1 **Integration of embryo-endosperm interaction into a holistic and dynamic picture of seed**
2 **development using a rice mutant with notched-belly grains**

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14 **ABSTRACT**

15 The interaction between the embryo and endosperm affects seed development, an essential
 16 process in yield formation in crops such as rice. Signals that communicate between embryo
 17 and endosperm are largely unknown. Here we use the notched-belly (NB) mutant with
 18 impaired communication between embryo and endosperm to evaluate 1) the impact of embryo
 19 on developmental staging of the endosperm; 2) signaling pathways emanating from the
 20 embryo that regulate endosperm development. Hierarchical clustering of mRNA datasets from
 21 embryo and endosperm samples collected through development in NB and wild type showed
 22 a delaying effect of the embryo on the developmental transition of the endosperm by
 23 extending the middle stage. K-means clustering further identified coexpression modules of
 24 gene sets specific for embryo and endosperm development. Combined gene expression and
 25 biochemical analysis showed that T6P-SnRK1, gibberellin and auxin signalling from the
 26 embryo regulate endosperm developmental transition. The data enable us to propose a new
 27 seed developmental staging system for rice and the most detailed signature of rice grain
 28 formation to date, that will direct genetic strategies for rice yield improvement.

29

30 **Keywords:** Seed development; embryo-endosperm interaction; developmental transition;
 31 transcriptome; sugar signaling; rice physiology

32

33 INTRODUCTION

34 Rice is a main contributor of dietary calories and nutrients for nearly half of the global
35 population (Wu et al., 2020). Rice grain yield has to be sustainably improved both in quantity
36 and quality, to solve formidable challenges including the ever-increasing population, climate
37 change, and the quest for high-quality rice that has accompanied the rise in living standards
38 (Sreenivasulu et al., 2015). A detailed understanding of the molecular and physiological
39 mechanisms underpinning seed development may enable the design of effective strategies to
40 boost rice quality and yield.

41 The seed (grain) of rice (*Oryza sativa*) is a complex but delicate biological system, containing
42 three genetically distinct tissues, the diploid embryo, the triploid endosperm, and the diploid
43 maternal tissues (An et al., 2020). Concomitant development of embryo and endosperm under
44 the constraint of maternal tissues requires coordination of the two compartments
45 (Lafon-Placette and Köhler, 2014; Doll et al., 2020). Growing evidence in rice as well as
46 *Arabidopsis* (*Arabidopsis thaliana*) and maize (*Zea mays*) support bidirectional
47 communication between embryo and endosperm. In brief, the endosperm plays crucial role in
48 nurturing the developing embryo, whereas the embryo exerts a “counter-acting regulation
49 force” on endosperm, as reviewed by An et al. (2020) and Ingram (2020).

50 Transcriptome analyses of seed development have already identified most genes expressed in
51 its different compartments (Olsen, 2020). These comprehensive studies provided insights into
52 the molecular networks and pathway interactions that function during the development of
53 individual seed compartments for *Arabidopsis* (Belmonte et al., 2013), maize (Chen et al.,
54 2014; Yi et al., 2019), wheat (*Triticum aestivum*; Xiang et al., 2019), and barley (*Hordeum*
55 *vulgare*; Bian et al., 2019). In rice, laser-capture microdissection (LCM) has been employed
56 to unravel the molecular mechanisms underpinning grain development and its response to
57 abiotic stress (Ishimaru et al., 2019; Ram et al., 2020; Wu et al., 2020). Regardless of a large
58 number of comprehensive studies on the transcriptome atlas for the developing seed with high
59 spatio-temporal resolutions, detailed information about the communications between embryo
60 and endosperm is still lacking, which is partly attributed to the complexity of seed structures
61 (Doll et al., 2020).

62 Notched-belly grain is a misshapen type of rice grain, having a notched-line on its ventral side
63 and thus being inferior in appearance and milling quality (Tong et al., 2018). Previously, we
64 identified a notched-belly mutant (NB) of a japonica rice Wuyujing 3 using a chemical
65 mutagen (Lin et al., 2014). It has high occurrence of white-belly grains, and the notched line
66 is visible at 5 DAF, which separates the endosperm into two sub-compartments, the

translucent upper part and the chalky bottom (Fig. 1). Since the formation of chalky tissue is a result of incomplete accumulation of starch and protein, it is tempting to speculate that the embryo plays a role in it, probably by depriving the nutrients from its proximate bottom endosperm (An et al., 2020). As revealed by our previous studies, the embryo demonstrated a negative effect on the contents of proteins, amino acids, and minerals in the chalky endosperm (Lin et al., 2016; Lin et al., 2017). Therefore, the NB mutant enables an integrated understanding of the cellular processes for rice grain formation from perspective of embryo-endosperm interaction.

In this study, we used the NB mutant to characterize the genome-wide gene expression profile of embryo and endosperm samples between 0-60 DAF. In combination with physiological and histological investigations, we aimed to: (i) manifest the effect of embryo on endosperm development; and (ii) frame a holistic and dynamic picture of seed development by integrating the information of embryo-endosperm crosstalk. Comprehensive comparison of mRNAs datasets revealed a dragging effect of embryo on the developmental transition of the endosperm, which may be mediated by hormonal (GA and IAA) and T6P-SnRK1 signaling pathways. A new staging system is subsequently proposed by integrating the information about embryo-endosperm bidirectional communications. The findings obtained should offer insights into the biological processes and signatures of grain formation, hence providing a valuable resource for the manipulation of molecular and physiological processes responsible for cereal grain yield and quality.

MATERIALS AND METHODS

Plant materials and sampling

The notched-belly mutant (NB) was obtained by EMS treatment of a japonica rice cultivar Wuyujing3 (WT). It possesses a high ratio of notched-belly grains with a white-belly mainly on the bottom endosperm (Lin et al., 2014). In 2018, six seedlings of WT and NB were transplanted into a plastic pot filled with 10 kg paddy soil. The plants were grown under natural conditions, and at two days before flowering were transferred to a 31 °/24 ° growth chamber (12h/12h day and night cycle), with light intensity of 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and relative humidity $70 \pm 5\%$. Flowering dates were carefully marked for the sampled caryopsis on the middle primary rachis. Grains were sampled at eight times, i.e. 5, 10, 15, 20, 25, 30, 45 and 60 DAF, with three biological replicates. Samples were quickly frozen in liquid nitrogen and then stored at -80°C until analysis. The developing grains were manually dissected into three subsamples, the embryo, and the upper and bottom part of the endosperm (Fig. 1). It is noteworthy that the endosperm samples contain the maternal tissues such as pericarp and seed

101 coat.

102 **Gene expression profiling using RNA-Seq**

103 About 0.1 g sample was used for total RNA extraction by TRIzol reagent (Invitrogen, USA).
 104 The extracted RNA was dissolved in 100 µl of RNase-free water, and then quantified using a
 105 NanoDrop spectrophotometer (Thermo Scientific, USA). RNA quality was evaluated using
 106 the 6000 Pico LabChip of the Agilent 2100 Bioanalyzer (Agilent, USA). The qualification and
 107 quantification of the sample library were performed using an Agilent 2100 Bioanalyzer and
 108 StepOnePlus Real-Time PCR System (Applied Biosystem, USA). The library products were
 109 sequenced with BGISEQ-500 (BGI, China). Nipponbare reference genome (IRGSP-1.0;
 110 <https://www.ncbi.nlm.nih.gov>) was used to process the RNA-Seq libraries into read mapping
 111 and analysis.

112 To ensure good quality and effective mapping, the low-quality reads were removed using the
 113 SOAPnuke (V1.4.0) and trimmomatic (V0.36) software (Chen et al., 2018b), and only clean
 114 reads were processed for mapping using HISAT2 (V2.1.0) (Kim et al., 2015). The gene
 115 expression level was measured by RSEM (V1.2.8) software (Li and Dewey, 2011), and
 116 fragments per kilobase of transcript per million mapped reads (FPKM) values were used for
 117 quantifying gene activity. We only considered a gene as expressed if its FPKM value ≥ 1 to
 118 inhibit the influence of transcriptomic noise. To confirm the accuracy and authenticity of the
 119 three biological repeats, we calculated the Pearson correlation coefficient among them with
 120 the normalized expression level of $\log_2$ (FPKM value +1) (Supplementary Fig. S1). To
 121 identify differentially expressed genes (DEGs), we considered a DEG when it exhibits an
 122 absolute value of $\log_2$ ratio ≥ 1 compared with an FDR corrected P-value of ≤ 0.001 (Wang et
 123 al., 2010).

124 **Validation of transcriptome data using qRT-PCR**

125 RNA-Seq data quality was validated by measuring the relative expression pattern of 12 genes
 126 functioning in C and N metabolism using qRT-PCR (Supplementary Fig. S2). Briefly,
 127 target-specific qRT-PCR primers were designed using the Primer5.0 software and synthesized,
 128 as presented in Supplementary Table S1. Each sample was represented by three biological and
 129 three technical replicates. The comparison of qRT-PCR results with RNA-Seq revealed the
 130 similar expression pattern between two datasets, thus validating the transcriptomic data
 131 (Supplementary Fig. S2). In addition, transcript abundance pattern of embryo (*OSHI* and
 132 *OsLECI*) and endosperm (*GluD-1* and *RPBF*) specific genes also confirmed the credibility of
 133 the gene expression data (Supplementary Fig. S3), indicating that the embryo and endosperm
 134 samples were processed well.

135 **PCA and hierarchical clustering**

136 To facilitate graphical interpretation of relatedness among the 48 tissue samples, we reduced
137 the dimensional expression data to two dimensions by PCA through Omicshare tools
138 (<https://www.omicshare.com/tools/Home/Soft/pca>). Hierarchical clustering (HCL) was
139 carried out through Omicshare tools (<https://www.omicshare.com/tools/Home/Soft/hca>).

140 **Gene coexpression and functional enrichment analysis**

141 The coexpression analysis for genes in embryo and endosperm was conducted using MeV
142 (Howe et al., 2010). Notably, we used the Z-score value to calculate the relative expression
143 levels before running the MeV for a tissue. These Z-score values were used as input for MeV
144 and each individual dataset was clustered using the k-means method and Pearson correlation
145 coefficients among the genes. Through Gene Ontology (GO) annotation (Ashburner et al.,
146 2000), genes were annotated into the functional categories. Functional enrichment analysis
147 was performed using the phyper function in R software with default setting (R Team, 2013).
148 P-value was used as a filter mode, and significant GO categories (P-value <0.01) were
149 identified. In addition, embryo or endosperm specific genes were identified by Z-score value.
150 A gene was declared as embryo or endosperm specific if it had a Z-score above 2 in at least
151 one of the embryo or endosperm samples as compared to all the same kind samples.

152 **Light microscopy observation of grain structure**

153 Grains samples were fixed immediately in 0.1 M phosphate buffer solution (pH 7.2),
154 containing 2.5% glutaraldehyde (v/v) and 2% paraformaldehyde (w/v). Afterwards, they were
155 dehydrated in a graded ethanol series, transferred to acetone, and embedded in low-glutinosity
156 Spurr's resin (Spurr Low-Viscosity Embedding Kit, Sigma-Aldrich, St. Louis, USA). The
157 sample blocks were polymerized at 70°C for 8 h. Semi-thin sections (2 µm) were cut at the
158 longitudinal plane (embryo) and the transversal plane (endosperm) of the grains, using a Leica
159 Ultrathin Microtome (EM UC7, Germany). Samples were stained with 1 % methyl violet,
160 observed and photographed under a BX53 light microscope (Olympus, Tokyo, Japan).

161 **Chemical analysis for metabolites**

162 *Sucrose, fructose, glucose, and starch*: Sugar contents were measured by ultra-high
163 performance liquid chromatography system (UHPLC) as described by Chen et al. (2018a).
164 Sucrose and hexoses were extracted with 80% (v/v) ethanol for 30 min at 80 °C, followed by
165 centrifugation at 5000g for 15 min. The supernatant was pooled and lyophilized using a
166 Maxi-Dry Lyo instrument (Heto-Holten, France), and resuspended in ultrapure water and
167 filtered through a 0.45 µm pore size filter. The resulting filtrate was loaded into a Dionex
168 Ultimate 3000 chromatography system. Starch content was measured using anthrone reagent

169 at wavelength of 620 nm, after extraction by ethanol and digestion by perchloric acid.

170 **T6P and SnRK1 activity:** T6P was extracted and purified according to the method of Delatte
171 et al. (2009), and quantified by ultra-performance liquid chromatography (ACQUITY UPLC
172 I-Class PLUS, Waters, USA) coupled with triple quadruple mass spectrometry system (Xevo
173 TQ-S micro, Waters, USA), following the method of Sastre Toraño et al. (2012). SnRK1 was
174 extracted and its activity was assayed by the method of Zhang et al. (2009), using AMARA
175 peptide as substrates.

176 **Amino acids and proteins:** Samples were boiled with 80% ethanol until totally bleached to
177 extract free amino acids (FAAs). Then, the total volume was kept constant with distilled water
178 and was centrifuged for 15 min. The supernatant was pooled and filtered through a 0.45 µm
179 pore size filter, and then used for FAAs determination by amino acid analyzer (L-8900,
180 Hitachi, Japan). Protein fractions including albumin, globulin, prolamin and glutelin were
181 extracted and measured by the methods of Ning et al. (2010).

182 **Phytohormones and minerals:** IAA, ABA, CKs (tZ and tZR) and GAs (GA₁, GA₃, GA₄, and
183 GA₇) were extracted and purified according to the method of Wu et al. (2016a). IAA, ABA
184 and CKs were quantified by ultra-performance liquid chromatography (ACQUITY UPLC
185 H-CLASS, Waters, USA) according to Chou et al. (2000). GAs were quantified by ultra-high
186 performance liquid chromatography (AGLIENT1290, Agilent Technologies, USA) coupled
187 with mass spectrometry system (SCIEX-6500Qtrap, AB SCIEX, USA). Minerals, including P,
188 K, Ca, Na, Mg, Fe, Zn, Mn, and Cu, were quantified by Inductively Coupled Plasma Optical
189 Emission Spectrometry (ICP-OES 710, Agilent Technologies, USA), according to the method
190 of Wang et al. (2018).

191 **Statistical analysis**

192 The data of metabolites presented in the study are averages of triplicate observations.
193 Statistical analysis was performed with SPSS (V19.0, IBM) statistical software. Multiple
194 comparisons were operated by Duncan's multiple range test ($P < 0.05$). Origin 9.0 was used
195 for plotting.

196 **RESULTS**

197 **The generation and analysis of transcriptome data of developing embryo and** 198 **endosperm**

199 To systematically investigate the dynamics of transcriptome over seed development, we
200 generated RNA-Seq libraries of three different seed tissues of the wild type WYJ3 (WT) and
201 its notched-belly mutant (NB) from 8 different time points, including 16 embryos, 16 upper
202 endosperms and 16 bottom endosperms (Fig. 1). In total, we detected 20820 genes expressed

in at least one of the 48 samples. Among them, 18171 genes were expressed in both embryo and endosperm, while only 1969 and 680 genes were specifically expressed in embryo and endosperm, respectively (Fig. 2A). Subsequently, mRNA datasets from different tissues were compared to get a more specific look into the individual characteristics of the embryo and endosperm during seed development. Results showed that most of genes were expressed in seed tissues during the early developmental phase (Fig. 2B). Within endosperm, both genotypes showed higher gene activity in upper part of endosperm (EnU) than the bottom part (EnB). In addition, embryo samples had higher number of expressed genes as compared to those of the endosperm throughout seed development (Fig. 2C).

Categorizing developmental processes of embryo and endosperm based on gene expression pattern

PCA distinguished the samples into two distinct groups based on tissue identity, validating the purity of the tissue samples (Fig. 2D). Particularly, the first component (PC1; 77.1% variance explained) clearly separated the embryo from endosperm samples, while the second component (PC2; 12.6% variance explained) discriminated the developmental stages. We further classified the transcriptomic data into a dendrogram by hierarchical clustering (HCL) analysis, and uncovered four distinguishing groups within embryo and endosperm, with each group corresponding to a specific developmental stage (Fig. 2E-H).

For embryo samples, both genotypes showed an almost identical dendrogram (Fig. 2E, F). The first cluster is formed at 5 DAF, representing the stage around differentiation (Em-S1). During this stage, coleoptile and SAM emerge and first leaf primordium becomes visible on the opposite side of the coleoptiles, as visualized by electron microscopy (Supplementary Fig. S4). The second cluster from 10-15 DAF represents the embryo enlargement stage (Em-S2), as characterized by the formation of second and third leaf primordia, and organ enlargement (particularly the scutellum). The third cluster at 20DAF corresponds to the maturation phase (Em-S3) that shows no obviously morphological change. The fourth cluster from 25 DAF to 60 DAF represents the period of seed dormancy (Em-S4).

By contrast, the duration of developmental stages in endosperm varied with genotypes. Firstly, endosperms from WT showed four primary clusters (Fig. 2G, H), but those from NB showed only three clusters, suggesting the abnormal development during middle and later stages (Fig. 2I, J). Secondly, within the endosperm, the upper and bottom parts of WT showed identical duration. However, for NB, the time span of endosperm filling in the bottom endosperm (NB_EnB) was prolonged (15-25 DAF), as compared to the upper endosperm (NB_EnU) that had the identical duration of second cluster (15-20 DAF) to the WT.

Consequently, the maturation and dormancy stage were delayed in the bottom endosperm and displayed as a merged cluster (30-60 DAF) of stages 3 and 4, as compared to the upper part, where stages 3 and 4 cluster had the same duration (25-60 DAF) as WT. Collectively, these findings indicate a disturbance in the developmental process of the bottom endosperm of NB by its proximity to the embryo.

Therefore, normal type of WT instead of the disturbed NB is used for a general description of the developmental signatures of rice grain. The four clearly defined clusters in WT endosperm show that the differentiation (En-S1) stage last from 5-10 DAF in which it completes the cellularization, with the aleurone cells starting to become morphologically distinct from the starchy endosperm (Supplementary Fig. S4). Then storage accumulation stage (En-S2) occurs at 15-20 DAF, followed by the maturation (En-S3) phase of the endosperm at 25-30 DAF. The biological processes involved in dormancy (En-S4) are induced from 45-60 DAF. Altogether, these results suggested a divergent staging mechanism of developmental characteristics between embryo and endosperm based on transcriptomic data.

Coexpressed gene sets of embryo and endosperm development

Using transcript datasets of WT, tissue-specific genes were clustered into coexpression modules by the k-means clustering algorithm (Howe et al., 2010). Gene ontology (GO) annotation was performed to assign genes to functional categories for each module (Ashburner et al., 2000). Twenty-two coexpression modules were generated for both embryo and endosperm (Fig. 3A, B). Among these modules, 11 and 9 modules were expressed broadly at more than one stage in embryo and endosperm, respectively, indicating some common cellular processes across several stages. Moreover, genes from the 11 modules of embryo and the 13 modules of endosperm were more prevalent at one out of the four developmental stages, indicating specific function of these modules at the corresponding stage.

Cellular processes in the developing embryo

Cellular processes characterizing each developmental stage were identified by GO terms that were overrepresented at particular coexpression modules (Fig. 3A; Supplementary Fig. S5A). The early stage of Em-S1, best represented by modules DP1-DP5, was typified by the overrepresentation for GO term Ran GTPase binding, mitotic cell cycle, nuclear division, nuclear DNA replication, cell wall organization or biogenesis, cell differentiation, mitochondrial protein complex and tricarboxylic acid cycle. Em-S2 featured modules DP6 and DP7 that were enriched in GO term characteristic of embryo enlargement, including the

cell division, cell wall organization or biogenesis and lipid droplet. The module DP8 presented an up-regulation of genes involved in nutrient reservoir activity and response to external biotic stimulus, suggesting the maturation of embryo characterized by Em-S3. Stage Em-S4, represented by modules DP9-DP11, was characterized by the overrepresentation of genes involved in response to biotic stimulus, structural constituent of ribosome, mitochondrial matrix, vacuole organization and photomorphogenesis. In addition, genes from modules DP12-DP22 expressed broadly in embryo across the sampled time points, which were related to protein complex, Golgi apparatus, protein kinase activity, cellular response to stimulus, RNA modification, ribosome biogenesis and endonuclease activity.

Cellular processes in the developing endosperm

The functional characterization of endosperm was found different from that of embryo as different number of modules presented a particular growth stage (Fig. 3B; Supplementary Fig. S5B). Modules DP1-DP8 were characterized as the stage of En-S1, including genes related to mitotic cell cycle, DNA replication, cell wall organization, tricarboxylic acid cycle and starch biosynthetic process. En-S2 featured the overrepresentation of GO term nutrient reservoir activity and transporter activity in DP9. Modules DP10-DP12 presented high expression of genes responsible for response to biotic stimulus and lipid storage, representing endosperm maturation at stage En-S3. Finally, the module DP13 presented an up-regulation of the genes involved in positive regulation of autophagy, organelle disassembly, metal ion transport, and cellular catabolic process, characteristic of the desiccation and dormancy stage of endosperm (En-S4). Notably, genes responsible for metal cluster binding, macromolecular complex, RNA processing and signal transducer activity were overrepresented in DP14-DP22 and were broadly expressed throughout development.

Metabolic dynamics of embryo and endosperm development

Sugars and starch

During embryo and endosperm development, sucrose content gradually increased, peaking at 15 DAF and then decreased thereafter (Fig. 4A; Supplementary Fig. S6A). Glucose and fructose levels decreased during seed development (Supplementary Figs. S7A, S7B, S8A, S8B). The ratio of glucose to sucrose mediates endosperm differentiation (Olsen, 2020). It was higher in the embryo and endosperm at 5-10DAF, and then decreased gradually (Supplementary Figs. S7C, S8C), corresponding to the transition from differentiation to storage accumulation. These sugars were unevenly distributed in rice seed, with the embryo generally having higher contents (Fig. 4A; Supplementary Figs. S6A, S7A, S7B, S8A, S8B). Genes participating in sugar metabolism were differentially expressed between embryo and

305 endosperm. For the five genes encoding sucrose synthase, two of them (*OsSUS1* and
306 *OsSUS6*) were mainly expressed in embryo, while another three (*OsSUS2*, *OsSUS3*, *OsSUS4*)
307 in endosperm (Fig. 4A; Supplementary Fig. S6A).

308 During grain filling, starch content increased gradually, peaking at 20 DAF and 30 DAF in the
309 embryo and endosperm, respectively (Fig. 4B; Supplementary Fig. S6B). Compared with
310 embryo, endosperm contained higher starch content, which is consistent with previous report
311 (Juliano and Bechtel, 1985). Starch synthesis-related genes were coordinately expressed in the
312 embryo and endosperm, similar to that in wheat (Xiang et al., 2019). Moreover, these genes
313 were primarily expressed in endosperm at 5-20 DAF. By contrast, they displayed a gradual
314 increase in embryo at 5-20 DAF.

315 *T6P and its target protein SnRK1*

316 Trehalose-6-phosphate (T6P) signals the availability of sucrose in plant cells through the
317 feast-famine kinase, SnRK1 (O'Hara et al., 2013; Paul et al., 2017; Dingkuhn et al., 2020).
318 T6P level in embryo and endosperm showed a gradual decrease in the development course.
319 Compared with endosperm, embryo has higher content at early stage (5-10DAF) (Fig. 4C;
320 Supplementary Fig. S6C). For example, at 5 DAF, T6P was 3.98 and 6.61 nmol g⁻¹ DW in
321 endosperm and embryo, respectively. In addition, *TPS* genes were expressed in both embryo
322 and endosperm, being predominantly expressed at 5-20 DAF.

323 The SnRK1 activity in endosperm maintained a low level at 5-20 DAF, and then increased
324 rapidly at later stages (25-60 DAF). Different from the endosperm, SnRK1 in the embryo was
325 at a lower level, being higher only at 5 DAF (Fig. 4D; Supplementary Fig. S6D). *SnRK1A* was
326 specifically expressed in the embryo, while two *SnRK1B* genes (*SnRK1B* and *OSK35*) were
327 mainly expressed in endosperm. In addition, *SnRK1A* was broadly expressed across the time
328 points in embryo. By contrast, *SnRK1B* and *OSK35* were more prevalent in endosperm at 5-20
329 DAF.

330 *Amino acids and proteins*

331 During embryo development, the level of total free amino acids (FAAs) gradually decreased
332 at 5-25 DAF and increased thereafter (Supplementary Figs. S7D, S8D). In contrast, FAAs in
333 endosperm decreased as grain filling progressed. In addition, FAAs were higher in embryo
334 than endosperm. Storage proteins were higher in embryo than endosperm (Fig. 4E;
335 Supplementary Fig. S6E). However, gene expression pattern of storage protein synthesis
336 showed an opposite correlation with storage proteins content, suggesting that their encoding
337 genes are specifically expressed in the endosperm, as in wheat (Xiang et al., 2019).

338 *Phytohormones*

339 GAs showed a gradual decrease over seed developmental course. Compared with endosperm,
340 embryo had higher content at 5 DAF, while exhibited an opposite trend at 10 DAF (Fig. 4F;
341 Supplementary Fig. S6F). GA-synthetic genes, *KS* (*OsKS1* and *OsKS4*), *GA20ox*
342 (*OsGA20ox2* and *OsGA20ox3*), and *GA3ox* (*OsGA3ox1* and *OsGA3ox2*), were uniquely
343 expressed in embryo at 5-20 DAF, implying that the embryo may be main site for GAs
344 synthesis (Xue et al., 2012; Zhang et al., 2020a). In addition, two GA-deactivating genes, *EUI*
345 and *OsGA2ox3*, were specifically enriched in endosperm at 5 DAF and 15-20 DAF,
346 respectively.

347 Auxin gradually increased until 10-15 DAF in embryo and endosperm, then decreased
348 thereafter (Fig. 4G; Supplementary Fig. S6G). Compared with endosperm, embryo had higher
349 content at 5-20 DAF. Notably, *OsTAA1;4* was mainly expressed in endosperm at 5 DAF.

350 ABA content in endosperm was half of that in embryo. It showed two peaks at 15 DAF and
351 25 DAF in embryo and endosperm (Fig. 4H; Supplementary Fig. S6H), in line with the
352 biphasic pattern of the embryonic development (Qin et al., 1990). Activity of ABA-synthesis
353 genes in embryo and endosperm displayed two peaks. In embryo, *NCED1*, *OsZEP1* and *ABA2*
354 were highly expressed at 5 DAF, while *NCED3* and *NCED5* at 25 DAF. In endosperm,
355 *NCED1*, *OsZEP1* and *ABA2* were highly expressed at 5 DAF, while *NCED1* and *OsZEP1* at
356 20 DAF.

357 *OsIPT4* were specifically expressed in endosperm, especially at 5-10 DAF (Fig. 4I;
358 Supplementary Fig. S6I). In embryo, CKs levels gradually increased, peaked at 10 DAF and
359 then decreased thereafter, consistent with the expression patterns of CKs-synthetic genes. The
360 CKs in endosperm was lower than those in embryo at 5-30 DAF and peaked at 5 DAF.

361 *Minerals*

362 In embryo, contents of K, Ca, Na, Fe, Mn, Cu and Zn decreased as development progressed,
363 while those of Mg and P gradually increased (Fig. 5; Supplementary Fig. S9). In endosperm,
364 contents of the nine mineral nutrients decreased as grain filling progressed, being higher at
365 early stage than the later stage. This could be attributed to the dilution effect of grain dry
366 matter, which had a higher rate of accumulation than the minerals (Wang et al., 2018).
367 Compared with endosperm, contents of all minerals were high in the embryo.

368 **Influence of embryo on the transition of developmental stages of endosperm**

369 *A novel method to qualify the effect of embryo on endosperm development*

370 As shown in Fig. 2, the duration of middle stage in the bottom part of the NB grain (Fig. 2J)
371 was prolonged relative to that of its upper counterpart (Fig. 2I) as well as those of the WT
372 (Fig. 2G, H), indicating a strong influence of the embryo on the bottom endosperm proximate

373 to it. To quantitatively evaluate the embryo effect, we developed a novel comparison system by
 374 comparing the upper and bottom endosperms of NB, using WT as a reference (Fig. 6).
 375 Briefly, this system has three key components: (i) Position effect. The comparison between
 376 the upper (WT_EnU) and bottom (WT_EnB) endosperms of WT reflects the difference in
 377 position between upper and bottom endosperms within the WT grain, where nutrients and
 378 signals move freely without being blocked by the notched line as in NB (Fig. 6A). (ii)
 379 Compound effect. Due to the notched line, movement of nutrients and signals are severely
 380 restricted between the two endosperms, trapping the influence of the embryo in the bottom
 381 endosperm. Comparison between the bottom part (NB_EnB) and upper part (NB_EnU) of the
 382 NB grain shows the compound effect of position and embryo (Fig. 6B). (iii) Embryo effect.
 383 Finally, by eliminating the position effect, we can precisely calculate the influence of embryo
 384 on the endosperm via $NB_{(EnB/EnU)} / WT_{(EnB/EnU)}$ (Fig. 6C).

385 To exemplify the working principle of this comparison method, we selected three
 386 metabolites including glutelin, asparagine (Asn), and Zn to show the influence of embryo
 387 (Fig. 6). Firstly, taking in account the position effect and compound effect, the up-regulation
 388 of Asn and down-regulation of glutelin and Zn was observed in both genotypes. However,
 389 when eliminating the effect of position, embryo has a positive effect on the content of Zn and
 390 a negative effect Asn and glutelin, verifying the influence of embryo on endosperm filling.

391 *T6P-SnRK1 signaling putatively mediates the crosstalk between embryo and endosperm*

392 Using the new comparison method, our results showed that T6P-SnRK1 signaling pathway
 393 was active in the endosperm, consistent with that of the wheat (Martínez-Barajas et al., 2011).
 394 As shown in Fig. 7, at early stage of 5 to 10 DAF, embryo had a negative effect on sucrose,
 395 glucose and fructose levels in endosperm during 5-10 DAF, probably due to its nutrient
 396 consumption. The decrease of sucrose content in endosperm was accompanied by the decline
 397 in T6P levels, which in return inhibited SnRK1 activity (Fig. 7A). SnRK1 promoted catabolic
 398 activity in the endosperm, as evidenced by the declining accumulation of starch and storage
 399 proteins (prolamin and glutelin), and the up-regulation of genes encoding amylase (*AMY3D*),
 400 lipase (*Os01g0651800*, *Os01g0710700*, and *Os05g0574100*), and protease (*OsSAG12* and
 401 *OsSCP28*) in endosperm. Consistently, genes participating in synthetic process of starch
 402 (*OsAGPL4*, *OsSSSIIIa*, *OsSSIVa*, *OsSSIVb*, and *Os02g0807100*) and storage protein
 403 (*OsEnS-115*, *Prol-14*, *Pro-15*, *Prol13b.20*, and *Glutelin type-B 2-like*) were all
 404 down-regulated at 5-10 DAF (Fig. 7B).

405 Conversely, at middle stage between 20-25 DAF, T6P-SnRK1 signaling showed an
 406 opposite trend relative to that between 5-10 DAF. Gene activities of sucrose metabolism

(*OsINV1* and *OsNIN6*) and T6P synthesis (*OsTPS9* and *Os08g0414700*) were up-regulated (Fig. 7). SnRK1 activity was inhibited synchronously in endosperm (Fig. 7A). Meanwhile, starch synthetic process was promoted and catabolic processes were inhibited by SnRK1, respectively, as reflected by the overrepresented starch-synthetic genes (*OsAGPL3* and *ISA1*) and underrepresented catabolic genes encoding amylase (*AMY3A* and *AMY3E*), and protease (*OsAPI1*, *Os04g0535200*, *Os08g0267300*, *Os05g0403000*, *Os04g0330900*, and *OsSAG12-1*) in endosperm (Fig. 7B).

In addition, this comparison method revealed that the influence of embryo on IAA was similar with T6P but converse with SnRK1 activity across the developmental stages, decreasing at 10 DAF while increasing at 15-25 DAF (Fig. 7A). Recently, by modulating T6P content in growing embryos of garden pea (*Pisum sativum*), Meitzel et al. (2021) found that auxin acts downstream of T6P to facilitate seed filling. Our finding of the synchronously dynamic pattern of IAA and T6P indicates a similar role of auxin-T6P pathway in grain-filling process of rice.

DISCUSSION

Embryo-endosperm relationship is one of the determining factors contributing to seed development and subsequently the yield and quality of cereal grains. However, signals or regulators that coordinate the developmental processes of the two compartments remain largely unknown. This study took advantages of the NB mutant to evolve a novel comparison method for quantifying the influence of embryo on endosperm development, revealing that the embryo has a dragging effect on the developmental transition of the endosperm. To our knowledge, it is the first attempt to directly disclose the evidence of embryo-endosperm interaction during rice seed development. The findings reveal new aspects of the role of embryo in the formation process of rice quality, and help to draw an integrative picture of seed development from the perspective of agronomy and crop physiology.

1. The dragging effect of embryo on endosperm development

Seed development is an orchestrated progression through a series of stages, which can be described by two types of 'time': the chronological time and the developmental time defined by the sequence of stages (Ebisuya and Briscoe, 2018). There is evidence of bidirectional interaction between embryo and endosperm throughout development (An et al., 2020). Notably, two recent studies in *Arabidopsis* suggest an independent relationship between the two tissues. By analyzing mutants with defective endosperm cellularization, O'Neill et al. (2019) found that this endosperm process is not required for the onset of embryo maturation. Using single-fertilization mutants, Xiong et al. (2021) demonstrated that in the absence of

embryo, endosperm develops the same way as in the wild type, suggesting that endosperm development is an autonomously programmed process independent of embryogenesis. These two studies in combination indicate that in terms of developmental time, the mechanisms controlling endosperm and embryo development act independently of each other (O'Neill et al., 2019). On the contrary, in terms of chronological time, the current study show that duration of endosperm filling in the bottom part of the NB grains was prolonged by the embryo (Fig. 2J), suggesting a substantial interaction between embryo and endosperm. Similarly, Xiong et al. (2021) showed that rapid embryo expansion can significantly accelerate endosperm breakdown, thus shortening the lifespan of the transient endosperm. Further, it should be noted that cereal seeds like rice have a persistent endosperm, whereas dicots like *Arabidopsis* have an ephemeral endosperm that degrades as the embryo grows. As a result, whether the independence of endosperm development from embryo in *Arabidopsis* is applicable to rice awaits further investigation.

Plants have evolved a variety of timing mechanisms that integrate chronological time with developmental time to ensure proper development (O'Neill et al., 2019). For the endosperm, these include internal timers of molecular oscillators based on hormones or metabolites, and external timers dependent on environmental signals or emanating from a different tissue like the embryo. This study reveals a dragging effect of embryo on endosperm development in chronological time, i.e. extending the storage accumulating stage whereas delaying the maturation stage. This finding provides direct evidence for the role of embryo as the external timer controlling endosperm development. In addition, hormones like GA, auxins, and ABA were unevenly distributed in rice seed, with the embryo generally having higher contents at the early and middle stages, as also reported by Zhang et al. (2020a). Interestingly, *GA20ox* (*GA20ox2* and *GA20ox3*), *GA3ox* (*OsGA3ox1* and *OsGA3ox2*), *KS* (*OsKSI* and *OsKS4*) were predominantly expressed in embryo at 5-20 DAF, indicating that embryo may be the GA-synthetic site whereas endosperm is the GA-acting site (Fig. 4F). It is well established that embryo-derived GA modulates the secretion of starch-degrading enzymes like α -amylase from the aleurone and scutellum upon germination. But it is still uncertain whether the degradation of starch during seed development is analogous to the germinating process (Sreenivasulu et al., 2015). Our results imply that one of the external timers coordinating rice grain development might be the hormone GA released from endosperm, which needs further investigation.

For internal timers modifying endosperm development, the T6P-SnRK1 signaling pathway may be the key component. At early stage of 5 to 10 DAF, sucrose in endosperm was lower,

probably due to the deprivation by the growing embryo. In response to the reduced sucrose content, T6P was decreased simultaneously in endosperm, thus relieving the inhibition of SnRK1 activity. The increased SnRK1 activities promoted the catabolism or suppressed the anabolism of starch and proteins, as reflected by the lower content of starch and prolamins as well as the enhanced gene activity of amylase, lipase, and protease in endosperm (Fig. 7). Conversely, at middle stage between 20 and 25 DAF, the T6P-SnRK1 signaling showed an opposite trend relative to that between 5 and 10 DAF, indicating it may be involved in the transition of developmental stages in endosperm (Fig. 7). Taken together, the anticorrelation between T6P and SnRK1 activity opens the possibility that the T6P-SnRK1 pathway may be a master regulator coordinating the communication between embryo and endosperm during rice grain formation.

2. The integrative landscape of rice seed development

One of the major goals of crop production is to produce more grains with less time, thus improving resource use efficiency. The duration of each stage of seed development and the timing of transition between them is of agronomical significance. As reviewed by Olsen (2020), mechanism regulating the timing of endosperm cellularization has attracted attention due to its positive correlation with endosperm and seed size. Consequently, a precise and integrative landscape of seed development is necessary and useful for both fundamental and applied studies on the mechanisms underpinning crop yield and quality. Previously, from viewpoint of crop physiology, we proposed a practical staging system with three phases: embryo morphogenesis, endosperm filling, and seed maturation (An et al., 2020). Here, we update this staging system by integrating of molecular, physiological, and anatomical evidence in the current study as well as from the literature. In particular, considering the dragging effect of embryo on endosperm development at the former second stage (endosperm filling), we highlight the importance of this stage as critical for grain filling and quality formation, and thus subdivide it into two stages: embryo enlargement and endosperm filling. Accordingly, we paint a holistic and dynamic picture of rice seed development (Fig. 8), and provide a brief description of each stage and its agronomical relevance as follows.

Stage I: Morphogenesis (0-10 DAF): After double fertilization, patterning and differentiation occurs simultaneously in the embryo and endosperm. At the end of this stage, most of morphogenetic events in embryo have already occurred. Endosperm has finished differentiation, forming two subregions, the aleurone and starchy endosperm, and starts storing starch and proteins.

Stage II: Embryo enlargement (10-20 DAF): Embryo greatly grows to its maximum volume

at 20 DAF (Itoh et al., 2005). Starchy endosperm attains its highest rate of storage accumulation (Zhu et al., 2011; Fu et al., 2013), while the aleurone cells is filled with aleurone particles and spherosomes at the end of this stage (Yu et al., 2014). This stage witnesses strong interactions between embryo and endosperm, hence being critical for rice grain filling and quality.

Stage III: Endosperm filling (20-30 DAF): Embryo becomes dormant and endosperm continues to accumulate starch and proteins, reaching its maximum weight at 30 DAF. By the end of this stage, most of the starchy endosperm and maternal tissues have undergone PCD, losing their biological activity, while the aleurone and embryo is still alive (Wu et al., 2016b, c).

Stage IV: Maturation (30 DAF-maturity): After completion of reserve accumulation, the embryo becomes tolerant of desiccation, and undergoes a developmentally programmed dehydration event leading to dormancy and a quiescent state (Manfre et al., 2009; Angelovici et al., 2010). The starchy endosperm cells die completely upon seed maturation and desiccation. At this stage, seeds are susceptible to germination under hot and humid conditions, thus being vulnerable to preharvest sprouting (Du et al., 2018).

Fig. 8 draws distinctive patterns of embryo and endosperm development, with endosperm ceasing storage accumulation at 30 DAF, ten days after the corresponding timepoint of embryo (20 DAF). Supplementary Fig. S10 shows that the capacity of embryo to germinate starts at 15 DAF and peaks at 20 DAF for both genotypes of WT and NB. It is thus inferred that the embryo has the priority of nutrient allocation over endosperm during seed development. Moreover, it appears that this asynchrony of embryo and endosperm development is conserved across modern cultivars. They share a common chronological time, with embryo developmentally maturing at 20 DAF (Itoh et al., 2016; Armenta-Medina et al., 2021), while endosperm matures at 30 DAF (Morita et al., 2005; Yang et al., 2006; Wang et al., 2008; Chen et al., 2013; Wu et al., 2016c; Wei et al., 2017; Xu et al., 2021). Therefore, the essential period of rice yield and quality formation is the Stages I-III (Fig. 8), from anthesis to 30 DAF. After that, rice seed enters the stage of desiccation and maturation, lasting for 20 to 40 days with no marked increase in grain weight. By dividing the 60-day period of grain filling into two separate months, our previous report showed that only 10 % of the grain yield was formed after 30 DAF (Xu et al., 2021). Under the intensive rice-wheat and rice-oilseed rape cropping systems in the lower reaches of Yangtze River, China, late maturity of the rice crop causes the late sowing of wheat and oilseed rape, adversely affecting the seedling growth and subsequently the grain or seed yield (Bai and Tao, 2017; Zhang et al., 2020b).

543 Accordingly, we suggest a genetical intervention to shorten the duration of the late maturation
544 stage of rice in order to adapt to the double cropping systems. In addition, future work should
545 exploit more genotypes to verify if this asynchrony of embryo and endosperm development in
546 chronological time is conserved in rice as well as other cereal crops like maize and wheat.

547 **CONCLUSIONS**

548 Despite a number of comprehensive studies on the transcriptome atlas of seed development
549 with high spatio-temporal resolutions, definite information about the interaction between the
550 two economically important tissues of embryo and endosperm is still lacking. In this study,
551 we successfully applied a novel comparison method based on the NB mutant, demonstrating a
552 direct effect of embryo on the developmental processes of endosperm. Integrated analysis of
553 transcriptome and metabolites identified putative regulatory timers that coordinate the
554 developmental process between embryo and endosperm. The external timers for endosperm
555 may be hormones like GA secreted from the embryo, while the internal timers be the
556 T6P-SnRK1 signaling pathway that mediates carbon allocation in endosperm. In combination
557 with results from molecular, physiological, and anatomical investigations, we framed a
558 holistic and dynamic landscape of rice seed development, and explained its agronomical
559 significance, in particular for the intensive systems of rice-wheat or rice-oilseed rape double
560 cropping. In contrast to the quantum leap in fundamental science of genetic control of seed
561 development, progress on the applied science like the physiology of grain filling and quality
562 has apparently lagged behind. The integrative picture of rice grain formation proposed here is
563 of value to bridge this knowledge gap, and will facilitate the designing of cogent strategies to
564 enhance rice yield and quality.

565 **SUPPLEMENTARY DATA**

566 The following supplementary data are available at *JXB* online.

567 Table S1. Details of primers used in this study.

568 Fig. S1. Correlation of gene expression level (FPKM) among biological replicates.

569 Fig. S2. qRT-PCR validation of RNA-Seq results.

570 Fig. S3. Expression profiles of tissue-specific genes in embryo and endosperm tissues.

571 Fig. S4. Morphology of the developing embryo and endosperm for WT and NB.

572 Fig. S5. GO functional categories enriched in different coexpression modules of embryo and
573 endosperm for WT and NB, respectively.

574 Fig. S6. Carbohydrates, proteins, SnRK1, and hormones, and their related genes in the
575 embryo and endosperm of NB throughout development.

576 Fig. S7. Fructose, glucose, and free amino acids in embryo and endosperm of WT.

577 Fig. S8. Fructose, glucose, and free amino acids in embryo and endosperm of NB.

578 Fig. S9. Minerals in the embryo and endosperm of NB throughout development.

579 Fig. S10. Germination rate of developing seed of WT and NB.

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587 **CONFLICT OF INTEREST**

588 The authors report no declarations of interest.

589 **AUTHOR CONTRIBUTIONS**

590 YT had the main responsibility for data collection and analysis. YT and LA made the figures.
591 FX, GL, YD and MP revised the manuscript. ZL had the overall responsibility for
592 experimental design, project management and manuscript preparation. All authors contributed
593 to the article and approved the submitted version.

594 **DATA AVAILABILITY STATEMENT**

595 All the analysed data related to this manuscript are present in this paper and its supplementary
596 data. All the raw data associated with this study are submitted to NCBI and are available with
597 accession number PRJNA722833.

598

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FIGURE LEGENDS

Fig. 1. Overview of the time series samples of embryo and endosperms. Dotted line indicates the manual dissection of endosperm, cutting it into upper and bottom endosperms. DAF, days after fertilization; E, embryo; En, endosperm; EnB, bottom part of endosperm; EnU, upper part of endosperm; NB, notched-belly rice mutant; WT, wild type of Wuyujing-3. Bar = 1 mm

Fig. 2. Global transcriptome relationships among tissues and developmental stages. (A) Venn diagram of the 20820 genes expressed in the embryo and endosperm. (B) Number of genes detected in each tissue. (C) Comparison of gene activity between embryo and endosperm. (D) PCA of the seed tissue mRNA populations. PCA plot shows two distinct groups of embryo and endosperm mRNA populations: group I for embryo and group II for endosperm. (E) and (F) Clustered dendrogram showing global transcriptome relationships of time series samples from the embryo of WT and NB, respectively. (G) to (H) Clustered dendrogram showing global transcriptome relationships of the upper and bottom endosperms of WT, respectively. (I) to (J) Clustered dendrogram showing global transcriptome relationships of the upper and bottom endosperms of NB, respectively. The bottom row indicates the developmental phases according to the cluster dendrogram of the time series data. Except in (A), data of 5, 10, 15..., and 60 represent sampling timepoints (days after fertilization).

Fig. 3. Expression patterns of genes in different coexpression modules for embryo and endosperm of WT (A, B) and NB (C, D). Coexpression modules are ordered according to the sample time points of their peak expression. For each gene, the FPKM value normalized by the maximum value of all FPKM values of the gene over all time points is shown. The numbers of genes and TFs in each module are shown on the right.

Fig. 4. Carbohydrates, proteins, SnRK1, and hormones, and their regulating genes in the embryo and endosperm of WT throughout development. Each value represents the mean $\pm$ SE of three replicates. Black, orange, and blue indicate non-tissue, embryo, and endosperm specific genes, respectively.

Fig. 5. Minerals in the embryo and endosperm of WT and NB across the developmental stages. Each value represents the mean $\pm$ SE of three replicates.

Fig. 6. Diagrammatic representation of the new comparison method for quantifying the embryo effect on endosperm development. This method assembles three key components: (A) Quantification of the position effect (PE) by comparison between the upper (WT_EnU) and bottom (WT_EnB) endosperms of WT. This reflects the difference in position between upper and bottom endosperms within the WT grain, where nutrients and signals move freely without being blocked by the notched line as in NB. (B) Quantification of the compound effect (CE) of position and embryo by comparison between the upper (NB_EnU) and bottom (NB_EnB) endosperms of NB. Due to the notched line, movement of nutrients and signals are severely constrained between the two endosperms. Therefore, the influence of the embryo is trapped and thus mainly expressed in the bottom endosperm. Note that 2/3 interface between the upper and bottom is cut down by the notched line. (C) Evaluation of the embryo effect (EE) by secondary comparison of CE/PE to exclude the position effect. Three metabolites are used to exemplify the working principle of this method. For example, Asn content is up-regulated in the bottom endosperm of both WT and NB. By contrast, comparison of NB and WT reveals that the embryo effect is down-regulating it in the endosperm. Red and navy blue squares indicate up and down-regulation in the bottom endosperm, respectively.

Fig. 7. Putative role of T6P-SnRK1 signaling in developmental transition of the endosperm as revealed by the proposed new comparison method. Effects of position, compound, and embryo are all shown. (A) Schematic diagram showing the putative T6P-SnRK1 signaling pathway. (B) Heatmap of differentially expressed genes associated with position, compound, and embryo effects. ADP-G, ADP-glucose; AGPase, ADP-glucose pyrophosphorylase; DBE, debranching enzyme; Fru, fructose; G6P/PT, glucose-6-phosphate/phosphate translocator; G1P, glucose 1-phosphate; GA, gibberellin; GBSS, granule-bound starch synthase; Glc, glucose; IAA, indoleacetic acid; INV, invertase; ISA, isoamylase; MST, monosaccharide transporter; PM, plasm membrane; SBE, starch branching enzyme; SnRK1, sucrose non-fermenting-1 related protein kinase 1; SSP, seed storage protein; SSS, soluble starch synthase; SuSy, sucrose synthase; SUT, sucrose transporter; T6P, trehalose 6-phosphate; TAR2, tryptophan aminotransferase 2; TPP, trehalose 6-phosphate phosphatase; TPS, trehalose 6-phosphate synthase; Trp, tryptophan; UDP-G, UDP-glucose; UGPase, UDP-glucose pyrophosphorylase; α -AMY, α -amylase; β -AMY, β -Amylase. Red and navy rectangles indicate up- and down-regulation, respectively.

Fig. 8. Holistic and dynamic picture of seed development. (A) Schematic illustration of the morphological changes in embryo (longitudinal section) and endosperm (transversal). Varying colors of the pericarp and testa show the progression of degradation in maternal tissues, while those of the starch endosperm show the grain-filling process. (B) Dynamic accumulation of storage materials (sucrose, starch, FAAs, storage proteins, and minerals) and the increase in grain weight, length, width, and thickness. (C) Molecular signatures of the embryo and endosperm at different developmental stages.

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