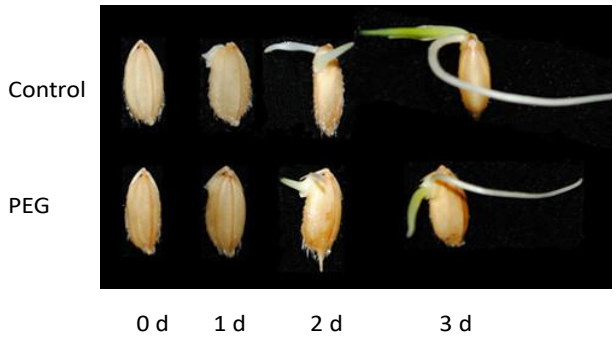

Fig. 1: The image of seed germination of upland rice IRAT109 under 15% PEG stress and H_2O (control, non-stress) at 0, 1, 2 and 3 days

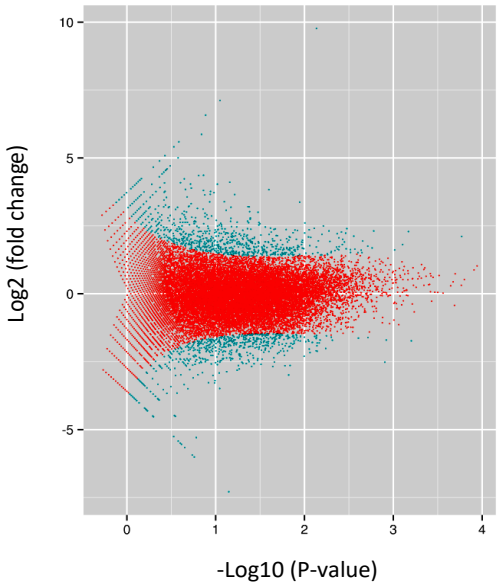

Fig. 2: Volcano plots of the transcriptome between PEG stressed and non-stressed seeds. The X-axis represents the negative log10-transformed P-values ($P < 0.05$) for differentially expressed genes between treatments. The Y-axis represents the fold change in PEG stress compared to non-stress (on a log2 scale). Green dots represent differential genes and red dots represent no significant differences

The 1149 DEGs were categorized into 52 functional groups, including 16 in cellular component, 13 in molecular function and 23 in biological process according to GO analysis (Fig. 3). The main secondary nodes of cellular component were cell part, cell, organelle, and membrane. In cellular component, 318 sub-categories were found of which nucleus (GO:0005634; 250), mitochondrion (GO:0005739; 246) and cytoplasmic membrane-bounded vesicle (GO:0016023; 233) had the highest number of DEGs. The main secondary functional nodes of molecular

Table 1: The germination vigor, shoot length, root length, GA content and ABA content of upland rice IRAT109 under 15% PEG stress and H_2O (control, non-stress)

Treatments	Germination vigor (%)	Shoot length (cm)	Root length (cm)	GA (ng/g. FW)	ABA (ng/g. FW)
Control	99.82 ± 4.61	3.13 ± 0.02	4.03 ± 0.21	4.65 ± 0.09	85.65 ± 5.08
PEG	72.15 ± 2.78**	0.96 ± 0.01**	2.81 ± 0.33**	3.92 ± 0.11*	94.86 ± 3.12*

Means ± SE (n = 3). *, ** represent significant difference at 0.05 and 0.01 levels, respectively

Table 2: Summary of transcriptome sequencing data of seeds after one day of germination under 15% PEG stress and H_2O (control, non-stress)

Samples	Total Reads	Mapped Reads	Unique Mapped Reads	Multiple Mapped Reads
Control	28,359,036	26,227,402	25,897,948	329,454
PEG	20,150,182	18,480,714	18,200,843	279,871

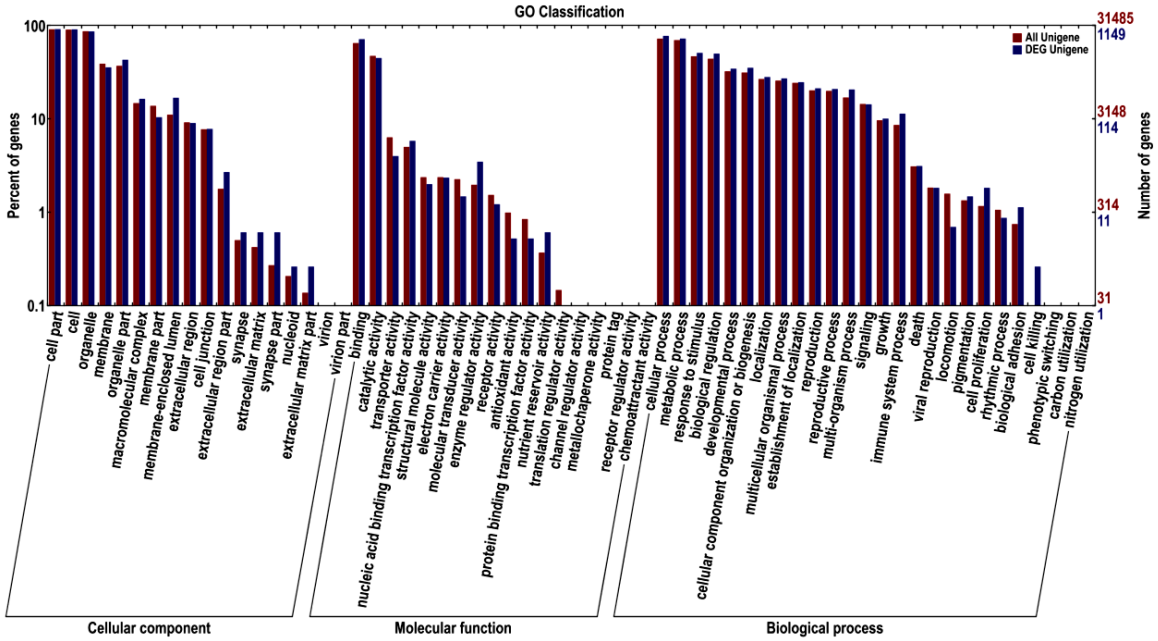

Fig. 3: GO annotation clusters of differentially expressed genes between PEG stressed and non-stressed seeds

function were binding, catalytic activity and transporter activity. In molecular function, 613 sub-categories were found of which protein binding (GO:0005515; 280), ATP binding (GO:0005524; 169), binding (GO:0005488; 168) and DNA binding (GO:0003677; 129) had the highest number of DEGs. The main secondary functional nodes of biological process were cellular process, metabolic process, response to stimulus, biological regulation and development process. In biological process, 1712 sub-categories were found. RNA processing (GO:0006396), defense response (GO:0006952), regulation of transcription, DNA-dependent (GO:0006355), and regulation of cellular process (GO:0050794) had the highest number of DEGs, which enriched 125, 86, 85 and 76, respectively. The GO sub-category associated with GA was enriched with 33 DEGs of which 28 were up-regulated (most genes involved in GA signal transduction) and 5 were down-regulated (4 genes, *Os07t0562800*, *Os02t0544951*, *Os01t0713900* and *Os04t0611800* involved in the GA biosynthetic process (GO:0009686)). Eight GO sub-categories associated with ABA were enriched with 109 DEGs of which 77 were up-regulated. Five DEGs involved in ABA biosynthetic process (GO:0009688) were up-regulated, while 2 DEGs involved in ABA metabolic process (GO:0009687) were down-regulated.

KEGG pathway analysis was performed to compare the metabolic pathways between stressed and non-stressed seeds and assigned 123 DEGs to 54 pathways. Plant hormone signal transduction pathway (ko04075) was enriched with 10 DEGs (Table 3) associated with auxin, ABA, ethylene, and jasmonic acid signal transduction processes that were up-regulated. Plant-pathogen interaction pathway (ko04626) was enriched with 8 DEGs that were up-regulated. Cysteine and methionine metabolism pathway (ko00270) were enriched with 7 DEGs. DNA replication pathway (ko03030) was enriched with 6 DEGs that were down-regulated.

Table 3: The top 10 KEGG pathways of differential expression genes between PEG stressed and non-stressed seeds

Pathway	Ko ID	Number of gene	Gene ID
Plant hormone signal transduction	ko04075	10	*Os02t0643800;Os03t0180800;Os03t0268750;Os04t0537100;Os06t0527800;Os06t0562200;Os07t0259100;Os09t0325700;Os10t0391400;Os10t0392400*
Plant-pathogen interaction	ko04626	8	*Os01t0949500;Os03t0180800;Os04t0492800;Os04t0618700;Os08t0144100;Os10t0391400;Os10t0392400;Os11t0140600*
Cysteine and methionine metabolism	ko00270	7	*Os01t0772900;Os03t0727600;Os03t0798300;Os05t0149450;Os06t0149801;Os07t0182900;Os12t0625102*
DNA replication	ko03030	6	*Os02t0511900;Os02t0797425;Os04t0588200;Os05t0235800;Os05t0476200;Os11t0484300*
Photosynthesis	ko00195	5	*Os01t0501800;Os01t0773700;Os06t0101600;Os07t0544800;Os08t0104600*
Photosynthesis-antenna proteins	ko00196	4	*Os07t0558400;Os07t0562750;Os09t0346500;Os11t0242850*
Ribosome biogenesis in eukaryotes	ko03008	4	*Os03t0333100;Os03t0343300;Os03t0824300;Os10t0456900*
Biosynthesis of unsaturated fatty acids	ko01040	4	*Os02t0716550;Os03t0748100;Os07t0170000;Os07t0561500*
Starch and sucrose metabolism	ko00500	4	*Os03t0212800;Os07t0681700;Os08t0449901;Os08t0450100*
Carotenoid biosynthesis	ko00906	4	*Os03t0645966;Os07t0154100;Os07t0154201;Os12t0617400*

Discussion

Seed germination is a complex physiological and biochemical process, which is influenced by internal material state and environmental factors (Shu *et al*., 2016). Endogenous hormones regulate a series of physiological changes in seed germination through signal transduction. GA is a hormone essential for seed germination, which plays a role in transmitting signals to initiate seed germination (Vishal and Kumar, 2018).

It enhances the expression of hydrolase, soften the tissue around the embryo, help overcome the limitation of the seed coat, and promote the growth of the embryo (Rajjou *et al*., 2012). ABA is the main hormone that induces seed dormancy and inhibits the effect of GA (Finkelstein *et al*., 2002; Finch-Savage and Leubner-Metzger, 2006). Because seed must absorb water from external environment during seed germination, drought affects seed germination. In this study, endogenous GA content decreased, ABA content increased, germination potential decreased and seed germination was inhibited under PEG stress. Upland rice is an ecological type that highly resistant to drought stress (Luo, 2010). Our study found that the germination vigor of IRAT109 was retained at a high level of 72.15% under 15% PEG stress and showed high drought tolerance during germination.

Differential transcriptome analysis showed that PEG induced up-regulation of 717 genes and down-regulation of 553 genes in upland rice. Four genes of GA biosynthetic process were down-regulated and GA content decreased. However, expression of 28 genes responding to GA signal was up-regulated. So, the functions of GA to promote seed germination may not be reduced. Under PEG stress, 77 genes of ABA-related GO sub-category were up-regulated, of which 5 genes were involved in ABA biosynthetic process. Meanwhile, ABA content increased, which help induce drought tolerance response. Eighty-six genes related to defense response biological process were differentially expressed. Majority of genes with the highest up-regulation such as *Os02t0716550*, *Os08t0191100*, *Os07t0170000*, *Os05t0104700* and *Os04t0412350* are involved in biochemical pathway of redox activity, cell metabolism, or hydrolytic enzymes. Some of these genes may be involved in the tolerance response induced by ABA. Therefore, the up-regulated expression of GA signal transduction genes and ABA-related genes under PEG stress may be the reason for the high germination under drought stress in upland rice. In addition, 10 DEGs related to auxin, ABA, ethylene and jasmonic acid signal transduction were up-regulated, which suggest that seed germination is regulated by multiple hormone interactions. Previous reports have indicated that a sophisticated molecular network of phytohormones regulate seed germination (Miransari and Smith, 2014; Llanes *et al*., 2016; Verma *et al*., 2016). GA and ABA have antagonistic functions in mediating plant development and abiotic stress responses (Shu *et al*., 2016, 2018). How hormones such as GA, ABA and auxin interact to regulate seed germination under drought stress in upland rice needs further study.

Conclusion

PEG reduced seed germination vigor of upland rice IRAT109 by 27.67%, with GA rise and ABA decline. Approximately, 56.46% of DEGs were up-regulated and 43.54% of DEGs were down-regulated under PEG stress. Many DEGs related to GA signal transduction, ABA biosynthesis and signal transduction and defense response pathways were up-regulated. These results provide candidate genes for molecular breeding in dry direct-seeded rice.

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