

Title: Alternative splicing and translation play important roles in parallel with transcriptional regulation during rice hypoxic germination

Running title: Proteogenomic analysis of rice hypoxic germination

Mo-Xian Chen^{a,b,1}, Fu-Yuan Zhu^{b,c,1}, Feng-Zhu Wang^{d,1}, Neng-Hui Ye^e, Bei Gao^f, Xi Chen^g, Shan-Shan Zhao^d, Tao Fan^a, Yun-Ying Cao^{f,i}, Tie-Yuan Liu^f, Ze-Zhuo Su^d, Li-Juan Xie^d, Qi-Juan Hu^c, Hui-Jie Wuⁱ, Shi Xiao^d, Jianhua Zhang^{b,j,2} and Ying-Gao Liu^{a,2}

^a State Key Laboratory of Crop Biology, College of Life Science, Shandong Agricultural University, Taian, Shandong, China.

^b Shenzhen Research Institute, The Chinese University of Hong Kong, Shenzhen, China.

^c College of Biology and the Environment, Nanjing Forestry University, Nanjing, Jiangsu Province, 210037, China.

^d State Key Laboratory of Biocontrol and Guangdong Provincial Key Laboratory of Plant Resources, School of Life Sciences, Sun Yat-sen University, Guangzhou, China.

^e Southern Regional Collaborative Innovation Center for Grain and Oil Crops in China, Hunan Agricultural University, Changsha, 410128, China.

^f School of Life Sciences, The Chinese University of Hong Kong, Shatin, Hong Kong.

^g Wuhan Institute of Biotechnology, Wuhan, China.

^h China National GeneBank, BGI-Shenzhen, Shenzhen 518120, China.

ⁱ College of Life Sciences, Nantong University, Nantong, Jiangsu, China.

^j Department of Biology, Hong Kong Baptist University, and State Key Laboratory of Agrobiotechnology, The Chinese University of Hong Kong, Shatin, Hong Kong.

¹These authors contributed equally to this work.

²To whom correspondence should be addressed. Email: liuyg@sdau.edu.cn and jzhang@hkbu.edu.hk

Corresponding authors

Liu Ying-Gao

State Key Laboratory of Crop Biology, College of Life Science, Shandong Agricultural University, Taian, Shandong, China

liuyg@sdau.edu.cn Tel.: (86) 538 8249767

Zhang Jianhua

Department of Biology, Hong Kong Baptist University, and State Key Laboratory of Agrobiotechnology, The Chinese University of Hong Kong, Shatin, Hong Kong.

jzhang@hkbu.edu.hk Tel.: (852) 3411 7011

39 Abstract

40 Post-transcriptional mechanisms, including alternative splicing (AS) and alternative
 41 translation initiation (ATI), have been used to explain the protein diversity involved in
 42 plant developmental processes and stress responses. Rice germination under hypoxia
 43 conditions is a classical model system for the study of low oxygen stress. It is known
 44 that there is transcriptional regulation during rice hypoxic germination, but the
 45 potential roles of AS and ATI in this process are not well understood. In this study, a
 46 proteogenomic approach was used to integrate the data from RNA sequencing,
 47 qualitative and quantitative proteomics to discover new players or pathways in the
 48 response to hypoxia stress. The improved analytical pipeline of proteogenomics led to
 49 the identification of 10,253 intron-containing genes, 1,729 of which were not present
 50 in the current annotation. Approximately 1,741 differentially expressed AS (DAS)
 51 events from 811 genes were identified in hypoxia-treated seeds in comparison to
 52 controls. Over 95% of these were not present in the list of differentially expressed
 53 genes (DEG). In particular, regulatory pathways such as spliceosome, ribosome, ER
 54 protein processing and export, proteasome, phagosome, oxidative phosphorylation
 55 and mRNA surveillance showed substantial AS changes under hypoxia, suggesting
 56 that AS responses are largely independent of traditional transcriptional regulation.
 57 Massive AS changes were identified, including the preference usage of certain
 58 non-conventional splice sites and enrichment of splicing factors in the DAS datasets.
 59 In addition, using self-constructed protein libraries by 6-frame translation, thousands
 60 of novel proteins/peptides contributed by ATI were identified. In summary, these
 61 results provide deeper insights towards understanding the underlying mechanisms of
 62 AS and ATI during rice hypoxic germination.

63 **Keywords:** alternative splicing, hypoxia, *Oryza sativa*, proteogenomics, seed
 64 germination, splicing factor, translation initiation.

65

66 INTRODUCTION

67 Rice is a staple food that provides dietary nutrition for more than two billion people
68 around the world (Yang and Zhang, 2006). In addition, rice is a model monocot plant
69 used in modern research. Rice has been reported to have the ability to survive periods
70 of submergence from seed germination to adult plants (Atwell et al., 2015). In
71 particular, it has been documented as one of the few species that can germinate under
72 anoxia by elongating its coleoptile to reach the water surface (Berta and Ismail, 2013).
73 This adaptation to oxygen deprivation caused by flooding can be used as a model to
74 study molecular mechanisms in response to hypoxic or anoxic conditions. Flooding is
75 becoming one of the most severe abiotic stresses worldwide (Sasidharan et al., 2017).
76 As the primary stresses due to flooding, hypoxia and anoxia have drawn much
77 attention in the past decade. Under normal oxygen concentrations, oxygen gradients
78 have been reported in dense plant organs, including seeds, fruits and tubers
79 (Sasidharan et al., 2017). Thus, studying the molecular mechanisms during hypoxic or
80 anoxic conditions may facilitate an understanding of the function of O₂ molecules in
81 both stress response and plant development. In recent years, a N-end rule protein
82 degradation pathway has been proposed to be an important oxygen sensing
83 mechanism in Arabidopsis (Gibbs et al., 2011; Licausi et al., 2011). Its downstream
84 components, plant ethylene-responsive transcription factors, are affected by this
85 pathway to activate or deactivate their target genes in response to hypoxia (Weits et al.,
86 2014; Giuntoli et al., 2017). Increasing numbers of loci involved in flooding responses
87 have been characterized, including those in lipid signalling (Xie et al., 2015),
88 jasmonic acid and antioxidant pathways (Yuan et al., 2017), protein kinase (Chang et
89 al., 2012) and transcription factor (Giuntoli et al., 2017). However, few studies are
90 related to functional characterization of rice genes during flooding germination. In

addition to the classical CIPK15-SnRK1A-MYBS1- mediated sugar-sensing pathway (Lu et al., 2007; Lee et al., 2009), a mitochondrion-localized protein (OsB12D1) has been reported to enhance flooding tolerance in rice germination and subsequent seedling growth (He et al., 2014). In addition, a rice trehalose-6-phosphate (T6P) phosphatase (OsTPP7) gene has been proposed to increase sink strength in response to flooding germination (Kretschmar et al., 2015).

With the development of large profiling techniques, considerable efforts have been made to study the global transcripts changes, protein abundance and metabolic variation during rice hypoxic germination (Lasanthi-Kudahettige et al., 2007; Narsai et al., 2009; Narsai et al., 2011; Sadiq et al., 2011; Narsai et al., 2015; Hsu and Tung, 2017). Although comparative analysis indicates that part of the hypoxic responsive pathways are conserved among several species (Narsai et al., 2011), the mechanism of adult flooding tolerance may be largely different from that of seed flooding tolerance (Lu et al., 2007; Lee et al., 2009). Furthermore, recent RNA seq analysis using eight *Arabidopsis* ecotypes suggests that alternative splicing could be another pivotal factor involved in hypoxic responses (Van et al., 2016). Alternative splicing results from post-transcriptional control of eukaryotic intron-containing genes. Recent advancement reveals that more than 95% of genes have splicing isoforms in mammals (Eckardt, 2013). Two major types of splicing complex have been documented that can determine the splicing site sequences. One is the U2 complex, which can splice at a 5'-GT-AG-3' exon-intron junction. The other is called the U12 complex and is able to utilize 5'-AT-AC-3' as a splicing junction (Zdraviko J et al., 2005; Will and Luhrmann, 2011). Alternative splicing from multiexonic genes has been regarded as a potential way to increase plant genome coding ability (James et al., 2012; Ruhl et al., 2012; Chang et al., 2014; Feng et al., 2015). In addition to alternative splicing, another type of post-transcriptional regulation defined as alternative translation initiation (ATI) is

involved in contributing to protein diversity (Sonenberg and Hinnebusch, 2009). Recent identification of translation initiation sites using advanced technology such as ribosome sequencing and MS-based proteomics reveals that a large number of these sites are not conventional AUG sequences (Sonenberg and Hinnebusch, 2009; Ingolia et al., 2011; Lee et al., 2012). In comparison to AS regulation (James et al., 2012; Ruhl et al., 2012; Chang et al., 2014; Feng et al., 2015; Wang et al., 2015; Zhan et al., 2015; Thatcher et al., 2016), the function of ATI has been seldom reported in plants (de Klerk and t Hoen, 2015). The above techniques have demonstrated that the eukaryotic genome has the ability to encode short peptides, including upstream open reading frames (uORFs) and other small ORFs, that are located in previously marked non-coding regions of the genome (Tavormina et al., 2015). Several peptides have been characterized to show crucial roles in regulating plant development and stress responses (Simon and Dresselhaus, 2015; Tameshige et al., 2016).

In summary, although stress-induced genome-wide AS changes have been extensively documented in various plant species (Yang et al., 2015; Thatcher et al., 2016; Van et al., 2016; Fesenko et al., 2017), the quantification of corresponding AS isoforms at the protein level have seldom been reported. In this study, a parallel RNA seq and proteomic approach defined as proteogenomic has been applied to achieve integrative analysis using both transcriptome and proteome data. Given our previous experience in ABA-regulated AS analysis (Zhu et al., 2017), we further improved our analytical pipeline for the determination of AS- and ATI-induced genome coding ability. The results from this study further expand our understanding of genome coding ability in rice seeds, suggesting an underlying regulatory network resulting from AS and ATI during rice hypoxic germination. Understanding this hidden network may facilitate the agricultural production of rice that is suitable for direct seeding systems and provide guidelines for improving hypoxic tolerance in other crop species.

143 **Materials and methods**

144 **Plant material, growth conditions and hypoxic treatment**

145 Seeds of *Oryza sativa* (Nipponbare) were surface-sterilized with 20% bleach and
 146 0.05% Tween-20 before treatments. Seeds (~30-50 individuals) were placed on petri
 147 dishes with wet filter paper and then were transferred to air control or hypoxia
 148 conditions under complete darkness. The hypoxia treatment was carried out using the
 149 Whitley H35 Hypoxystation (Don Whitley Scientific Limited, UK) with 3% O₂ level
 150 at 28°C. Seed samples were harvested at 6 h after treatments and used for further
 151 transcriptomic and proteomic analysis.

152

153 **Rice seed RNA extraction and RNA sequencing**

154 Rice seed total RNA were ground in liquid nitrogen and extracted using a Plant
 155 RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.
 156 RNA-sequencing (RNA-seq) experiments were conducted as previously described
 157 with minor modifications (Zhu et al., 2017). The resulting cDNA library constructed
 158 from rice seed RNA samples (Air_6 h and Hypoxia_6 h) were used for paired end (2 x
 159 125 bp) sequencing on an Illumina HiSeq 4000 platform by Annoroad Gene
 160 Technology Co. Ltd. (Beijing, China). Three replicates for each sample were trimmed
 161 to obtain clean reads for subsequent analysis (Supplemental Table 1).

162

163 **Analysis of RNA sequencing and proteomic data**

164 The rice (Nipponbare) reference genome annotation file (Oryza_sativa.IMGSP-1.0.32)
 165 was downloaded from the Ensembl website (<http://www.ensembl.org/index.html>).
 166 Clean reads mapping and subsequent bioinformatic analysis was as described
 167 previously (Zhu et al., 2017). The analytical pipeline is summarized in Supplemental
 168 Fig. 1. As mentioned previously (Zhu et al., 2017), significant changes of

169 differentially expressed genes (DEG) (Supplemental Table 2) and differentially
 170 expressed alternative splicing events (DAS) (Supplemental Table 3) were determined
 171 as $\text{Log}_2\text{FC} > 2$ and $q\text{-value}$ (false discovery rate, $\text{FDR} < 5\%$). Identification and
 172 quantification of AS events were conducted by using the software ASprofile
 173 (<http://ccb.jhu.edu/software/ASprofile>) (Florea et al., 2013). Splicing junctions
 174 reported in this study was generated by default settings of TopHat v2.1 aligner. The
 175 AS events with no expression values were filtered out before subsequent analysis
 176 (Zhu et al., 2017). Gene ontology analysis (GO, <http://geneontology.org/>) and Kyoto
 177 encyclopedia of genes and genomes (KEGG, <http://www.kegg.jp/>) enrichment
 178 classification were carried out using both DEG and DAS datasets. Heatmaps were
 179 generated using the BAR HeatMapperPlus tool
 180 (http://bar.utoronto.ca/ntools/cgi-bin/ntools_heatmapper_plus.cgi). The splicing sites
 181 conservation analysis was performed using WebLogo v3
 182 (<http://weblogo.threeplusone.com/>) (Crooks et al., 2004).

183

184 **Total protein extraction, digestion and qualitative identification**

185 Total protein of rice seeds was extracted and digested as described previously (Chen
 186 et al., 2014) with minor modifications. In general, approximately 5 g of rice seed
 187 tissues of each sample were ground in liquid nitrogen for subsequent proteomic
 188 analysis. The precipitated protein pellets were digested by trypsin and desalted using a
 189 Sep-Pak C₁₈ column (Waters). The resulting peptides were then separated and
 190 characterized in a TripleTOF 5600⁺ (AB SCIEX) splitless Ultra 1D Plus (Eksigent)
 191 system (Andrews et al., 2011).

192

193 **Peptide dimethyl labelling and quantitative proteomics**

194 The quantitative proteomics was conducted as described previously with minor

modifications (Zhou et al., 2015). Digested peptides were dissolved with 0.1 M sodium acetate (pH≈6, best below 6) (*i.e.*, 500 µg peptides per 0.25 mL sodium acetate). Either 4% formaldehyde or formaldehyde-d2 (40 µL per 500 µg peptides) were added and mixed. Then, 40 µL / 500 µg peptides of 0.6 M NaBH₃(CN) were added. The solution mixture was shaken for 0.5 h. Furthermore, 160 µL / 500 µg peptides of 1% NH₄OH, was added and mixed for 5 min. Then, 5% formic acid (160 µL per 500 µg peptides) was added and mixed. The solution was placed in 4°C for at least 1 h. The light and heavy dimethyl labelling peptides were combined in a 1:1 ratio and desalted using a Sep-Pak C₁₈ column (Waters).

Mixed peptides were subsequently fractionated by using a C₁₈-ST column (2.0 mm × 150 mm, 5 µm particle size) (TechMate) on the Agilent 1260 system (Agilent Technologies). An elution gradient of 60 min was used for peptide separation with 20 mM ammonium formate in H₂O (adjusted to pH 10 by 25% NH₃.H₂O) as solvent A and 20 mM ammonium formate in 80% ACN (adjust pH to 10 by 25% NH₃.H₂O) as solvent B. The gradient elution profile was composed of 5%-25% B for 20 min, 25-45% B for 15 min, 45-90% B for 1 min, then maintained at 90% B for 4 min, followed by 10-95% A for 1 min, and ending with 95% A for 14 min. The flow rate was 0.2 mL/min. UV absorbance was monitored at 216 nm. A total of 60 0.2 mL fractions were collected, then concatenated and mixed to obtain 20 fractions. Fractions were dried *via* speed-vacuum and desalted by the StageTip C₁₈ method.

RPLC-ESI-MS/MS was used to detect the sample. LC-MS/MS detection was carried out on a hybrid quadrupole-TOF LC/MS/MS mass spectrometer (TripleTOF 5600⁺, AB Sciex) equipped with a nanospray source. Peptides were first loaded onto a C₁₈ trap column (5 µm, 5 x 0.3 mm, Agilent Technologies) and then eluted into a C₁₈ analytical column (75 µm × 150 mm, 3 µm particle size, 100 Å pore size, Eksigent).

Mobile phase A (3% DMSO, 97% H₂O, 0.1% formic acid) and mobile phase B (3%

221 DMSO, 97% ACN, 0.1% formic acid) were used to establish a 100 min gradient,
 222 which consisted of 0 min of 5% B, 65 min of 5-23% B, 20 min of 23-52% B, 1 min of
 223 52–80% B, and the gradient was maintained in 80% B for 4 min, followed by 0.1 min
 224 of 80–85% B, and a final step in 5% B for 10 min. A constant flow rate was set at 300
 225 nL/min. MS scans were conducted from 350 to 1500 amu, with a 250 ms time span.
 226 For MS/MS analysis, each scan cycle consisted of one full-scan mass spectrum (with
 227 m/z ranging from 350 to 1500 and charge states from 2 to 5) followed by 40 MS/MS
 228 events. The threshold count was set to 120 to activate MS/MS accumulation, and
 229 former target ion exclusion was set for 18 s.

230

231 **Library construction and mass spectrometry database searching**

232 An AS junction library (576,570 entries) was constructed as described previously
 233 (Sheynkman et al., 2013; Castellana et al., 2014; Walley and Briggs, 2015) with minor
 234 modifications. In brief, six-frame translations, including 3 frames on the forward
 235 strand and 3 frames on the reverse complement strand, were used construct the AS
 236 junction library. Additionally, a frame library was constructed using all transcripts
 237 annotated in the reference annotation file by 6 frames. The redundant sequences were
 238 then removed from translated sequences at the first step. Peptide sequences longer
 239 than 6 amino acids were attached to the UniProt rice japonica database for subsequent
 240 database search. Raw spectrum data generated from both qualitative and quantitative
 241 proteomics were searched with the ProteinPilot software (v5.0, AB SCIEX) using
 242 preset parameters. All data were filtered at 1% FDR with at least 1 peptide at 95%
 243 confidence level calculated automatically by the ProteinPilot software (Zhu et al.,
 244 2017). For quantitative proteomics, data were searched against UniProt and
 245 self-constructed databases using the following parameters: sample type, dimethyl (0,
 246 +4) quantitation; cys alkylation, iodoacetamide, digestion, trypsin. The search effort

was set to rapid ID. For DEP analysis, proteins with a fold change of >1.2 or <0.8 (P value <0.05) are considered as DEP in this study.

Quantitative real-time PCR validation of AS transcripts

Total RNA ($\sim 5 \mu\text{g}$) was reverse-transcribed into cDNA by using the Superscript First-Strand Synthesis System (Invitrogen, USA) following the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was conducted as described previously (Zhu et al., 2013). Resulting products of qRT-PCR were subjected to DNA sequence analysis. Isoform-specific primers used for AS isoforms identification are listed in Supplemental Table 5.

Data submission.

The rice transcriptome data have been uploaded to Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>) under Bioproject PRJNA451248. The raw data of qualitative and quantitative proteomics have been submitted to the PRIDE PRoteomics IDentifications (PRIDE) database with accession number PXDxxxxxx and PXDxxx, respectively.

Results

Improvement of analytical pipeline and experimental conditions

The analytical pipeline used in this study is presented in Supplemental Fig. 1. Improvements have been made since the last bioinformatic flowchart (Zhu et al., 2017). The identification and quantification procedures of AS events were simplified for subsequent GO and KEGG analysis. In addition, refinement of redundancy and error check steps further improved the accuracy of identification. In this study, AS events such as AFE (alternative first exon) and ALE (alternative last exon) purely

caused by alternative transcription start and poly adenylation has been removed to further differentiate AS modification from other transcriptional or post-transcriptional mechanisms. To distinguish 5' donor sites and 3' acceptor sites, we further divided AE (alternative exon) events into AE5' and AE3' for further bioinformatic analysis. Furthermore, incorporation of quantitative proteomics yielded more information on steady protein levels in comparison to qualitative proteomic profiling, which can only identify the presence of translated peptides (Zhu et al., 2017). For testing samples, we chose dry seeds of japonica rice (Nipponbare) treated with hypoxia (3% O₂) for 6 h in comparison to air controls under complete darkness. This treatment will help us to understand the short-term responses at both transcripts and protein levels during hypoxia when seeds start to germinate. Plenty of samples were harvested for the following three profiling experiments: short-read RNA sequencing (RNA seq), qualitative and quantitative proteomics. Prior to these experiments, we have compared 49 up-regulated anaerobic marker genes highlighted in previous publications to our dataset (Lasanthi-Kudahettige et al., 2007; Narsai et al., 2009). Among 21 genes detected in this study, 19 of these genes showed consistency on their differential regulation, but at a lower magnitude (Supplemental Fig. 2A). We used qRT-PCR to further validate those expressions. In total, 18 of 19 genes showed similar expression pattern as the result of our RNA seq data (Supplemental Fig. 2B), indicating the efficacy of hypoxic treatment using 3% O₂ in our system.

293

294 **Completely different set of genes undergo alternative splicing (AS) in response to** 295 **hypoxia during rice seed germination**

Approximately 1.32 billion raw reads in total averaging 200 million reads per sample were obtained from RNA sequencing (Supplemental Table 1). Among these, 1.25 billion clean reads were subjected to the mapping process. On average, approximately

95% were uniquely mapped to the genome and used for subsequent bioinformatic analysis (Supplemental Table 1). For AS identification, each sample identified over 75,000 AS events. In total, 10,253/26,848 (38.2%) annotated intron-containing genes in rice seed were observed to exist as AS events in rice seeds. Approximately 6.4% (1,729/26,848) more intron-containing genes were observed in comparison to the original annotation file. Slightly differ from previous AS analysis in ABA-treated Arabidopsis seedlings (Zhu et al., 2017), alternative first exons (AFE), alternative last exons (ALE) and intron retention (IR) remained as the most abundant three AS events through all the samples (Fig. 1A). Among these three AS event types, AFE and ALE caused variable 5'- and 3'-untranslated ends, which may affect the efficiency of translation or stability of corresponding transcripts (Andreassi and Riccio, 2009; Sonmez et al., 2011; Jenal et al., 2012). For example, hidden small open reading frames (sORF) from the 5'-end of transcripts encoding short peptides have the ability to regulate translational efficiency of target transcripts (Laing et al., 2015), whereas polyadenylation at the 3'-end of transcripts is well known to affect the localization and stability of the transcripts (De et al., 2017). When the dataset of differentially expressed genes (DEG) (Supplemental Table 2) was compared to the dataset of differentially expressed AS genes (DAS) (Supplemental Table 3), over 95% were not the same (Fig. 1B). Only 23 genes were differentially regulated at both transcription and post-transcriptional levels (Fig. 1B). This suggests that alternative splicing may play an important and distinctive role during rice hypoxic germination. Subsequent gene ontology enrichment analysis also confirmed the result from the Venn diagram (Fig. 1B, Supplemental Fig. 3). In several cases, DEG and DAS genes did not coexist in the same secondary GO category (Supplemental Fig. 3). Fourteen isoforms of 7 genes in the DAS dataset were assembled and validated by quantitative real-time PCR (qRT-PCR). In total, 6 of these genes were consistent to the data from RNA seq

analysis, suggesting the reliability of AS identification and quantification from the analytical pipeline (Supplemental Fig. 4). Except for categories related to linoleic acid metabolism, the majority of DEG and DAS genes were not enriched in the same KEGG category (Supplemental Fig. 3), suggesting that DAS category is a different group of genes in response to hypoxic germination. The majority of pathways enriched in DEG dataset were closely related to cellular metabolisms (*e.g.* pentose phosphate pathway, glycolysis/gluconeogenesis, fructose and mannose metabolism *etc.*) and cell growth (meiosis, DNA replication and cell cycle *etc.*). Whereas some regulatory pathways were specifically over-represented in DAS dataset, such as spliceosome, ribosome, ER protein processing, protein export, proteasome, phagosome, oxidative phosphorylation and mRNA surveillance pathway, implying that these pathways may play essential role in AS-mediated responses under rice hypoxic germination. Gene members in several pathways have been selected for RT-PCR and qRT-PCR validation (Fig. 1D and Fig. 2). Some splicing isoforms of corresponding gene showed differential expression under hypoxic treatment, indicating their potential role in response to rice hypoxic germination.

341

342 **Qualitative proteomic identification reveals that hypoxia-regulated AS events are** 343 **more likely to be translated**

344 To further characterize the translational products of identified AS events, we carried
345 out a qualitative proteomic profiling using tandem mass spectrometry (MS/MS) for
346 both control and hypoxia-treated samples (Alfaro et al., 2014; Tavares et al., 2015;
347 Zhu et al., 2017). Proteomic analysis this time generated 547,545 and 485,392
348 high-quality spectra for control and hypoxia-treated samples, respectively.
349 Approximately 5,549 and 5,385 proteins were identified using the UniProt database
350 (Fig. 3A). Among these, 18.6% and 16.1% of identified proteins were uniquely

351 present in control or hypoxia-treated samples, respectively, serving as good candidates
 352 for further functional characterization. Subsequent AS junction library search
 353 identified 4,431 / 4,313 peptides from AS events (41,887) and 510 / 490 peptides from
 354 DAS events (1,742) for control / hypoxia-treated samples, respectively (Fig. 3A, B).
 355 Among these, approximately 70% of peptides were shared by both samples.
 356 Intriguingly, much fewer AFE events could be detected at peptide level in comparison
 357 to ALE events (Fig. 2B). Furthermore, 13.5% of the total AS events (5,652/41,887)
 358 were translated into peptides, suggesting that the majority of AS transcripts may be
 359 degraded by RNA surveillance mechanisms such as nonsense-mediated mRNA decay
 360 (NMD) (Nicholson et al., 2010; Drechsel et al., 2013). In contrast, an elevated
 361 percentage (38.3%) of DAS events could be translated into peptides in all AS types
 362 (Fig. 3B), indicating their potential role in response to hypoxic stress during rice
 363 germination. Similar observations have been reported in ABA-treated Arabidopsis
 364 seedlings, which indicates that thousands of AS proteins are translated under hypoxic
 365 conditions during rice germination, and most of these were not present in the DEG
 366 dataset analysed by a conventional RNA seq pipeline.

367 In addition, approximately 68.3% of AS events identified in this study were not
 368 annotated in the genome and thus were marked as new features for rice genome
 369 annotation (Fig. 3B). Additionally, 40.9% of the DAS peptides were not present in the
 370 current version of the annotation, which suggests the translation of new protein
 371 isoforms during rice germination in response to hypoxia. DAS peptides were
 372 subjected to KEGG enrichment analysis (Fig. 3C). For example, some KEGG terms
 373 including ribosome, proteasome and oxidative phosphorylation, were repeatedly
 374 enriched in both RNA seq and qualitative proteomic datasets, giving protein evidence
 375 of these splicing isoforms in response to hypoxia treatment.

376

377 **Quantitative proteomics indicates that the expression of protein and transcripts** 378 **are correlated at the AS level**

379 To find relationship between the protein abundance and corresponding transcripts at
380 the AS level, quantitative proteomics were conducted using the dimethyl labelling
381 method. In total, 10,946 proteins were identified from this approach and 4566 of them
382 were quantified (Supplemental Table 4). Among these, 278 differentially expressed
383 proteins (DEP) and 29 differentially regulated AS peptides (DASP) were identified
384 (Fig. 4A-B). Thirteen DASP were found to be differentially expressed in quantitative
385 proteomics and referred as DASDP. Amongst these, none of them were shared with
386 DEP dataset (Fig. 4C). Similar to previous parallel analysis (Bai et al., 2015;
387 Marmioli et al., 2015), much less overlap was observed between DEP and DEG, DAS
388 and DASP as well as DEP and DASDP (Fig. 4A-C). Only 11 genes were identified as
389 both DEG and DEP with low correlation ($R^2=0.18$) of their expression levels (Fig. 4D,
390 E), suggesting the existence of post-transcriptional regulation for most of the
391 transcripts. Although 2 genes were detected in both DAS and DASP datasets, the
392 expression of their transcripts and proteins were at the same trend (Fig. 4F), indicating
393 that quantification at AS isoform level may provide more accurate data representation
394 for both transcripts and proteins than conventional quantification method used in RNA
395 seq and proteomics. However, more data is required to confirm this hypothesis. In
396 addition to the effect of post-transcription, the low overlap of DEP/DASDP with
397 DEG/DAS datasets may be explained by the relatively low throughput and coverage
398 of the MS-based proteomic method.

399

400 **Construction of a customized protein library leads to novel proteins** 401 **identification and quantification during rice hypoxic germination**

402 Similar to previous findings (Zhu et al., 2017), the spectra usage for protein

403 identification was approximately 40-50% in this study (Fig. 5A) using both UniProt
 404 and AS junction libraries as input files. An increasing number of publications suggest
 405 that single transcripts are able to be translated into multiple proteins by using
 406 alternative translation initiation (ATI) sites (Brar and Weissman, 2015). This indicates
 407 that a large number of novel proteins or short peptides are yet to be identified, and this
 408 is caused by incomplete genome annotation (Kim et al., 2014). Thus, a 6-frame
 409 translation library was constructed using the combination of assembled cufflink files
 410 during RNA seq analysis and reference annotation files based on previously published
 411 methods (Castellana et al., 2008; Zhu et al., 2017). The outcomes from the database
 412 searching identified thousands of novel proteins and peptides, with 74.6% of proteins
 413 longer than 80 amino acids (a. a.), 24.0% of proteins/peptides from 11-80 amino acids
 414 and 1.4% of peptides from 6-11 amino acids (Fig. 5B, C). Among these, 2294 / 1432
 415 novel proteins (> 80 a.a.) and 310 / 774 novel proteins or peptides (6-80 a. a.) were
 416 identified in control / hypoxia-treated samples, respectively (Fig. 5D). This
 417 observation provides further evidence of increment coding ability for proteins and
 418 short peptides by using ATI sites. Additionally, an increasing number of short peptides
 419 (774) were detected in hypoxia-treated samples in comparison to air controls (310),
 420 suggesting that short peptides may play an important role in response to hypoxia
 421 during rice germination. Intriguingly, 137 novel proteins were quantified at a second
 422 frame of known transcripts. Few of these overlapped with DEG and DEP datasets,
 423 indicating that most of these proteins can only be detected by proteomic analysis
 424 using the customized library. This set of genes served as a source of novel candidates
 425 for further investigation of hypoxic responses during rice germination.

426

427 **The conventional 5'-splicing sites are less conserved in rice seeds at normal**
 428 **condition and under hypoxia treatment**

To further investigate the splicing characteristics between total AS and hypoxia-affected DAS datasets, statistical analysis of splicing sites (ss) conservation was performed. Conventionally, U2-type splicing sites (5'-GT-AG-3') are conserved and account for 90% of total splicing sites among plant species (Will and Luhrmann, 2011). In this study, the 3'-splicing site (AG) was relatively conserved and accounted for over 80% in both control and hypoxia-treated samples (Fig. 6A). An extra 'C' was identified as a conserved sequence in both AS and DAS datasets (Fig. 6B). Thus, 3'-splicing sites were identified as 'CAG' in rice seeds, and the hypoxia treatment did not change this signature (Fig. 6B). However, there was a decrease in the 'AG' proportion in hypoxia-treated samples, which was associated with the increase in the proportions of several other ss sequences especially 'AC'. In contrast, the conventional 5'-splicing site (GT) accounted for only 50% of total AS and was increased to approximately 60% in the hypoxia-affected DAS dataset (Fig. 6A). Meanwhile, non-conventional 5'-splicing sites such as 'AA' and 'CT' was largely reduced in the DAS dataset by comparing with AS dataset, suggesting its role in response to hypoxia stress (Fig. 6A). In addition, similar results were obtained by conservation analysis; 'GGT' signature was obtained in both AS and DAS datasets (Fig. 6B). Further investigation of ss among AS types demonstrated that AFE was responsible for the 'GT' reduction in both AS and DAS datasets (Supplemental Fig. 5). Although 3'-ss were more conserved, certain types of non-conventional splicing sites were induced among the specific AS types in the DAS dataset in comparison to the AS dataset such as 3'-TG and 3'-TT in AE5', 3'-AC and 3'-GG in AE3', 3'-GC and 3'-TG in IR and 3'-GC in SKIP (Supplemental Fig. 5). This result indicates that the AS regulation under hypoxia stress may be caused by alternative recognition of sequence of splicing sites.

454

455 **Splicing factors are enriched in differentially expressed AS events**

456 To further understand the underlying mechanism of DAS regulation under hypoxia
 457 stress, splicing factors in rice were summarized and subjected to further analysis.
 458 Three genes were found in the DEG dataset (Fig. 7A). In contrast, a total of 105 AS
 459 events from 21 splicing factor-related proteins were observed in the DAS dataset (Fig.
 460 7A, B), and none of them were found in the DEG dataset. Among these, 60 AS events
 461 were up-regulated, whereas 45 AS events were down-regulated (Fig. 7B). In detail,
 462 43.8% of AS events were AFE and ALE accounting for 28.6% (Fig. 7C). The
 463 remaining three AS types accounted for 27.7% of the total AS events (Fig. 7C).
 464 According to the classification in the splicing-related gene database (SRGD,
 465 <http://www.plantgdb.org/SRGD/index.php>), the 21 genes observed in the DAS dataset
 466 were classified into 11 subgroups (Fig. 7D) from core splicing components to
 467 auxiliary factors. And those SFs enriched in KEGG term of spliceosome (Fig. 1C)
 468 were chosen for qRT-PCR validation (Fig. 7E). Some isoforms of selected SFs were
 469 differentially expressed under hypoxia treatment, suggesting that the change of AS in
 470 splicing components may be crucial in response to hypoxia stress during rice
 471 germination.

472

473 **Discussion**

474 **The discovery of a hidden network of AS in response to hypoxic stress during**
 475 **rice germination provides additional targets in the study of hypoxia**

476 Alternative splicing produces multiple RNA isoforms for each locus. Each isoform
 477 may encode one protein isoform as well, which greatly expands the genome coding
 478 ability. Additionally, the discovery of two new AS types, AFE and ALE, has revealed
 479 the great potential to generate AS isoforms (Yan and Marr, 2005; de Klerk and t Hoen,
 480 2015; Zhu et al., 2017). In this study, approximately 97.2% (787/810) of the DAS

481 genes had no differences at the gene expression level, suggesting AS control of
 482 transcripts is completely separated from the conventional DEG group (Fig. 1B).
 483 Moreover, increasing evidence reveals that protein isoforms generated by AS
 484 transcripts have the ability to alter protein subcellular localization, protein-protein
 485 interaction networks and protein stability due to the presence or absence of certain
 486 motifs (Buljan et al., 2012; Ellis et al., 2012). Thus, the 667 AS peptides identified in
 487 both control and hypoxia-treated samples provided protein evidence of AS transcripts
 488 and may serve as good candidates for further functional characterization (Fig. 3A, B).
 489 These genes were distributed in a variety of biological pathways, including amino
 490 acid biosynthesis, ribosome and proteasome pathway, pantothenate and CoA
 491 biosynthesis and oxidative phosphorylation, and were not selected for further
 492 investigation by the first round of screening using DEG as criteria amongst large scale
 493 transcriptome analysis, suggesting that AS responses are embedded in various
 494 biochemical processes under hypoxia stress.

495 Transcriptomic studies have shown that low oxygen induces a myriad of gene
 496 responsiveness in terms of transcript abundance (Lasanthi-Kudahettige et al., 2007;
 497 Narsai et al., 2009; Narsai et al., 2011; Sadiq et al., 2011; Narsai et al., 2015; Hsu and
 498 Tung, 2017). Accordingly, transcriptional regulation in oxygen sensing pathways has
 499 been extensively studied in plants. Key regulators, such as ERFVII transcription
 500 factors, have been substantially characterized (Fukao et al., 2009; Hattori et al., 2009;
 501 Hinz et al., 2010; Licausi et al., 2010). However, few studies have been carried out to
 502 unravel the AS regulation under hypoxia. In the current study, AS analysis indicates
 503 that the conventional splicing sites are not conserved at the 5' position in rice seeds
 504 (Fig. 6A, B and Supplemental Fig. 5). Major regulators defined as splicing factors
 505 within assembled spliceosome have been characterized to participate in AS site
 506 determination (Golovkin and Reddy, 1996; Kalyna et al., 2006; Krummel et al., 2009;

Will and Luhrmann, 2011; Kondo et al., 2015; Yoshida et al., 2015). Although several splicing factors have been reported to be involved in stress responses (Ruhl et al., 2012; Feng et al., 2015), none of them are related to hypoxia responses. In our results, a 7-fold increase in the number of splicing factors (21 in DAS to 3 in DEG) were found in comparison to the DEG dataset (Fig. 7), suggesting the importance of those proteins in splicing site recognition. Over 100 AS events in these 21 splicing factors were affected during hypoxia treatment, which may greatly alter the protein isoforms of these proteins in comparison to the control group. Subsequently, hypoxia may change the composition and conformation of spliceosomes by recruiting different protein isoforms of splicing factors, which may in turn lead to a different choice of splicing site sequence recognition. This may explain the increment of the proportion of certain non-conventional splicing sites during rice hypoxic germination (Supplemental Fig. 5). Furthermore, the integration of qualitative proteomic data implies that hypoxia-responsive AS events are more likely to be translated in comparison to non-responsive ones (Fig. 3B, lower panel), providing protein evidence for the potential role of these AS isoforms in response to hypoxia stress. Therefore, our results suggest that alternative splicing is an independent pathway other than transcriptional repression in response to hypoxia during rice germination. The majority of members in this pathway remain to be elucidated.

526

527 **Alternative cellular pathways are activated by AS under hypoxia treatment**

Several pathways were found to be over-represented under AS-mediated responses during rice hypoxic germination. mRNA surveillance, such as NMD, has long been demonstrated to play an important role in controlling mRNA stability and abundance before translation (Nicholson et al., 2010; Drechsel et al., 2013). It has been reported that NMD is closely related to exon junction complex (EJC) of splicing machinery in

both animals and plants (Shaul, 2015). In Arabidopsis, hypoxia-responsive ERFs, HRE1 and HRE2, have been proposed to be likely regulated by post-transcriptional mechanisms for their mRNA stability (Licausi et al., 2010). From our dataset, isoforms of several components belong to EJC complex (*e.g.* Os08g0305300, *OsSMG7* and Os05g0140500, *OsY14a*) were observed to be differentially regulated (Nyikó et al., 2013), indicating their potential function in surveillance of newly spliced RNA isoforms under hypoxia. Evidence shows that the status of spliceosome will be affected under hypoxia in animal tissues (Schmidtkastrner et al., 2008). Splicing factors like serine-arginine (SR) proteins is activated under hypoxic condition by phosphorylation (Jakubauskiene et al., 2015). However, the responsiveness of spliceosome under hypoxia treatment remains to be elucidated *in planta*. In this study, a variety of splicing components have been identified to show differential expression under hypoxia treatment. Among these, six isoforms from two SR proteins (Os03g0344100, *SR32* and Os02g0610600, *RSZ23*) were induced by hypoxia treatment (Fig. 7E). Although multiple isoforms of SR proteins have been detected in different rice tissues (Peng et al., 2013), no evidence links them to hypoxia stress responsiveness before. Here, we hypothesize that SF changes under hypoxia is crucial for downstream AS regulation under hypoxia. However, less information can be found by annotation and datamining of these SFs that we have identified in this study. Further functional characterization is required to confirm their roles in response to hypoxia. Besides post-transcriptional regulatory pathways, processes related to protein export, lysosome and proteasome were observed to play a role during hypoxic germination (Figs. 1 and 2). The enhancement of some splicing isoforms in protein export process (Fig. 2C) may effectively help plants to survive during hypoxia conditions. Furthermore, lysosome is a place where cell to recycle building materials or detoxification (Chen et al., 2015). Recent study shows that hypoxia may rapidly

559 induce autophagy, which is a highly conserved mechanism in eukaryotes to target
560 cellular components to lysosome for recycling purpose (Chen et al., 2015). Thus, the
561 newly formed isoforms of lysosomal gene may be responsible for the survival under
562 hypoxia stress. Similarly, protein degradation has been considered as a major
563 responsive mechanism in response to hypoxia in both animals and plants (Huang et al.,
564 1998; Gibbs et al., 2011; Licausi et al., 2011). Significant protein will be degraded as
565 an alternative energy source and remodelling during hypoxia treatment. Isoforms
566 formed in this process may efficiently degrade misfolded proteins for synthesis of
567 proteins isoforms that can confer hypoxia tolerance. Intriguingly, transcriptional
568 regulation focused on the control of cellular metabolic levels and growth factors,
569 whereas alternative splicing aims to produce new protein isoforms that is mainly
570 involved in degradation, post-transcriptional regulation and transport processes. These
571 two complementary mechanisms may facilitate rice seeds to survive under hypoxia
572 during germination.

573

574 **Thousands of novel proteins or peptides resulting from alternative translation**
575 **participate in the hypoxia response during rice germination**

576 In addition to AS-resulting protein diversity, proteins encoded from a second frame of
577 the same transcript or from annotated non-coding regions contribute to genome
578 coding ability as well (Jensen et al., 2013; Wade and Grainger, 2014). Specifically, a
579 considerable number of unannotated proteins were detected using a customized
580 library by six-frame translation (*i.e.*, 3 in the forward strand and 3 in the reverse
581 complement strand). The coding ability of one transcript using a second frame has
582 been widely studied in animals but is rarely reported in plants (de Klerk and t Hoen,
583 2015). One example from plant systems is an alpha-enolase gene (*LOS2*) in
584 Arabidopsis that encodes an MBP-like protein by alternative translation. This

585 MBP-like protein affects ABA responses and its protein level is regulated by E3 ligase
586 SAP5 (Kang et al., 2013). Furthermore, the existence of uORFs in the 5'-untranslated
587 regions of certain transcripts may lead to a feedback regulation of translation
588 efficiency (Laing et al., 2015). From our results, a total of 2,660 putative proteins over
589 80 amino acids and 904 proteins/peptides ranging from 6 to 80 amino acids have been
590 identified (Fig. 5D). A total of 960 of these proteins/peptides were specifically
591 induced under hypoxia treatment, suggesting that they are new players involved in
592 hypoxia responses. Furthermore, a total of 137 novel proteins were quantified by
593 proteomic analysis, 128 of which were not present in the DEG and DEP lists (Fig. 5E),
594 demonstrating that the usage of a customized library combined with quantitative
595 proteomics is essential for this kind of novel protein/peptide identification.

596

597 **Proteogenomic approach evolves as a new generation method to analyse** 598 **omics-based datasets**

599 Large profiling methods have been applied in plant research to study various
600 developmental processes or stress responses. However, individual approaches such as
601 transcriptome or proteome analysis are restricted by their defects in experimental
602 conditions and analytical pipelines. For example, pure transcriptome analysis is
603 affected by the corresponding reference genome annotation. Pure proteomic methods
604 are limited by currently available protein libraries, which were generated based on
605 incomplete genome information (Zhu et al., 2017). Thus, proteogenomics, a method
606 incorporating transcriptomic and proteomic datasets, represents a new generation of
607 analytical approaches for deeper understanding of the functional importance of
608 potential genome coding ability (Castellana et al., 2008; Kumar et al., 2016). First,
609 this analytical approach is able to determine which AS isoforms will be translated into
610 proteins and thus can differentiate between mRNA degradation regulation and

translational control (Nicholson et al., 2010; Drechsel et al., 2013). Second, in combination with quantitative proteomics, proteogenomic analysis links the protein evidence to their transcript changes to give an accurate footprint for each transcript isoform during the analysis. Low correlation of the expression levels between proteins and transcripts will be improved when using this type of analytical pipeline (Fig. 4D, E). This in turn will reveal valuable targets that are truly regulated at transcript and protein levels in the same trend. At last, coupled with a self-constructed protein library, this method enhances the identification of novel proteins/peptides (Fig. 5) that are potential hidden regulatory components in plant development or stress responses. However, this approach can be further improved from its current version. For example, using strand-specific library construction in short-read RNA seq analysis can enhance the accuracy and reduce redundancy of subsequent protein library construction. Furthermore, using the 3rd generation of sequencing methods, such as single molecule long-read sequencing, can aid in the precise identification of full-length transcripts for accurate AS identification (Zhu et al., 2017). In addition, the low overlap between DEG and DEP or DAS and DASP can be improved by increasing the throughput and coverage of proteomic analysis. The incorporation of SWATH (sequential window acquisition of all theoretical spectra-mass spectrometry)-based quantitative proteomics (Zhu et al., 2016; Zhu et al., 2016) and two or more enzyme digestion steps may achieve better results than those of the current study.

631

632 CONCLUSION

In conclusion, this study expands our understanding of the genome coding ability of rice under hypoxic germination. Two post-transcriptional mechanisms, alternative splicing and alternative translation initiation, have major contributions to protein diversity during hypoxia (Figure 8). Alternative splicing may function in parallel with

transcriptional control in response to hypoxia stress during rice germination. Specifically, low oxygen conditions extensively affect AS and ATI patterns in parallel with conventional transcriptional regulation during rice germination. The compositional change of spliceosomes may result in the preferred usage of non-canonical splicing sites under hypoxia treatment. In this case, the conservation of 5'-splicing sites was largely affected by the hypoxia treatment. In addition, hypoxia-affected DAS events were more likely undergo protein translation in comparison to AS events identified under normal conditions. The above results indicate the existence of a large underground network of hypoxia responses at the post-transcriptional level. This newly discovered underlying response mechanism is mediated by AS and ATI. The members of this network need to be further characterized. This case study using hypoxic germination as a model demonstrates how modern technology and bioinformatic analysis improves our understanding of the plant genome coding ability and its features during stress responses.

651

652 **Supplemental Data**

653 **Supplemental Fig. 1.** Analytical pipeline of AS identification, quantification and
654 validation in this study.

655 **Supplemental Fig. 2.** Comparison of previous published datasets and qRT PCR
656 validation.

657 **Supplemental Fig. 3.** GO enrichment analysis between DAS and DEG datasets from
658 RNA sequencing.

659 **Supplemental Fig. 4.** qRT-PCR validation of selected genes from DAS events.

660 **Supplemental Fig. 5.** Comparison of splicing sites (ss) recognition between AS and
661 DAS events.

662

663 **Supplemental Table 1.** Summary of the basic parameters in RNA sequencing dataset.

664 **Supplemental Table 2.** List of differentially expressed genes.

665 **Supplemental Table 3.** List of the differentially expressed AS events.

666 **Supplemental Table 4.** Summary of quantified proteins in proteomic analysis.

667 **Supplemental Table 5.** Primers used in this study.

668

669 **ACKNOWLEDGMENTS**

670 This work was supported by the Natural Science Foundation of Shandong Province
671 (BS2015NY002), Funds of Shandong “Double Top” Program, Natural Science
672 Foundation of China (NSFC31101099), the China Postdoctoral Science Foundation
673 (2017M622801), Science and Technology Program of Nantong (MS12016044), the
674 National Natural Science Foundation of China (NSFC31101099, 31771701,
675 31701341), Innovative Training Program of Nantong University College Students
676 2017 (201710304049Z), National Key Basic Research Program of China
677 (2012CB114300), the Natural Science Foundation of Guangdong Province
678 (2014A030313794), Shenzhen Overseas Talents Innovation and Entrepreneurship
679 Funding Scheme (The Peacock Scheme, KQTD201101) and Hong Kong Research
680 Grant Council (AoE/M-05/12, AoE/M-403/16, CUHK 14122415, 14160516,
681 14177617).

682

683 **AUTHOR CONTRIBUTIONS**

684 M.X.C., F.Y.Z., J.H.Z., Y.G.L. designed experiments. M.X.C., F.Y.Z., F.Z.W., N.H.Y.,
685 T.F., Y.Y.C., T.Y.L. S.S.Z performed experiments. M.X.C., F.Y.Z., B.G., K.L.M.,
686 G.Y.F., Z.Z.S., L.J.X., Q.J.H., H.J.W. analysed data. F.Y.Z., M.X.C., N.H.Y. wrote the
687 manuscript. S.X., J.H.Z., Y.G.L. critically commented and revised it.

688

689 **COMPETING FINANCIAL INTERESTS**

690 The authors declare no competing financial interests.

691

692 **REFERENCES**

693 **Alfaro JA, Sinha A, Kislinger T, Boutros PC** (2014) Onco-proteogenomics: cancer proteomics joins
694 forces with genomics. *Nat Methods* **11**: 1107-1113

695 **Andreassi C, Riccio A** (2009) To localize or not to localize: mRNA fate is in 3' UTR ends. *Trends Cell*
696 *Biol* **19**: 465-474

697 **Andrews GL, Simons BL, Young JB, Hawkridge AM, Muddiman DC** (2011) Performance
698 characteristics of a new hybrid quadrupole time-of-flight tandem mass spectrometer
699 (TripleTOF 5600). *Anal Chem* **83**: 5442-5446

700 **Atwell BJ, Greenway H, Colmer TD** (2015) Efficient use of energy in anoxia-tolerant plants with
701 focus on germinating rice seedlings. *New Phytol* **206**: 36-56

702 **Bai Y, Wang S, Zhong H, Yang Q, Zhang F, Zhuang Z, Yuan J, Nie X, Wang S** (2015) Integrative
703 analyses reveal transcriptome-proteome correlation in biological pathways and secondary
704 metabolism clusters in *A. flavus* in response to temperature. *Sci Rep* **5**: 14582

705 **Berta M, Ismail AM** (2013) Tolerance of anaerobic conditions caused by flooding during germination
706 and early growth in rice (*Oryza sativa*L.). *Front Plant Sci* **4**: 269

707 **Brar GA, Weissman JS** (2015) Ribosome profiling reveals the what, when, where and how of protein
708 synthesis. *Nat Rev Mol Cell Biol* **16**: 651-664.

709 **Buljan M, Chalancon G, Eustermann S, Wagner GP, Fuxreiter M, Bateman A, Babu MM** (2012)
710 Tissue-specific splicing of disordered segments that embed binding motifs rewires protein
711 interaction networks. *Mol Cell* **46**: 871-883

712 **Castellana NE, Payne SH, Shen Z, Stanke M, Bafna V, Briggs SP** (2008) Discovery and revision of
713 Arabidopsis genes by proteogenomics. *Proc Natl Acad Sci U S A* **105**: 21034-21038

714 **Castellana NE, Shen Z, He Y, Walley JW, Cassidy CJ, Briggs SP, Bafna V** (2014) An automated
715 proteogenomic method uses mass spectrometry to reveal novel genes in *Zea mays*. *Mol Cell*
716 *Proteomics* **13**: 157-167

717 **Chang CY, Lin WD, Tu SL** (2014) Genome-Wide Analysis of Heat-Sensitive Alternative Splicing in
718 *Physcomitrella patens*. *Plant Physiol* **165**: 826-840

719 **Chang R, Jang CJ, Branco-Price C, Nghiem P, Bailey-Serres J** (2012) Transient MPK6 activation in
720 response to oxygen deprivation and reoxygenation is mediated by mitochondria and aids
721 seedling survival in Arabidopsis. *Plant Mol Biol* **78**: 109-122

722 **Chen L, Liao B, Qi H, Xie LJ, Huang L, Tan WJ, Zhai N, Yuan LB, Zhou Y, Yu LJ** (2015)
723 Autophagy contributes to regulation of the hypoxia response during submergence in
724 Arabidopsis thaliana. *Autophagy* **11**: 2233-2246

725 **Chen X, Chan WL, Zhu FY, Lo C** (2014) Phosphoproteomic analysis of the non-seed vascular plant

726 model *Selaginella moellendorffii*. *Proteome Sci* **12**: 16

727 **Crooks GE, Hon G, Chandonia JM, Brenner SE** (2004) WebLogo: a sequence logo generator.

728 *Genome Res* **14**: 1188-1190

729 **de Klerk E, t Hoen PA** (2015) Alternative mRNA transcription, processing, and translation: insights

730 from RNA sequencing. *Trends Genet* **31**: 128-139

731 **De LL, Sorenson R, Baileyserres J, Hunt AG** (2017) Noncanonical Alternative Polyadenylation

732 Contributes to Gene Regulation in Response to Hypoxia. *Plant Cell*: **29**: 1262-1277

733 **Drechsel G, Kahles A, Kesarwani AK, Stauffer E, Behr J, Drewe P, Ratsch G, Wachter A** (2013)

734 Nonsense-mediated decay of alternative precursor mRNA splicing variants is a major

735 determinant of the Arabidopsis steady state transcriptome. *Plant Cell* **25**: 3726-3742

736 **Eckardt NA** (2013) The plant cell reviews alternative splicing. *Plant Cell* **25**: 3639

737 **Ellis JD, Barrios-Rodiles M, Colak R, Irimia M, Kim T, Calarco JA, Wang X, Pan Q, O'Hanlon**

738 **D, Kim PM, et al.** (2012) Tissue-specific alternative splicing remodels protein-protein

739 interaction networks. *Mol Cell* **46**: 884-892

740 **Feng J, Li J, Gao Z, Lu Y, Yu J, Zheng Q, Yan S, Zhang W, He H, Ma L, et al.** (2015) SKIP

741 Confers Osmotic Tolerance during Salt Stress by Controlling Alternative Gene Splicing in

742 Arabidopsis. *Mol Plant* **8**: 1038-1052

743 **Fesenko I, Khazigaleeva R, Kirov I, Kniazev A, Glushenko O, Babalyan K, Arapidi G, Shashkova**

744 **T, Butenko I, Zgoda V** (2017) Alternative splicing shapes transcriptome but not proteome

745 diversity in *Physcomitrella patens*. *Sci Rep* **7**: 2698

746 **Florea L, Song L, Salzberg SL** (2013) Thousands of exon skipping events differentiate among

747 splicing patterns in sixteen human tissues. *F1000Research* **2**: 188

748 **Fukao T, Harris T, Baileyserres J** (2009) Evolutionary analysis of the Sub1 gene cluster that confers

749 submergence tolerance to domesticated rice. *Ann Bot* **103**: 143-150

750 **Gibbs DJ, Lee SC, Isa NM, Gramuglia S, Fukao T, Bassel GW, Correia CS, Corbineau F,**

751 **Theodoulou FL, Bailey-Serres J** (2011) Homeostatic response to hypoxia is regulated by the

752 N-end rule pathway in plants. *Nature* **479**: 415-418

753 **Gibbs DJ, Lee SC, Isa NM, Gramuglia S, Fukao T, Bassel GW, Correia CS, Corbineau F,**

754 **Theodoulou FL, Baileyserres J** (2011) Homeostatic response to hypoxia is regulated by the

755 N-end rule pathway in plants. *Nature* **479**: 415-418

756 **Giuntoli B, Licausi F, Van VH, Perata P** (2017) Functional Balancing of the Hypoxia Regulators

757 RAP2.12 and HRA1 Takes Place in vivo in Arabidopsis thaliana Plants. *Front Plant Sci* **8**: 591

758 **Golovkin M, Reddy AS** (1996) Structure and expression of a plant U1 snRNP 70K gene: alternative

759 splicing of U1 snRNP 70K pre-mRNAs produces two different transcripts. *Plant Cell* **8**:

760 1421-1435

761 **Hattori Y, Nagai K, Furukawa S, Song XJ, Kawano R, Sakakibara H, Wu J, Matsumoto T,**

762 **Yoshimura A, Kitano H** (2009) The ethylene response factors SNORKEL1 and SNORKEL2

763 allow rice to adapt to deep water. *Nature* **460**: 1026-1030

764 **He D, Zhang H, Yang P** (2014) The Mitochondrion-Located Protein OsB12D1 Enhances Flooding
765 Tolerance during Seed Germination and Early Seedling Growth in Rice. *Int J Mol Sci* **15**:
766 13461-13481

767 **Hinz M, Wilson IW, Yang J, Buerstenbinder K, Llewellyn D, Dennis ES, Sauter M, Dolferus R**
768 (2010) Arabidopsis RAP2.2: an ethylene response transcription factor that is important for
769 hypoxia survival. *Plant Physiol* **153**: 757-772

770 **Hsu SK, Tung CW** (2017) RNA-Seq Analysis of Diverse Rice Genotypes to Identify the Genes
771 Controlling Coleoptile Growth during Submerged Germination. *Front Plant Sci* **8**: 762

772 **Huang LE, Gu J, Schau M, Bunn HF** (1998) Regulation of hypoxia-inducible factor 1 α is
773 mediated by an O₂-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc*
774 *Natl Acad Sci U S A* **95**: 7987-7992

775 **Ingolia NT, Lareau LF, Weissman JS** (2011) Ribosome profiling of mouse embryonic stem cells
776 reveals the complexity and dynamics of mammalian proteomes. *Cell* **147**: 789-802

777 **Jakubauskiene E, Vilys L, Makino Y, Poellinger L, Kanopka A** (2015) Increased Serine-Arginine
778 (SR) Protein Phosphorylation Changes Pre-mRNA Splicing in Hypoxia. *J Biol Chem* **290**:
779 18079-18089

780 **James AB, Syed NH, Bordage S, Marshall J, Nimmo GA, Jenkins GI, Herzyk P, Brown JW,**
781 **Nimmo HG** (2012) Alternative splicing mediates responses of the Arabidopsis circadian clock
782 to temperature changes. *Plant Cell* **24**: 961-981

783 **Jenal M, Elkon R, Loayza-Puch F, van Haften G, Kuhn U, Menzies FM, Oude Vrielink JA, Bos**
784 **AJ, Drost J, Rooijers K, et al.** (2012) The poly(A)-binding protein nuclear 1 suppresses
785 alternative cleavage and polyadenylation sites. *Cell* **149**: 538-553

786 **Jensen TH, Jacquier A, Libri D** (2013) Dealing with pervasive transcription. *Mol Cell* **52**: 473-484

787 **Kalyna M, Lopato S, Voronin V, Barta A** (2006) Evolutionary conservation and regulation of
788 particular alternative splicing events in plant SR proteins. *Nucleic Acids Res* **34**: 4395-4405

789 **Kang M, Abdelmageed H, Lee S, Reichert A, Mysore KS, Allen RD** (2013) AtMBP \square 1, an
790 alternative translation product of LOS2, affects abscisic acid responses and is modulated by
791 the E3 ubiquitin ligase AtSAP5. *Plant J* **76**: 481-493

792 **Kim MS, Pinto SM, Getnet D, Nirujogi RS, Manda SS, Chaerkady R, Madugundu AK, Kelkar**
793 **DS, Isserlin R, Jain S, et al.** (2014) A draft map of the human proteome. *Nature* **509**: 575-581

794 **Kondo Y, Oubridge C, van Roon AM, Nagai K** (2015) Crystal structure of human U1 snRNP, a small
795 nuclear ribonucleoprotein particle, reveals the mechanism of 5' splice site recognition. *Elife* **4**:
796 e04986

797 **Kretschmar T, Pelayo MAF, Trijatmiko KR, Gabunada LFM, Alam R, Jimenez R, Mendioro**
798 **MS, Slametloedin IH, Sreenivasulu N, Baileyserres J** (2015) A trehalose-6-phosphate
799 phosphatase enhances anaerobic germination tolerance in rice. *Nat Plants* **1**: 15124

800 **Krummel DAP, Oubridge C, Leung AK, Li J, Nagai K** (2009) Crystal structure of human
801 spliceosomal U1 snRNP at 5.5 Å resolution. *Nature* **458**: 475-480

802 **Kumar D, Yadav AK, Jia X, Mulvenna J, Dash D** (2016) Integrated transcriptomic-proteomic
803 analysis using a proteogenomic workflow refines rat genome annotation. *Mol Cell Proteomics*
804 **15**: 329-339

805 **Laing WA, Martínez-sánchez M, Wright MA, Bulley SM, Brewster D, Dare AP, Rassam M, Wang**
806 **D, Storey R, Macknight RC** (2015) An upstream open reading frame is essential for feedback
807 regulation of ascorbate biosynthesis in Arabidopsis. *Plant Cell* **27**: 772-786

808 **Lasanthi-Kudahettige R, Magneschi L, Loreti E, Gonzali S, Licausi F, Novi G, Beretta O, Vitulli F,**
809 **Alpi A, Perata P** (2007) Transcript profiling of the anoxic rice coleoptile. *Plant Physiol* **144**:
810 218-231

811 **Lee KW, Chen PW, Lu CA, Chen S, Ho TH, Yu SM** (2009) Coordinated responses to oxygen and
812 sugar deficiency allow rice seedlings to tolerate flooding. *Sci Signal* **2**: ra61

813 **Lee S, Liu B, Lee S, Huang S-X, Shen B, Qian S-B** (2012) Global mapping of translation initiation
814 sites in mammalian cells at single-nucleotide resolution. *Proc Natl Acad Sci U S A* **109**:
815 2424-2432

816 **Licausi F, Kosmacz M, Weits DA, Giuntoli B, Giorgi FM, Voosenek LA, Perata P, van Dongen JT**
817 (2011) Oxygen sensing in plants is mediated by an N-end rule pathway for protein
818 destabilization. *Nature* **479**: 419-422

819 **Licausi F, Van Dongen JT, Giuntoli B, Novi G, Santaniello A, Geigenberger P, Perata P** (2010)
820 HRE1 and HRE2, two hypoxia-inducible ethylene response factors, affect anaerobic responses
821 in Arabidopsis thaliana. *Plant J* **62**: 302-315

822 **Lu CA, Lin CC, Lee KW, Chen JL, Huang LF, Ho SL, Liu HJ, Hsing YI, Yu SM** (2007) The
823 SnRK1A protein kinase plays a key role in sugar signaling during germination and seedling
824 growth of rice. *Plant Cell* **19**: 2484-2499

825 **Marmiroli M, Imperiale D, Pagano L, Villani M, Zappettini A, Marmiroli N** (2015) The Proteomic
826 Response of Arabidopsis thaliana to Cadmium Sulfide Quantum Dots, and Its Correlation with
827 the Transcriptomic Response. *Front Plant Sci* **6**: 1104

828 **Narsai R, Edwards JM, Roberts TH, Whelan J, Joss GH, Atwell BJ** (2015) Mechanisms of growth
829 and patterns of gene expression in oxygen-deprived rice coleoptiles. *Plant J* **82**: 25-40

830 **Narsai R, Howell KA, Carroll A, Ivanova A, Millar AH, Whelan J** (2009) Defining core metabolic
831 and transcriptomic responses to oxygen availability in rice embryos and young seedlings.
832 *Plant Physiology* **151**: 306-322

833 **Narsai R, Rocha M, Geigenberger P, Whelan J, Dongen JTV** (2011) Comparative analysis between
834 plant species of transcriptional and metabolic responses to hypoxia. *New Phytol* **190**: 472-487

835 **Nicholson P, Yepiskoposyan H, Metze S, Zamudio Orozco R, Kleinschmidt N, Muhlemann O**
836 (2010) Nonsense-mediated mRNA decay in human cells: mechanistic insights, functions
837 beyond quality control and the double-life of NMD factors. *Cell Mol Life Sci* **67**: 677-700

838 **Nyikó T, Kerényi F, Szabadkai L, Benkovics AH, Major P, Sonkoly B, Mérai Z, Barta E, Niemiec**
839 **E, Kufel J** (2013) Plant nonsense-mediated mRNA decay is controlled by different

840 autoregulatory circuits and can be induced by an EJC-like complex. *Nucleic Acids Res* **41**:
841 6715-6728

842 **Peng Z, Deng H, Xiao FM, Liu YS** (2013) Alterations of Alternative Splicing Patterns of
843 Ser/Arg-Rich (SR) Genes in Response to Hormones and Stresses Treatments in Different
844 Ecotypes of Rice (*Oryza sativa*). *J Int Agri* **12**: 737-748

845 **Ruhl C, Stauffer E, Kahles A, Wagner G, Drechsel G, Ratsch G, Wachter A** (2012) Polypyrimidine
846 tract binding protein homologs from Arabidopsis are key regulators of alternative splicing
847 with implications in fundamental developmental processes. *Plant Cell* **24**: 4360-4375

848 **Sadiq I, Fanucchi F, Paparelli E, Alpi E, Bachi A, Alpi A, Perata P** (2011) Proteomic identification
849 of differentially expressed proteins in the anoxic rice coleoptile. *J Plant Physiol* **168**:
850 2234-2243

851 **Sasidharan R, Baileyserres J, Ashikari M, Atwell BJ, Colmer TD, Fagerstedt K, Fukao T,**
852 **Geigenberger P, Hebelstrup KH, Hill RD** (2017) Community recommendations on
853 terminology and procedures used in flooding and low oxygen stress research. *New Phytol* **214**:
854 1403-1407

855 **Schmidtkastrner R, Yamamoto H, Hamasaki D, Yamamoto H, Parel JM, Schmitz C, Dorey CK,**
856 **Blanks JC, Preising MN** (2008) Hypoxia-regulated components of the U4/U6.U5 tri-small
857 nuclear riboprotein complex: possible role in autosomal dominant retinitis pigmentosa. *Mol*
858 *Vision* **14**: 125-135

859 **Shaul O** (2015) Unique Aspects of Plant Nonsense-Mediated mRNA Decay. *Trends Plant Sci* **20**:
860 767-779

861 **Sheynkman GM, Shortreed MR, Frey BL, Smith LM** (2013) Discovery and mass spectrometric
862 analysis of novel splice-junction peptides using RNA-Seq. *Mol Cell Proteomics* **12**:
863 2341-2353

864 **Simon R, Dresselhaus T** (2015) Peptides take centre stage in plant signalling. *J Exp Bot* **66**:
865 5135-5138

866 **Sonenberg N, Hinnebusch AG** (2009) Regulation of translation initiation in eukaryotes: mechanisms
867 and biological targets. *Cell* **136**: 731-745

868 **Sonmez C, Baurle I, Magusin A, Dreos R, Laubinger S, Weigel D, Dean C** (2011) RNA 3'
869 processing functions of Arabidopsis FCA and FPA limit intergenic transcription. *Proc Natl*
870 *Acad Sci U S A* **108**: 8508-8513

871 **Tameshige T, Okamoto S, Lee JS, Aida M, Tasaka M, Torii K, Uchida N** (2016) A Secreted Peptide
872 and Its Receptors Shape the Auxin Response Pattern and Leaf Margin Morphogenesis. *Curr*
873 *Biol* **26**: 2478-2485

874 **Tavares R, Scherer NM, Ferreira CG, Costa FF, Passetti F** (2015) Splice variants in the proteome: a
875 promising and challenging field to targeted drug discovery. *Drug Discov Today* **20**: 353-360

876 **Tavormina P, De CB, Nikonorova N, De SI, Cammue BP** (2015) The Plant Peptidome: An
877 Expanding Repertoire of Structural Features and Biological Functions. *Plant Cell* **27**:

2095-2118

Thatcher SR, Danilevskaya ON, Meng X, Beatty M, Zastrow-Hayes G, Harris C, Van Allen B, Habben J, Li B (2016) Genome-Wide Analysis of Alternative Splicing during Development and Drought Stress in Maize. *Plant Physiol* **170**: 586-599

Van VH, Vashisht D, Akman M, Girke T, Mustroph A, Reinen E, Hartman S, Kooiker M, Van TP, Schranz ME (2016) Transcriptomes of Eight *Arabidopsis thaliana* Accessions Reveal Core Conserved, Genotype- and Organ-Specific Responses to Flooding Stress. *Plant Physiol* **172**: 668-689

Wade JT, Grainger DC (2014) Pervasive transcription: illuminating the dark matter of bacterial transcriptomes. *Nat Rev Microbiol* **12**: 647-653

Walley JW, Briggs SP (2015) Dual use of peptide mass spectra: Protein atlas and genome annotation. *Curr Plant Biol* **2**: 21-24

Wang Z, Ji H, Yuan B, Wang S, Su C, Yao B, Zhao H, Li X (2015) ABA signalling is fine-tuned by antagonistic HAB1 variants. *Nat Commun* **6**:8138

Weits DA, Giuntoli B, Kosmacz M, Parlanti S, Hubberten HM, Riegler H, Hoefgen R, Perata P, Dongen JTV, Licausi F (2014) Plant cysteine oxidases control the oxygen-dependent branch of the N-end-rule pathway. *Nat Commun* **5**: 3425

Will CL, Luhrmann R (2011) Spliceosome structure and function. *Cold Spring Harb Perspect Biol* **3**: a003707

Xie LJ, Chen QF, Chen MX, Yu LJ, Huang L, Chen L, Wang FZ, Xia FN, Zhu TR, Wu JX (2015) Unsaturation of very-long-chain ceramides protects plant from hypoxia-induced damages by modulating ethylene signaling in *Arabidopsis*. *PloS Genet* **11**: e1005143

Yan J, Marr TG (2005) Computational analysis of 3'-ends of ESTs shows four classes of alternative polyadenylation in human, mouse, and rat. *Genome Res* **15**: 369-375

Yang J, Zhang J (2006) Grain filling of cereals under soil drying. *The New phytologist* **169**: 223-236

Yang M, Xu L, Liu Y, Yang P (2015) RNA-Seq Uncovers SNPs and Alternative Splicing Events in Asian Lotus (*Nelumbo nucifera*). *PLoS One* **10**: e0125702

Yoshida H, Park SY, Oda T, Akiyoshi T, Sato M, Shirouzu M, Tsuda K, Kuwasako K, Unzai S, Muto Y, et al. (2015) A novel 3' splice site recognition by the two zinc fingers in the U2AF small subunit. *Genes Dev* **29**: 1649-1660

Yuan LB, Dai YS, Xie LJ, Yu LJ, Zhou Y, Lai YX, Yang YC, Xu L, Chen QF, Xiao S (2017) Jasmonate Regulates Plant Responses to Reoxygenation through Activation of Antioxidant Synthesis. *Plant Physiol* **173**: 1864-1880

Zdraviko J L, Reinhard L, Christina F, Andrea B (2005) Evolutionary conservation of minor U12-type spliceosome between plants and humans. *RNA* **11**: 1095-1107

Zhan X, Qian B, Cao F, Wu W, Yang L, Guan Q, Gu X, Wang P, Okusolubo TA, Dunn SL (2015) An *Arabidopsis* PWI and RRM motif-containing protein is critical for pre-mRNA splicing and ABA responses. *Nature Commun* **6**:8139

- 916 **Zhou MT, Qin Y, Li M, Chen C, Chen X, Shu HB, Guo L** (2015) Quantitative Proteomics Reveals
917 the Roles of Peroxisome-associated Proteins in Antiviral Innate Immune Responses. *Mol Cell*
918 *Proteomics* **14**: 2535-2549
- 919 **Zhu FY, Chan WL, Chen MX, Kong RP, Cai C, Wang Q, Zhang JH, Lo C** (2016) SWATH-MS
920 Quantitative Proteomic Investigation Reveals a Role of Jasmonic Acid during Lead Response
921 in Arabidopsis. *J Proteome Res* **15**: 3528-3539
- 922 **Zhu FY, Chen MX, Su YW, Xu X, Ye NH, Cao YY, Lin S, Liu TY, Li HX, Wang GQ** (2016)
923 SWATH-MS Quantitative Analysis of Proteins in the Rice Inferior and Superior Spikelets
924 during Grain Filling. *Front Plant Sci* **7**: 1926
- 925 **Zhu FY, Chen MX, Ye NH, Shi L, Ma KL, Yang JF, Cao YY, Zhang Y, Yoshida T, Fernie AR**
926 (2017) Proteogenomic analysis reveals alternative splicing and translation as part of the
927 abscisic acid response in Arabidopsis seedlings. *Plant J* **91**: 518-533.
- 928 **Zhu FY, Li L, Lam PY, Chen MX, Chye ML, Lo C** (2013) Sorghum extracellular leucine-rich repeat
929 protein SbLRR2 mediates lead tolerance in transgenic Arabidopsis. *Plant Cell Physiol* **54**:
930 1549-1559

933 **Figure Legends**

934 **Figure 1 Identification and comparison between the datasets of differentially**
935 **expressed genes (DEG) and differentially expressed alternative splicing (DAS)**
936 **events during rice hypoxic germination.** (A) Statistics of the identified alternative
937 splicing (AS) events and types. ALE, alternative last exon; AFE, alternative first exon;
938 SKIP, exon skipping; IR, intron retention; AE5', alternative donor; AE3', alternative
939 acceptor. (B) The Venn diagram represents unique and shared genes between DEG
940 and DAS datasets. (C) Gene ontology enrichment analysis between DEG and DAS
941 datasets. (D) RT-PCR validation of the DAS events in mRNA surveillance pathway.
942 Air: air control; Hyp: Hypoxia; ND: not detected. Gene models of each isoform are
943 indicated (blue: coding region; white: non-coding UTRs; not to scale).

944

945 **Figure 2 qRT-PCR validations of DAS events.** Validation of DAS events detected in
946 KEGG enrichment analysis. DAS events involved in categories of (A) mRNA
947 surveillance pathway and lysosome, (B) lysosome, (C) protein export, (D) proteasome
948 was verified by qRT-PCR analysis from three biological replicates. *OsACTIN1* was
949 used as an internal reference gene. Mean values \pm SD are presented (n=3). ‘***’ and ‘**’
950 represent mean values of hypoxia-treated (Hyp) group is significantly higher or lower
951 than that in air control (Air), $P < 0.01$ and $P < 0.05$, respectively. Gene models of each

952 isoform are indicated (blue: coding region; white: non-coding UTRs; not to scale).

953

954 **Figure 3 Qualitative proteomic identification of AS peptides.** (A) Venn diagram
955 representation of qualitative proteomic identification using UniProt, AS and DAS
956 databases. (B) AS peptides identification and classification (upper panel). ALE,
957 alternative last exon; AFE, alternative first exon; SKIP, exon skipping; IR, intron
958 retention; AE5', alternative donor; AE3', alternative acceptor. Summary of identified
959 AS/DAS events and peptides (lower panel). (C) KEGG pathway enrichment analysis
960 of DAS peptides in qualitative proteomics. '*' marked pathway is repeatedly found in
961 both transcriptome and qualitative proteomic datasets.

962

963 **Figure 4 Comparison between proteomic and transcriptomic datasets.** Venn
964 diagram representation of (A) differentially expressed genes (DEG) vs. differentially
965 expressed proteins (DEP), (B) differentially expressed AS events (DAS) vs.
966 differentially expressed AS peptides (DASDP), (C) DEP vs. DASDP. Heatmap
967 representation (D) and correlation analysis (E) of overlapping genes between DEG
968 and DEP. (F) Heatmap representation of overlapping genes between DAS and DASDP.
969 ALE, alternative last exon; '*' represents the regulation of transcripts and proteins at
970 the same trend in corresponding datasets; H: L, hypoxia vs. air control.

971

972 **Figure 5 Novel protein/peptide identification.** (A) Summary of the spectrum usage
973 of the data from qualitative proteomics using UniProt, AS and DAS databases. (B)
974 Spectrum usage of the data from qualitative proteomic using the 6-frame translated
975 protein database. (C) Pie diagram represents percentage distribution of identified
976 novel proteins/peptides. (D) Venn diagram representation of identified novel
977 proteins/peptides in control and hypoxia-treated samples. (E) Venn diagram represents
978 the shared and unique genes among differentially expressed genes (DEG),
979 differentially expressed proteins (DEP) and differentially expressed frame proteins
980 (DE FP).

981

982 **Figure 6 Splicing sites recognition under hypoxia stress.** (A) Statistical analysis of
983 the splicing sites (ss) between the total AS events and the hypoxia-affected DAS
984 events. (B) Conservation analysis using sequence located at exon-intron junctions.

985

986 **Figure 7 Splicing factors involved in hypoxia responses during rice germination.**
987 (A) The Venn diagram represents identified splicing factors between DEG and DAS

988 datasets. (B) Statistics of hypoxia-affected DAS genes and events of splicing factors.
989 (C) Pie chart distribution of the DAS events belonging to splicing factors. (D)
990 Subgroup classification of splicing factors identified in DAS events. (E) qRT-PCR
991 validation of DAS events detected in spliceosome. DAS events in the category of
992 spliceosome were verified by qRT-PCR analysis from three biological replicates.

993 *OsACTIN1* was used as an internal reference gene. Mean values \pm SD are presented
994 (n=3). ‘***’ and ‘*’ represent the mean values of hypoxia-treated (Hyp) group is
995 significantly higher or lower than that in air control (Air), $P<0.01$ and $P<0.05$,
996 respectively. Gene models of each isoform are indicated (blue: coding region; white:
997 non-coding UTRs; not to scale).

998

999 **Figure 8 Model of alternative splicing and alternative translation initiation**
1000 **involved in the hypoxic germination pathway.**

1001 Summary model of the rice genome using its coding ability to produce diverse
1002 functional proteins during hypoxic germination. The traditional transcriptional
1003 pathway (blue) has been well studied. The parallel pathway of alternative splicing (AS,
1004 orange) is able to generate AS isoforms, which in turn can be translated into protein
1005 isoforms in response to hypoxia treatment. In the third pathway of alternative
1006 translation initiation (ATI, violet), upstream open reading frames (uORFs) and small
1007 ORFs (sORFs) can further expand the protein diversity under hypoxia treatment.

1008

1009 **Figure S1 Analytical pipeline of AS identification, quantification and validation**
1010 **in this study.**

1011

1012 **Figure S2 Comparison of previous published datasets and qRT PCR validation.**

1013 (A) Heatmap comparison of previous published microarray datasets (Pub) to our RNA
1014 seq analysis (Our). (B) qRT-PCR validations of the selected and marker genes during
1015 hypoxic germination from three biological replicates. *OsACTIN1* was used as an
1016 internal reference gene. ‘*’ and ‘***’ denote that the relative mRNA level is
1017 significantly higher in hypoxia-treated samples (grey bars) in comparison to air
1018 control (black bars) in complete darkness, $P<0.05$ and $P<0.01$, respectively. **Locus**
1019 **IDs (Bolded)** represent genes have similar expression pattern in qRT-PCR analysis in
1020 comparison to previous transcriptome or microarray analysis.

1021

1022 **Figure S3 GO enrichment analysis between DAS and DEG datasets from RNA**

1023 **sequencing.**

1024

1025 **Figure S4 qRT-PCR validation of selected genes from DAS events.** Primers used in
1026 the experiment are listed in Supplemental Table 5. *OsACTIN1* was used as an internal
1027 reference gene. ‘*’ and ‘**’ denote that the relative mRNA level is significantly higher
1028 or lower in hypoxia-treated samples in comparison to air control, $P<0.05$ and $P<0.01$,
1029 respectively. AS events in bold form represent the consistency between RNA seq and
1030 qRT-PCR data.

1031

1032 **Figure S5 Comparison of splicing sites (ss) recognition between AS and DAS**
1033 **events.** ALE, alternative last exon; AFE, alternative first exon; SKIP, exon skipping;
1034 IR, intron retention; AE5’, alternative donor; AE3’, alternative acceptor.

1035

1036

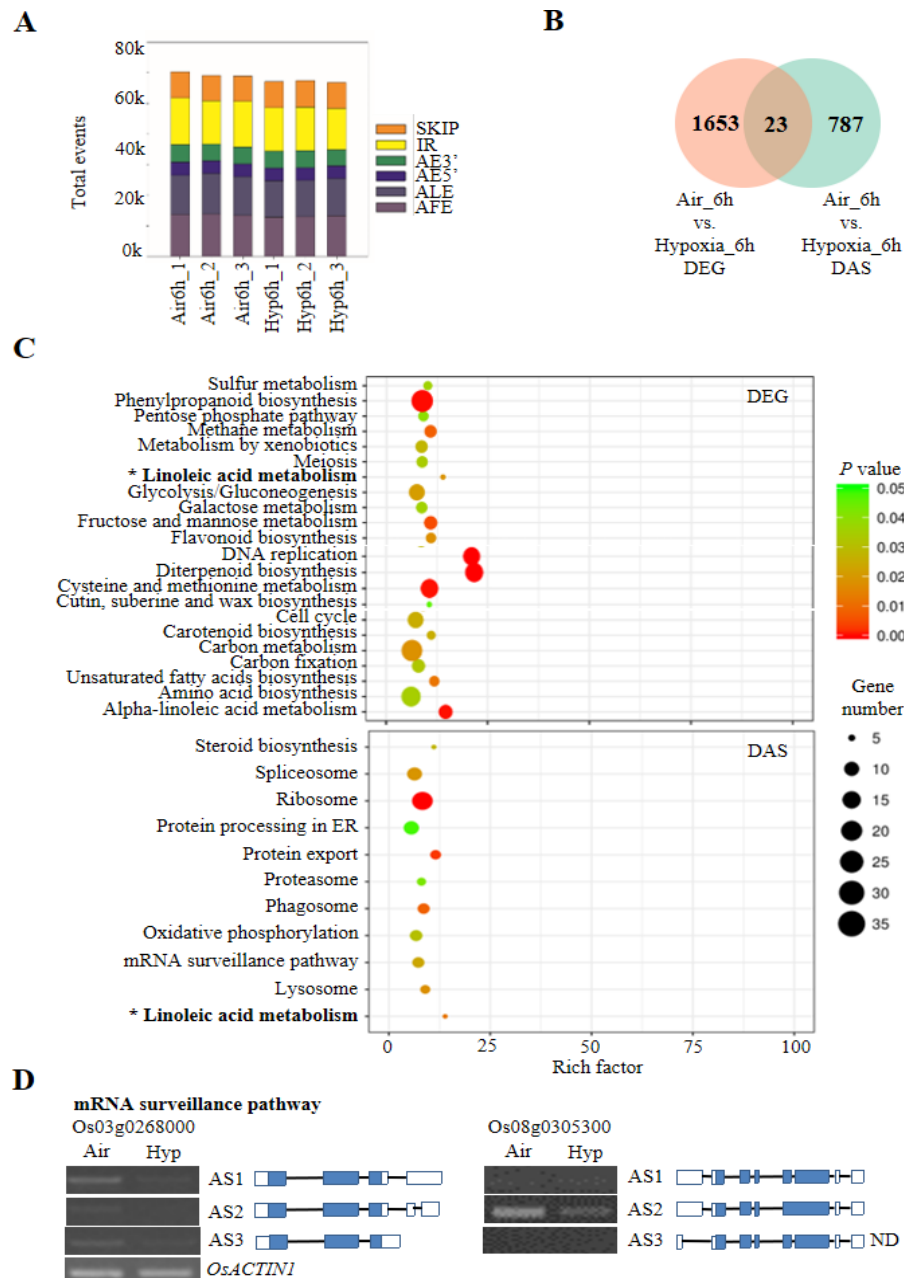


Figure 1 Identification and comparison between the datasets of differentially expressed genes (DEG) and differentially expressed alternative splicing (DAS) events during rice hypoxic germination. (A) Statistics of the identified alternative splicing (AS) events and types. ALE, alternative last exon; AFE, alternative first exon; SKIP, exon skipping; IR, intron retention; AE5', alternative donor; AE3', alternative acceptor. (B) The Venn diagram represents unique and shared genes between DEG and DAS datasets. (C) Gene ontology enrichment analysis between DEG and DAS datasets. (D) RT-PCR validation of the DAS events in mRNA surveillance pathway. Air: air control; Hyp: Hypoxia; ND: not detected. Gene models of each isoform are indicated (blue: coding region; white: non-coding UTRs; not to scale).

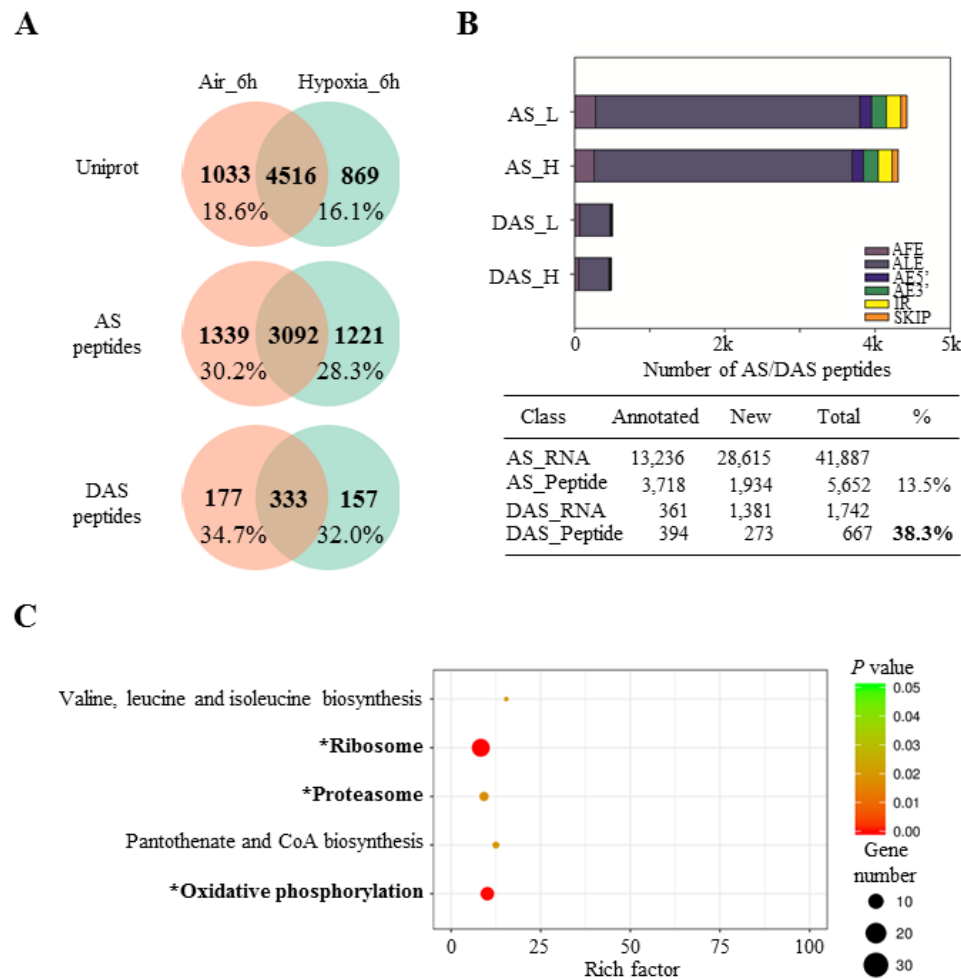


Figure 3 Qualitative proteomic identification of AS peptides. (A) Venn diagram representation of qualitative proteomic identification using UniProt, AS and DAS databases. (B) AS peptides identification and classification (upper panel). ALE, alternative last exon; AFE, alternative first exon; SKIP, exon skipping; IR, intron retention; AE5', alternative donor; AE3', alternative acceptor. Summary of identified AS/DAS events and peptides (lower panel). (C) KEGG pathway enrichment analysis of DAS peptides in qualitative proteomics. "*" marked pathway is repeatedly found in both transcriptome and qualitative proteomic datasets.

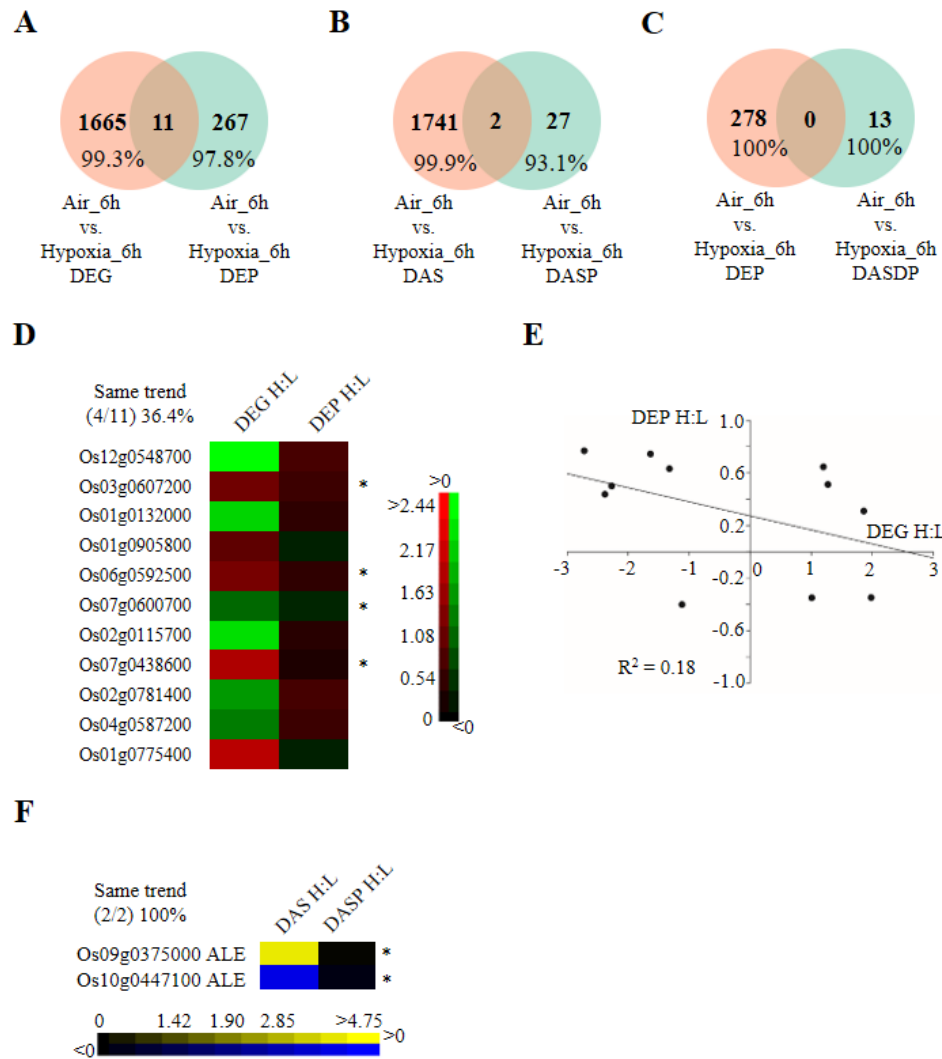


Figure 4 Comparison between proteomic and transcriptomic datasets. Venn diagram representation of (A) differentially expressed genes (DEG) vs. differentially expressed proteins (DEP), (B) differentially expressed AS events (DAS) vs. differentially expressed AS peptides (DASDP), (C) DEP vs. DASDP. Heatmap representation (D) and correlation analysis (E) of overlapping genes between DEG and DEP. (F) Heatmap representation of overlapping genes between DAS and DASDP. ALE, alternative last exon; '*' represents the regulation of transcripts and proteins at the same trend in corresponding datasets; H: L, hypoxia vs. air control.

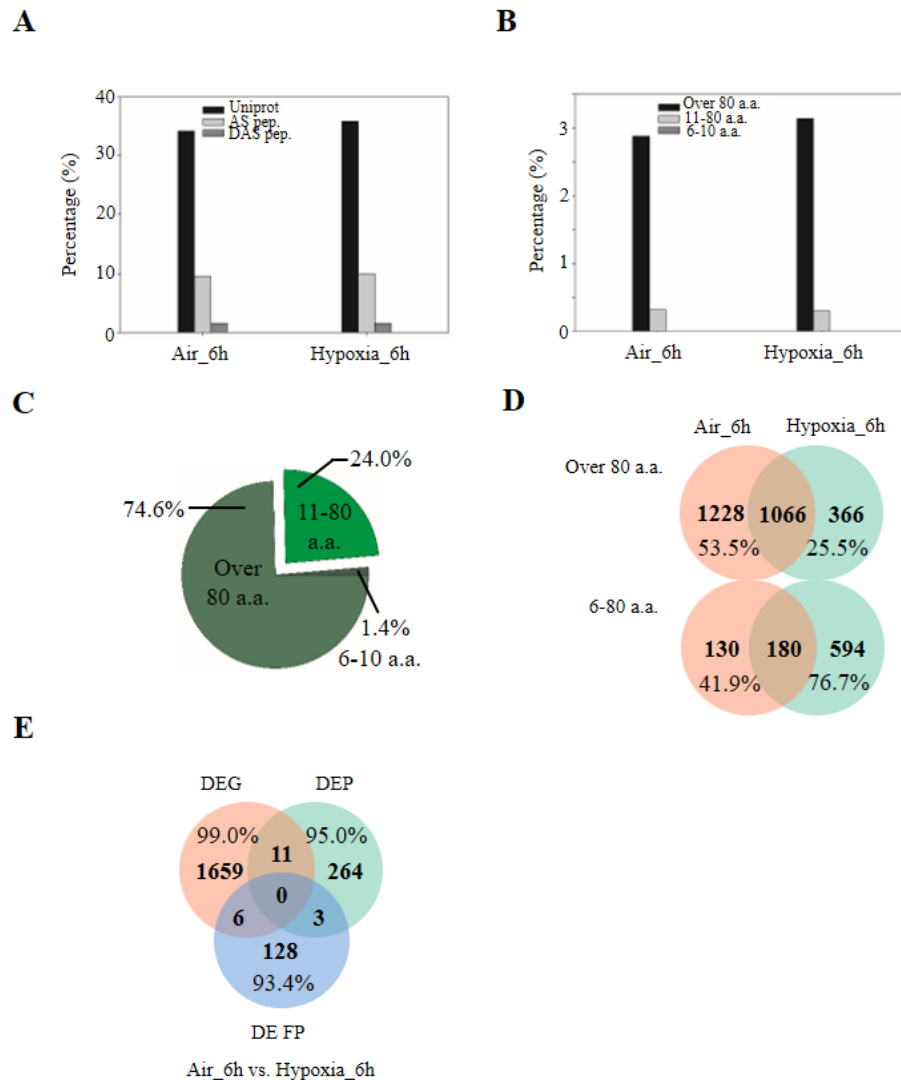


Figure 5 Novel protein/peptide identification. (A) Summary of the spectrum usage of the data from qualitative proteomics using UniProt, AS and DAS databases. (B) Spectrum usage of the data from qualitative proteomic using the 6-frame translated protein database. (C) Pie diagram represents percentage distribution of identified novel proteins/peptides. (D) Venn diagram representation of identified novel proteins/peptides in control and hypoxia-treated samples. (E) Venn diagram represents the shared and unique genes among differentially expressed genes (DEG), differentially expressed proteins (DEP) and differentially expressed frame proteins (DE FP).

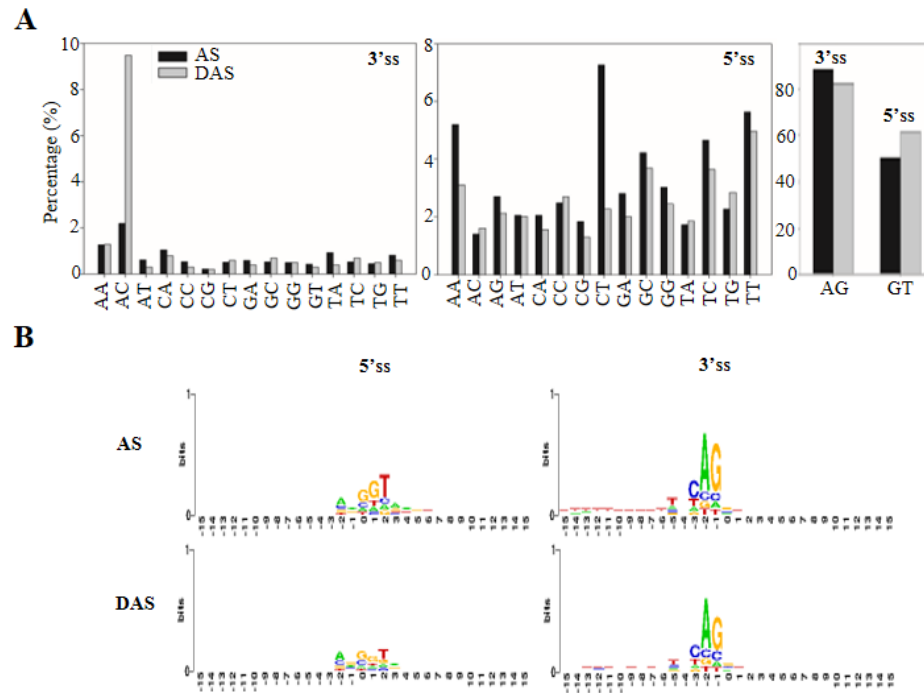


Figure 6 Splicing sites recognition under hypoxia stress. (A) Statistical analysis of the splicing sites (ss) between the total AS events and the hypoxia-affected DAS events. (B) Conservation analysis using sequence located at exon-intron junctions.

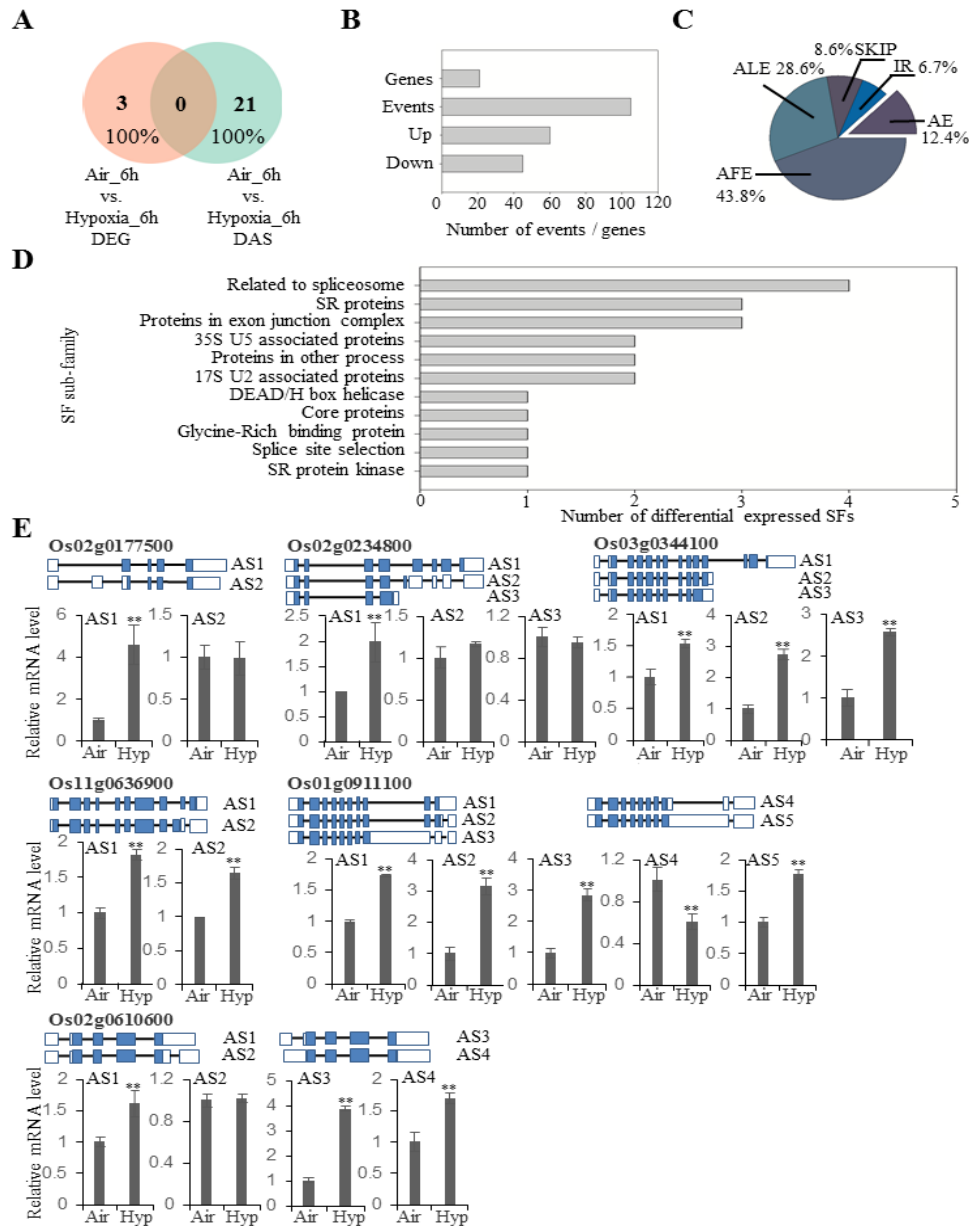


Figure 7 Splicing factors involved in hypoxia responses during rice germination.

(A) The Venn diagram represents identified splicing factors between DEG and DAS datasets. (B) Statistics of hypoxia-affected DAS genes and events of splicing factors. (C) Pie chart distribution of the DAS events belonging to splicing factors. (D) Subgroup classification of splicing factors identified in DAS events. (E) qRT-PCR validation of DAS events detected in spliceosome. DAS events in the category of spliceosome were verified by qRT-PCR analysis from three biological replicates.

OsACTIN1 was used as an internal reference gene. Mean values \pm SD are presented ($n=3$). ‘***’ and ‘*’ represent the mean values of hypoxia-treated (Hyp) group is significantly higher or lower than that in air control (Air), $P<0.01$ and $P<0.05$, respectively. Gene models of each isoform are indicated (blue: coding region; white:

non-coding UTRs; not to scale).

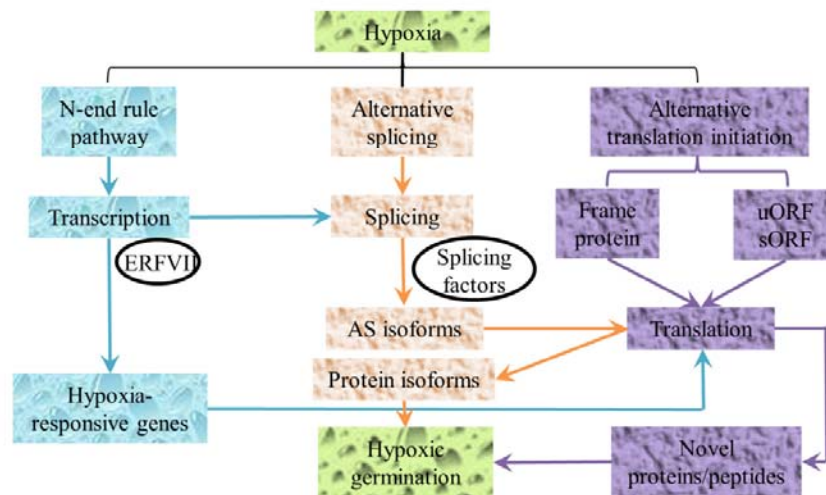


Figure 8 Model of alternative splicing and alternative translation initiation involved in the hypoxic germination pathway.

Summary model of the rice genome using its coding ability to produce diverse functional proteins during hypoxic germination. The traditional transcriptional pathway (blue) has been well studied. The parallel pathway of alternative splicing (AS, orange) is able to generate AS isoforms, which in turn can be translated into protein isoforms in response to hypoxia treatment. In the third pathway of alternative translation initiation (ATI, violet), upstream open reading frames (uORFs) and small ORFs (sORFs) can further expand the protein diversity under hypoxia treatment.