A CDKB/KRP/FB3 cell cycle core complex functions in rice gametes and zygotes

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Abstract

The cell cycle controls division and proliferation of all eukaryotic cells and is tightly regulated at multiple checkpoints by complexes of core cell cycle proteins. Due to the difficulty in accessing female gametes and zygotes of flowering plants, little is known about the molecular mechanisms underlying initiation embryogenesis despite the crucial importance of this process for seed crops. In this study, we reveal four levels of factors involved in rice zygotic cell cycle control and characterize their functions and regulation. Protein-protein interaction studies, including within zygote cells, and *in vitro* biochemical analyses delineate a model of the zygotic cell cycle core complex for rice. In this model, CDKB1, a major regulator of plant mitosis, is a cyclin (CYCD5)-dependent kinase; its activity is coordinately inhibited by two cell cycle inhibitors, KRP4 and KRP5; and both KRPs are regulated via F-box protein 3 (FB3)-mediated proteolysis. Supporting their critical role in controlling the rice zygotic cell cycle, mutations in KRP4, KRP5, and FB3 result in the compromised function of sperm cells and abnormal organization of female germ units, embryo and endosperm, thus significantly reducing seed-set rates. This work helps reveal regulatory mechanisms controlling the zygotic cell cycle toward seed formation in angiosperms.

Key words: Cell cycle, coordinate inhibition, core complex, F-Box mediated degradation, Kip-Related Protein, *Oryza sativa,* rice zygote, seed formation.

Introduction

Rice provides nearly half of daily human nutrition, and rice's outstanding genetic and genomic resources render it a model monocotyledonous plant. Therefore, understanding the mechanisms underlying the initiation of rice embryogenesis may improve cereal production for the increasing global population (Godfray et al., 2010).

Seeds of monocot plants such as rice consist of embryo and endosperm, derived from double fertilization. The embryo is a diploid embryonic structure with genetic content derived roughly evenly from the sperm and egg; the endosperm is a triploid fusion product of a sperm (n) and a maternal central cell (2n), providing nutrition for the embryo during seed development and germination (Russell, 1992; Hu, 1998; Dresselhaus et al., 2016; Sprunck, 2020). The zygote is the founder cell of the embryo. Its first division forms a smaller cytoplasm-rich apical cell and a larger vacuolated basal cell, which then divides at multiple planes and develops into the pro-embryo and ultimately the embryo (Khanday and Sundaresan, 2021).

Embryogenesis is the product of coordinated events of cell division that result in the formation of a mature embryo. The cell cycle needs to be re-established in every generation of sexually reproducing organisms within the nascent zygote. The progression through the cell division phases [gap 1 (G1), DNA synthesis (S), gap 2 (G2), and mitosis (M)] is tightly controlled by multiple regulatory proteins at each checkpoint, especially in the transitions from G1 to S and G2 to M. Cell cycle control coordination failures can be lethal, leading either to embryo abortion or to development of tumors (Williams and Stoeber, 2012; Otto and Sicinski, 2017).

Across eukaryotes, Cyclins (CYC) and Cyclin-Dependent Kinases (CDKs) constitute the central core cell cycle control proteins. Flowering plants possess a large set of CDKs and CYCs that allowing numerous core complex combinations (Stals and Inze, 2001; De Veylder, 2007; Van Leene, 2011; Cheng et al., 2013; Dante, 2014; Pedroza-Garcia, 2016; Wang, 2004; La, 2006; Dudits, 2007; Hu et al., 2010; Ma et al., 2013; Lin et al., 2014). Furthermore, both CDK and CYC are conjugated with a third level partner, an Inhibitor of Cyclin- Dependent Kinase (ICKs), which inhibits CDK activity (Dudits, 2016; Lin et al., 2014), and in plants are called Kip-Related Proteins (KRPs; Kip, a mammalian tumor suppressor). KRPs are nuclear proteins of 100 to 300 amino acid residues, with 2 motifs at the C-terminal that interact with CDK and CYC (Stals and Inze, 2001; Wang, 1997; De Veylder et al., 2001). In Arabidopsis, plant sperm cell formation is

101 controlled by two cell cycle inhibitors, AtKRP6 and AtKRP7, which are degraded by the FB
102 Like 17-associated SCF complex (Kim et al., 2008). Rice has six KRPs, most of which play a
103 critical role in rice seed development and vegetative growth. For instance, KRP1 functions in
104 rice plant growth and seed formation (Barrôco et al., 2006). Overexpression of KRP1 and KRP2
105 results in smaller seeds (Ajadi et al., 2020). KRP3 controls rice syncytial endosperm formation
106 (Mizutani et al., 2010), and over-expressing KRP4 results in abnormal rice growth and reduced
107 seed-set rate (Yang et al., 2011). But, to date, no KRPs have been specifically connected to rice
108 zygotic cell cycle control.
109 To enter the S or M-phase, the inhibitory KRPs must be removed so that CDKs can be activated.
110 Members of the F-box protein (FBP) family play a central role by mediating the degradation of
111 KRPs. Numbers of FBPs vary greatly among species (Kipreos and Pagano, 2000; Xu et al.,
112 2009; Zhang S et al., 2019; Zhang X et al., 2019). In rice, for example, 678 FBPs have been
113 predicted. Across eukaryotes, FBPs typically associate with two other factors to form an SCF
114 complex (Skp1-Cullin1-F-box) (Kipreos and Pagano, 2000; Pagano et al., 1995; Bai et al., 1996;
115 Schulman et al.,2000; Zheng et al., 2002; Malik et al., 2020), which acts as an E3 ligase that
116 targets protein ligands for proteolysis via the 26S proteasome pathway (Zhang et al., 2019).
117 Due to the inaccessibility of the zygote, which is deeply embedded within multiple layers of
118 tissue, no core complex (CYC-CDK and KRP) has been identified that controls rice zygotic
119 division. Here, we report a rice cell cycle core complex consisting of CYCD5 and CDKB1 that is
120 coordinately arrested by two inhibitory proteins, KRP4 and KRP5, and, in turn, activated by
121 FB3-mediated proteolysis. This complex is likely involved in rice zygotic cell cycle control.
122
123 ## Results
124
125 **Rice KRP4 and KRP5 are preferentially expressed in egg cells and zygotes**
126 Cell cycle control core complexes include three components: CDKs, CYCs and KRPs.
127 Interactions among these components have been comprehensively studied in Arabidopsis (Leene
128 et al., 2011). However, in rice, we have limited knowledge of these interactions. Particularly,
129 little is known of their function in female gametes and zygotes of Arabidopsis or other flowering
130 plants. Due to the well-known difficulty in accessing these single cells that are deeply embedded
131 within multiple layers of tissue, there is no research on the functions of cell cycle core complex

in plant female gametes or zygotes for the initiation of embryogenesis following fertilization. To address this void, we mined the relevant literature (Guo et al., 2007) and genomic and transcriptomic (Anderson et al., 2013; Anderson et al., 2017) databases, and identified 9 *CDK*, 6 *KRP*, and 24 *CYC* genes expressed in rice flowers (Supplemental Table S1). Among them, 3 *KRPs* (*KRP1, KRP4* and *KRP5*), 6 *CDKs* (*CDKA1;1, CDKA2;1, CDKB1;1, CDKB2;1, CDKC1;*2 and *CDKE1*), and 10 *CYCs* were cloned for further functional study as candidate core complex factors in rice zygotic cell cycle.

Transcriptome data revealed preferential expression of *KRP4* and *KRP5* in rice egg cells and zygotes, compared to other *KRP* genes (Anderson et al., 2013; Anderson et al., 2017) (Figure 1 A). We further confirmed by RT-qPCR that the expression of *KRP4* and *KRP5* is significantly higher in rice pistil compared to other tissures (leaf, stem, root anther, and lemma/palea, Figure 1, B and C).

Yeast two hybridization (Y2H)-based interactome of cell cycle core complex proteins of rice egg cells and zygotes

To elucidate the proteins potentially composing the cell cycle core complex, we conducted Y2H assays using KRP4 and KRP5 proteins as baits, and CDK and CYC proteins as preys. Using a strict selective media, we detected that KRP5 interacts with all 6 CDKs (CDKA1;1, CDKA2;1, CDKB1;1, CDKB2;1, CDKC1;1 and CDKE1, Figure 2A). Similarly, KRP4 interacts with all CDKs except CDKE1. Notably, we observed a preferential interaction between KRP4 and CDKC1;2. As expected, truncated KRP4 (KRP4m) and KRP5 (KRP5m), lacking the domain for interaction with CDK, did not interact with any of the six CDKs tested (Figure 2A).

As CDKB proteins are unique to plants and essential for plant cell division during mitosis (Leene et al., 2011; Boudolf et al., 2004; Andersen et al., 2008; Atkins and Cross, 2018), we verified the interaction of CDKB1 and CDKB2 with KRP4 and KRP5 in a co-immunoprecipitation assay. Both c-Myc-tagged KRP4 and KRP5 and HA-tagged CDKB1 were precipitated from total yeast nuclear proteins with an anti-Myc antibody [Figure 2B (upper panel)], demonstrating the interactions of CDKB1 with both KRP4 and KRP5 in yeast nuclei. Similarly, CDKB2 also interacts with both KRP4 and KRP5 [Figure 2B (lower panel)].

Since both KRP4 and KRP5 interact with most selected CDKs (Figure 2, A and B), we traded their orientation (as bait or prey) in Y2H to assess specificity and also used these constructs to

163 test interactions between the KRPs. Results were similar regardless of orientation, and the KRP
164 proteins strongly interacted with each other (Figure 2, C and D). To our knowledge, this is the
165 first interaction detected between the two putative inhibitory KRP proteins in eukaryotes.
166 Importantly, subsequent experiments verified the coordination between KRP4 and KRP5.
167 In addition, all six rice CDKs were also used as baits to detect potential interactions with ten
168 different rice CYCs. We found that CDKA2;1 interacts with five CYCs (CYCB2;2, CYCD2;1,
169 CYCD5;1, CYCD6;1 and CYCT1;4), CDKB1;1 interacts only with CYCD5;1; and that
170 CDKB2;1 interacts with seven Cyclins (CYCA2;1, CYCA3;1, CYCB1;1, CYCB2;2, CYCD2;1,
171 CYCD5;1 and CYCD6;1) (Figure 3A). No interaction was detected from the other three CDKs
172 and ten CYCs. KRP1, KRP4 and KRP5 were also used as baits for interactions with different
173 CYCs (Figure 3B). We detected interactions only between KRP5 and CYCD5;1 or CYCT1;4
174 (weakly). All these detected interactions are summarized as Figure 3C.
175
176 **KRP4 and KRP5 coordinately interact with CDKBs to inhibit yeast growth and kinase**
177 **activity**
178 Figure 4, A and B, show that transfection with a single rice cell cycle control protein, either
179 CDKB1, CYCD5, KRP4 or KRP5, does not significantly affect yeast growth. However,
180 co-transformation of CDKB1 (Figure 4A), or CDKB1 and CYCD5 (Figure 4B; Supplemental
181 Figure S1), with either KRP4 and/or KRP5, singly or together, significantly reduces the yeast
182 growth rate. This observation suggests that the complexation of CDKB1 (and CYCD5) with
183 KRP4 or KRP5 can negatively regulate the yeast cell cycle. The lower growth rate when both
184 KRP4 and KRP5 are present suggests a cooperative relationship between KRP4 and KRP5 in
185 their interactions with CDKB1 (and CYCD5).
186 Both rice KRP4 and KRP5 are predicted to function as CDK inhibitors based on sequence
187 homology. We verified their inhibitory function on CDK kinase activity with rice proteins
188 expressed and immunopurified from yeast nuclei. In the assay that detects ADP formation as a
189 readout of kinase activity, base activity of CDKB1 alone was ~52,797 relative fluorescence units
190 (RFU), which increased by ~20% upon the addition of CYCD5 (Figure 4C). On the other hand,
191 CDKB1 activity decreased by 23% and 31% upon incubation with KRP4 and KRP5,
192 respectively. Strikingly, CDKB1 activity dropped by 62% when both KRP4 and KRP5 were
193 added (CDKB1 + KRP4 + KRP5). This additive inhibition effect on the activity of CDKB1 is

194 reversed back by replacing one of the two KRPs with its truncated form (i.e., KRP4m or
195 KRP5m), lacking the CDK-interaction domain. The inhibitory effect of KRP4 and KRP5 was
196 also observed on the activity of CDKB2 (Figure 4D). The kinase activity of CDKB2, estimated
197 at 35,390 RFU, increased by 15% upon incubation with CYCD5, and decreased by 32% and
198 30% by incubating with KRP4 and KRP5, separately. CDKB2 activity decreased by 40% upon
199 incubation with both KRP4 and KRP5 (CDKB2 + KRP4 + KRP5), supporting the additive
200 inhibition of KRP4 and KRP5 on the CDKB activity. The above assays demonstrate the
201 coordinate relationship of KRP4 and KRP5 in their inhibition of CDKB activity.
202
203 **Bimolecular Fluorescence Complementation (BiFC) shows interactions between core cell**
204 **cycle proteins in isolated living rice egg cells and zygotes**
205 Overcoming technical challenges of limited accessibility and cell fragility, we next verified
206 interactions among the putative core rice cell cycle proteins via BiFC in rice egg cells and
207 zygotes, themselves. As shown in Figure 5, A and B, fluorescent signals (green) that appear with
208 the native promoter-driven BiFC constructs evidence *in vivo* interactions of KRP4 and KRP5
209 with CDKB1 in rice egg cells and zygotes. These interactions take place within the nucleus as
210 indicated by the overlap of EYFP signal with a mCherry protein fused with a nuclear localization
211 signal (NLS). This result corroborates previous studies showing the nuclear localization of the
212 KRP proteins (Yang et al., 2011). To examine if both KRP4 and KRP5 can interact with the
213 same CDKB1 *in vivo* as suggested by our yeast assays, we conducted a multi-color BiFC assay
214 (mcBiFC) (Li and Gelvin, 2014) in isolated egg cells. In this assay, the signal is detected in
215 separate channels for interactions of CDKB1-cCFP with KRP4-nVenus and KRP5-nCerulean.
216 As shown by the positive fluorescent signal in Figure 5C, both KRP4 and KRP5 interact with
217 CDKB1 in rice egg cells with very similar patterns, consistent with simultaneous interaction
218 among CDKB1, KRP4, and KRP5.
219 As in the yeast and kinase activity assays, KRP4 and KRP5 also interact in native promoter-
220 BiFC assays in rice egg cells and zygotes (0 and 9 HAP, Figure 5D). Remarkably, we noticed
221 that the apparent relative fluorescent units (RFU) of the transfected cells varies with the stages of
222 zygotic development at harvest (Figure 5E). Compared to the egg cells, the intensity was slightly
223 higher (~ 6.9%) in the zygotes at 2 HAP, but apparently lower (~ 32%, $P<0.05$) in zygotes at 9
224 HAP. Meanwhile, the interaction of KRP5 and CDKB1 with development presents the similar

225 pattern (Figure 5E). This observation seems fit for a model in which both KRP4 and KRP5
226 function as CDKB inhibitors as the cell cycle proceeds to the crucial stage: the zygotic division
227 (at ~12 HAP). However, since the fluorescent intensity observed in BiFC is influenced by
228 different stimuli (Kerppola, 2008), the pattern (Figure 5E) remains to be further confirmed with
229 advanced methods.
230
231 **FB3 protein interacts with KRP4 and KRP5**
232 Based on cell cycle regulation in other organisms, we next sought to determine if a rice F-box
233 protein might interact with KRP4 and KRP5, potentially targeting them for degradation. Of the
234 678 F-box genes annotated in rice, only seven (referred to as *FB1 to FB7* in Supplemental Table
235 S2) are expressed in rice gametes and zygotes according to RNAseq data (Anderson et al., 2013;
236 Anderson et al., 2017). Among them, *FB3* is preferentially expressed in rice egg cells and
237 zygotes (Supplemental Figure S2). We confirmed this result by RT-PCR in which we detected
238 the highest transcript abundance in the rice pistil (0 HAP) compared with other organs (Figure
239 6A). Moreover, Y2H assays revealed that the FB3 protein interacts moderately with KRP4 and
240 strongly with KRP5 (Figure 6B).
241 To test whether interactions between FB3 and KRP4 or KRP5 take place in rice cells, we
242 conducted BiFC, first in rice seedling leaf protoplasts and then in isolated rice egg cells and
243 zygotes. The clear EYFP signal in protoplasts expressing FB3-cEYFP and KRP4-nEYFP (Figure
244 6C) indicates the interaction between FB3 and KRP4 and the nuclear location of the two
245 proteins. Truncated FB3 proteins, i.e., N-terminal -100 amino acids [including the N-terminal F-
246 box domain (ΔF-box)] or C-terminal -111 amino acids [including the C-terminal leucine-rich
247 region domain (ΔLRR)] also interact with KRP4 but at apparently lower level, as shown via
248 fluorescence image quantification (Figure 6C). Similarly, BiFC assays in protoplasts indicate a
249 similar pattern of nuclear-localized interactions between KRP5-nEYFP and FB3-cEYFP,
250 including the detectable interaction between KRP5-nEYFP and the truncated FB3 polypeptides
251 (ΔF-box and ΔLRR)-cEYFP (Figure 6D).
252 BiFC experiments in isolated egg cells and zygotes using constructs with native promoters
253 indicate a similar result. Detection of a nuclear-localized EYFP signal in experiments with egg
254 and zygote cells transfected with $FB3_{pro}$-*FB3-cEYFP* and $KRP5_{pro}$-*KRP5-nEYFP* supports both
255 the co-expression and protein interactions of FB3 and KRP5 (Figure 6F). Similarly, we also

256 detected interactions within nuclei of egg cells and zygotes transfected with *FB3*$_{pro}$*-FB3-cEYFP*
257 and *Ubi*$_{pro}$*-KRP4-nEYFP* (Figure 6E). We conclude that the rice FB3 protein interacts with the
258 KRP4 and KRP5 proteins in the nuclei of rice egg cells and zygotes. This is consistent with a
259 model in which FB3 regulates the inhibitory activities of KRP4 and KRP5 permitting cell cycle
260 progression in rice early zygotes.
261
262 **FB3 degrades both KRP4 and KRP5 via the proteasome pathway**
263 To further test the role of FB3 in controlling the activity of KRP4 and KRP5, we conducted
264 kinase and protein degradation assays with extracts of transformed rice protoplasts, overcoming
265 the technical challenge of very low expression of FB3 in yeast and bacteria. Transformation of
266 rice protoplasts with HA-tagged FB3-CFP and GFP-tagged FB3 under the control of a
267 β-estrogenregulated, inducible promoter system (Sreekala et al., 2005; Hirose et al., 2012) gave
268 proteins of the expected size, 89 kDa for GFP-FB3 and 75 kDa for HA-FB3-cCFP, via western
269 blot (Supplemental Figure S3).
270 Applying the fluorescent ADP detection assay on mixtures of immunopurified protein
271 complexes, we found that the inhibitory effect of KRP4 and KRP5 on CDKB1 and CDKB2
272 kinase activity can be reversed by FB3 (Figure 7A). CDKB1 activity with both inhibitors
273 presents (CDKB1 + KRP4 + KRP5) was estimated at ~20k RFU; however, it increased by
274 ~2.9-fold upon the addition of purified FB3 (i.e., CDKB1 + KRP4 + KRP5 + FB3, Figure 7A).
275 Similarly, inhibited CDKB2 activity (CDKB2 + KRP4 + KRP5) increased by 1.7-fold, its
276 original level, upon addition of the purified FB3 (CDKB1 + KRP4 + KRP5 + FB3). Both
277 scenarios indicate that the two KRPs interact with FB3 and are likely degraded via FB3 mediated
278 proteasome pathway.
279 In addition to interacting with each other, we obtained evidence that KRP4 and KRP5 are
280 degraded by the 26S proteasome. After 30 to 60 min of incubation of purified KRP4 and KRP5
281 with purified FB3 in rice ovary extracts, western blotting with anti-Myc and anti-HA antibodies
282 was used to detect the KRPs. Figure 7B shows that myc-tagged KRP5 and HA-tagged KRP4
283 protein is degraded in rice ovary extracts (DMSO control), but that this degradation is slowed by
284 MG132, a specific small-molecular proteasome inhibitor (Kim et al., 2008). Hence, we conclude
285 that KRP4 and KRP5 degradations are proteasome-dependent, consistent with the involvement
286 of an E3 ligase, such as one that includes FB3.

Intriguingly, the published expression profiles of these genes provide further support for the regulation of KRP4 and KRP5 by FB3. The expression of *KRP4 and KRP5* declines or holds steady after pollination at ~ 2 HAP, and becomes relatively lower by 9 HAP, whereas *FB3* transcript increases (Supplemental Figure S4). These *in vivo* dynamics of transcripts are consistent with degradation of KRP4 and KRP5 by an FB3-mediated proteasome path at or after 9 HAP as the first zygotic division around 12 HAP approaches.

Effects of the cell-cycle gene alteration on rice seed formation

To further investigate the *in vivo* functions of *KRP4*, *KRP5*, and *FB3* in rice embryogenesis and seed formation, we characterized five T-DNA or transposon insertion lines for the corresponding genes, consisting of two T-DNA insertion alleles for *KRP5*, a transposon insertion and a T-DNA activation tagged-allele for *KRP4*, and a transposon insertion allele for *FB3* (Figure 8; Supplemental Table S3 and S4; Supplemental Figure S5-S9). Upon genotyping of the T2 generation, we identified very few homozygous insertion mutants for *krp5-1*, *krp5-2*, and *KRP4-D1*$^{OE/+}$ and thus focused on the characterization of the heterozygotes; whereas, the *krp4-1* line was already homozygous upon the first characterization.

Seed-set rates were significantly reduced relative to the original parental cultivars in all mutants examined (Figure 8), demonstrating the importance of these genes in rice gamete or zygotic development. While the seed-set rate for all parental cultivars under our growth conditions was about 90%, the seed-set rate for *fb3-1*$^{+/-}$ in the T_2 generation was reduced to 46%, which is expected for a gene required either for sperm or egg viability (0.9*50% = 45% expected). The lower seed-set rates in *krp5-1*$^{+/-}$, *krp5-2*$^{+/-}$, *KRP4-D1*$^{OE/+}$ (33%, 28% and 34%, respectively) are consistent with haploid lethality combined with a reduction of zygote viability with unbalanced KRP abundance, i.e., halved protein abundance in the heterozygous *krp5-1*$^{+/-}$, *krp5-2*$^{+/}$ and increased in the *KRP4-D1*$^{OE/+}$. Gametes of these lines could also have decreased viability. Consistent with KRP4 imbalance being detrimental, Yang et al (2011) also observed that overexpression of KRP4 in *indica* rice gave a low seed-set rate of 28 ± 4 (%). On the other hand, the slightly higher seed-set rate for homozygous *krp4*$^{-/-}$ (56%) may be due to a partial ability of its coordinate partner, KRP5, to compensate for KRP4 deficiency in gamete cells. To address the mechanism of reduced seed-set, we examined the mutant line pollen grains and ovaries with light microscopy. No differences were apparent in *krp5-1*$^{+/-}$, *KRP4-D1*$^{OE/+}$, *krp4-1* $^{-/-}$

and fb3-1$^{+/-}$ genotypes *versus* WT in FDA staining for pollen viability and sperm cell morphology via DAPI staining (Supplemental Figure S10). Whereas Kim et al (2008) observed that only a single germ cell nucleus was present in the pollen of the F-box protein mutant *fbl17*, and Yang et al (2011) reported that a 33-kDa secretory protein gene (33SP) promoter driving KRP4 over expression in *indica* rice dramatically reduced pollen viability, this does not seem to be the case in the *japonica* rice mutants characterized here. Still, the sperm cells of these mutants were abnormal. In reciprocal pollination (Figure 8D), we found that WT, used as either mother or father crossed with the mutants, significantly restored seed-set rates in contrast to mutant self-pollination (Figure 8D). Moreover, compared with using mutant pollen to cross-pollinate WT, using WT as the pollen donor with mutant ovaries increased seed-set by 20% for *krp5-1*$^{+/-}$, 24% for *krp4-1* $^{-/-}$ and 38% for *KRP4-D1*$^{OE/+}$ (Figure 8D), suggesting that mutation in KRP4 and KRP5 compromises rice sperm cell functions in fertilization and/or zygote formation and development.

Furthermore, examination of the morphology of the female germ unit and embryo revealed that unlike the normal cellular structure in the WT cultivars [1 egg cell, 2 synergids and 1 central cell (green arrows in Figure 9), in the mutants (*krp5-1*$^{+/-}$, *KRP4-D1*$^{OE/+}$, *krp4-1* $^{-/-}$ and *fb3-1*$^{+/-}$), female germ units were collapsed at the 0 DAP and embryos were abnormally developed at 1 and 3 DAP (red arrows in Figure 9). Putting the above together, either functionally compromised sperm cells, structurally collapsed female germ unit cells or irregularly developed embryos may negatively impact the cell cycle during embryogenesis thus significantly lower rice see-set rate.

To follow up, we conducted additional cross-pollination between two rice mutant lines for two purposes, examining the phenotype of a *krp4krp5* double mutant and *krp4-1* complementation. A double mutant line, *krp4-1*$^{-/-}$*krp5-1*$^{+/-}$**,** would have allowed further exploration of the functional redundancy of the two inhibitors during the rice zygotic cell cycle. However, though all of the few seeds obtained germinated, none of the double mutant plants survived past early seedling stage (Table 1). The low seed-set is consistent with a crucial role of coordination between KRP4 and KRP5 in rice zygotic development and grain formation. The failure of the obtained seedlings to develop suggests that the KRP4-KRP5 complex may have other functions in vegetative development not revealed by the study of hemizygous mutants. The other objective was to complement the mutant *krp4-1*$^{-/-}$ with the *KRP4-D1*$^{OE/+}$ activation tagged line. In this case, cross-pollination was successful though only 3 filled seeds were harvested. We were able to

349 grow these to maturity and found that all the progeny carry KRP4 OE allele by genotyping and
350 their seed-set rate was restored to 74% (Table 1), providing evidence for partial complementation
351 of the KRP4 abundance inbalance in the progeny. Since the mutant krp4-/- plants have a partial
352 seed set of ~ 50% (Figure 8D), only ~ 24% was rescued from the remaining 50% (non-seed set).
353 Therefore, the rescue frequency is actually 50% (ratio of 1:1), as expected from segregation of
354 the complementing *KRP4-D1*^{OE} transgene.
355
356 **Discussion**
357
358 The relationship of KRP4 and KRP5 in plant zygotic cell cycle control and their degradation by
359 rice F-box proteins suggests the important role of the cell cycle proteins in the formation of
360 zygotes and seeds. Based on our research, a model of rice zygotic cell cycle control can be
361 described (Figure 10). The core complex consists of four members: CDKB1, CYCD5, KRP4 and
362 KRP5. This model shows that the activity of CDKB1 is CYCD5-dependent but inhibited by
363 KRP4 and KRP5 prior to zygotic division. KRP4 and KRP5 physically interact to cooperate in
364 inhibiting CDKB1 activity. The advantages of such a relationship for cell cycle control might be
365 improved stabililty and redundancy if one of the two KRPs is dysfunctional. KRP4-KRP5
366 complex formation is supported by multiple lines of evidence, including yeast two hybridization,
367 co-immunoprecipitation, yeast growth, *in vitro* CDK activity, BiFC in rice zygotes and mutant
368 analysis. To our knowledge, this is the first report of a coordinate relationship of CDK inhibitors.
369 Based on coexpression evidence, it seems likely that coordinated function is a universal
370 mechanism for KRP-mediated cell cycle control in plants. For example, both AtKRP6 and
371 AtKRP7 are expressed during spermatogenesis of Arabidopsis (Kim et al., 2008), and both
372 KRP1 and KRP2 are preferentially expressed in rice sperm cells as (Figure 1A).
373 As cell cycle inhibitors, if KRP4 or KRP5 hyper accumulated, zygotes could be prevented from
374 entering G2 or Mitosis, which is supported by our results with *KRP5* overexpression. On the
375 other hand, if KRP4 or KRP5 abundance is reduced, zygotes might have been expected to
376 prematurely advance to G2 or Mitosis, which could result in bigger seeds. Unfortunately, this
377 yield advantage is not observed (Figure 8). Instead, our results suggest that an abundance balance
378 between the KRPs and other cell cycle proteins is crucial for the development of gametes,
379 zygotes, embryo, and endosperm.
380 FBPs compose a superfamily in plants. Through the proteasome pathway, they are involved in

381 many different processes in plant growth and development including cell cycle control, signal
382 transduction, metabolic regulation, floral organogenesis, and senescence. Another important
383 reproductive event, plant sperm cell formation is under the control of two cell cycle inhibitors,
384 AtKRP5 and AtKRP6, which are degraded by the FBL17-associated SCF complex (Kim et al.,
385 2008). Here, we identify FB3 as a novel rice F-box protein preferentially expressed in rice egg
386 cells and zygotes and with a role in degrading both KRP4 and KRP5 via the 26S proteasome
387 pathway. Experimental evidence for this function consists of the protein degradation assay,
388 reversal of KRP-mediated inhibition of CDKB activity, and interaction of FB3 with KRP4 and
389 KRP5 as indicated in Y2H, BiFC, and cellular localization in rice zygotes at different stages. Our
390 results demonstrate that FB3 is a regulator of the two KRP inhibitors for rice zygotic cell cycle
391 control.
392 We also confirmed that mutations in KRP4, KRP5 and FB3 result in the compromised function
393 of sperm cells and abnormal structure of the female germ unit and embryo in the mutants, and
394 hence leading to significantly reduced seed-sets. These results support that balanced ratio of
395 KRP4, KRP5 and FB3 proteins are important for zygotic development. Since the zygote is
396 formed from the combination of one male and one female gamete, defective gametes fail to
397 contribute the three proteins to the zygote. Imbalances of these proteins in the zygote appear to
398 frequently lead to the failure of embryogenesis and seed formation (Figure 9), though more
399 evidence to understand whether these effects are quantitative or qualitative is necessary. Still, our
400 data also provides insight into zygotic cell cycle control in plants.
401 In addition, RNAseq data shows that CDKBs (Supplemental Figure S11A) are present in rice
402 egg cells but preferentially expressed in rice zygotes (9 and 14 HAP), and CYCD5
403 (Supplemental Figure S11B) is preferentially expressed in rice egg cells and zygotes, supporting
404 them as the components in our cell cycle model; meanwhile, some other transcripts like
405 CDKC1;2 and CYCT1;4 are also abundant in rice egg cell and zygotes (Supplemental Figure
406 S11). Further study is required to reveal their functions.
407

408 ## Materials and Methods

409

410 **Plant material**

411 Rice seeds were germinated in the dark at room temperature for 5 days. Seedlings were transplanted to
412 soil and maintained in a greenhouse (27°C during daytime, 12 hours, and 25°C for nighttime, 12 hours)

413 until blooming. The daytime lighting was automatically controlled to 500 μmol $m^{-2}s^{-1}$. Plants were
414 irrigated with deionized water and fertilized twice weekly by filling the headspace in pots with 300-350
415 ppm nitrogen (Jack's 20-10-20). Mutant lines (Table S4) were identified from the Rice Functional
416 Genomic Express Database (http://signal.salk.edu/cgi-bin/RiceGE) and obtained from South Korea
417 (Jeong et al., 2002 and 2006) and UC-Davis (Kumar et al., 2005).
418
419 **RNA isolation and RT-qPCR**
420 For RNA extraction, samples (100 mg each) were collected from 8-week-old plants. Fresh florets were
421 collected and immersed in 0.3 M mannitol for dissecting anthers, lemma/palea, and pistil followed by
422 snap freezing in liquid nitrogen. Total RNA was extracted using Qiagen RNAeasy Plant Mini Kit.
423 Reverse transcription (RT) was performed with RevertAid First Strand cDNA Synthesis Kit (Thermo
424 Scientific). RT-PCR was conducted on BioRad C1000 Touch Thermal Cycler system with Phusion High
425 Fidelity (Thermo Scientific). For RT-qPCR, PowerUp™SYBR™ Green Master Mix Kit (Thermo
426 Scientific) was employed and 18S rRNA gene was used as the internal control based on consistent
427 expression in eight different rice samples of all three biological replicates. The relative transcript
428 abundances from RT-qPCR were calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).
429 Primers are listed in Supplemental Table S5.
430
431 **Yeast two hybridization (Y2H) assays**
432 Y2H was conducted using Matchmaker Gold Yeast Two-Hybrid System (Clontech). The coding
433 sequences (CDS) of rice *KRP1, KRP4, KRP5* and 5`-most-315 bp of *KRP4* (encoding KRP4m, which has
434 90 aa deleted from C-terminus of KRP4) and 5`-most 530 bp of *KRP5* (encoding KRP5m, which has 48
435 aa deleted from the C-terminus of KRP5) were amplified and cloned into vector pGBKT7 as bait
436 constructs; CDSs of CDKs, CYCs and F-box genes (Supplemental Table S2) were cloned into pGADT7 as
437 prey constructs. The transformed or hybrid cells were cultured on the selective medium of DDO
438 (SD/-Trp-Leu) or QDO (SD/-Trp-Leu-His-Ade) both containing X-α-gal and Aureobasidin A (AbA) at
439 30°C for 3 days. Protein-protein interactions between baits and preys are indicted by blue stained patches
440 of yeast growth.
441
442 **Isolation of yeast nuclei for nuclear protein KRP4 and KRP5 assays**
443 The isolation of yeast nuclei was as previously described (Reese et al., 2008) with the following
444 modifications. The yeast cells containing c-Myc or HA-tagged constructs (c-Myc-KRP5, cMyc-KRP4,
445 HA-CDKB1, and HA-CDKB2) were cultured on -Leu-Trp plate at 30°C for 3 days, then cultured in
446 -Leu-Trp media (up to 200 ml). The collected yeast cells were treated with cell-wall enzyme solution

447 containing 2-3 mg Zymolyase 100T (USB) (30°C, 2 hs). Spheroplasts were pelleted (4500 ***g***, 4°C, 5
448 minutes) in 10 ml -Leu-Trp/S, washed twice in 25 ml cold -Leu-Trp/S and once in 20 ml 1M Sorbitol (ice
449 cold), then homogenized on ice with Nuclei buffer (30 mM Hepes pH7.6, 0.05 mM $CaCl_2$, 5 mM
450 $MgSO_4.7H_2O$, 1 mM EDTA, 10% Glycerol, 0.5% NP40, 7.2 mM Spermidine Hexahydrate with protease
451 inhibitors (Sigma), PMSF (1 mM) and ß-me (2 mM). After removing cellular debris, nuclei were
452 collected from the supernatant (14000 ***g***, 4°C, and 10 min) and stored at -20°C.
453
454 **Co-immunoprecipitation (Co-IP) assay**
455 Pierce™ Co-Immunoprecipitation Kit (Thermo Scientific™) was used. Frozen yeast nuclei were
456 suspended in Lysis Buffer containing 25 mM Tris (pH7.5), 150 mM NaCl, 1 mM EDTA, 1% NP40, 5%
457 glycerol 10 mM NaF, 1 mM Na_3VO_4 and freshly added with protease inhibitor cocktail (Sigma) plus
458 PMSF (1 mM) and incubated on ice for 10-15 minutes with agitation. The cell debris was removed by
459 centrifugation (13,500 ***g***, 4°C, and 8 min), and resulting supernatant was saved on ice: 1 µl was used to
460 determine protein concentration and the rest was precleared with 40 µl control agarose beads. About 25
461 µg of anti-HA (GenScript) or anti-c-Myc mAb (GenScript) were coupled with 50 µl beads and incubated
462 with precleared proteins at 4°C for overnight with gentle mixing. Finally, proteins were eluted with
463 elution buffer (pH 2.8) containing primary amine.
464
465 **Western blotting**
466 The eluted protein (~10 µg) was separated within 12% SDS - PAGE and transferred to PVDF membrane
467 (Bio-Rad). The membrane was blocked for 1 hour, then incubated with primary antibody (Anti-HA or
468 anti-c-Myc, mouse mAb) at 4°C for overnight, washed for three times and incubated with horseradish
469 peroxidase (HRP) - conjugated secondary antibodies (goat anti-mouse, Invitrogen) for 1 hour. After
470 washing the membrane was detected with One Step Ultra TMB-blotting Solution (Thermo Fisher) or
471 SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific).
472
473 **CDK activity assay**
474 Kinase activity was measured using ADPsensor™ Universal Kinase Activity Assay Kit (BioVision).
475 HA-tagged CDKB1, CDKB2 and CYCD5 and c-Myc-tagged KRP4 and KRP5 were purified from yeast
476 nuclei, and FB3-GFP from suspension-cultured rice protoplasts, by immunoprecipitation in antibody
477 (each 20 µg) immobilized agarose beads (each 50 µl) from Pierce™ Co-Immunoprecipitation Kit
478 (Thermo Scientific™). For each assay, 1 µg of purified protein was used; for 1 µg of FB3, 6 µl of rice
479 ovary extract (containing 26S proteasome) was also added. The fluorescence was scanned at Ex/Em =
480 535/587 nm in a multimode plate reader (BioTek, Synergy™ HT).
481

Rice cell isolation and transfection for BiFC and cellular localization

Isolation and transfection of rice leaf protoplasts were conducted as previously described (Wang et al, 2013). Isolation of rice egg cells and zygotes was conducted as previously described (Li et al., 2019, 2020 and 2022). Transfection of isolated rice egg cells and zygotes was based on previous reports (Koiso et al., 2017; Toda et al., 2019) with modifications. First, several isolated cells were transferred on to a micro slide (within a moisture chamber) in 8 µl MMG (4 mM Mes-KOH, pH5.7, 15 mM $MgCl_2$ and 0.4 M mannitol), added with 1-2 µg plasmid DNA and 10 µl of 30% PEG with 100 mM $CaCl_2$ in 0.4 M mannitol, followed by gently mixing with a micro-pipet. After incubation for 10 min, the cells were carefully washed 3 times with fresh MMG (capillary replacement of ~ 10 µl each time), followed by 3 more washes in modified W5 solution (W5: 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES; plus 500 mM Glucose). Then, the cells were cultured with ~ 10 µl of the modified W5 solution containing Ampicillin (100 µg/ml) at 25°C overnight in a dark moist chamber.

FDA and DAPI staining of and rice cell and pollen

To examine viability, isolated rice cells or pollen were treated with FDA (Fluorescein diacetate, 0.2 µg in 1 ml of 0.6 M Mannitol) in the dark for 5-10 minutes. To observe cellular nuclei, isolated rice cells or pollen were stained with DAPI (4',6-diamidino-2-phenylindole, 0.4 µg in 1 ml of 0.1 M sodium phosphate pH 7.0, 1 mM EDTA, 0.1% Triton X-100 and 0.6 M Mannitol) in the dark for 10-30 minutes.

Constructs with CaMV 35S promoter for cellular localization in rice cells

The CDSs of *KRP4*, *KRP5*, and *FB3* were obtained via PCR with specific primers (Table S5) and Q5 High Fidelity DNA Polymerase (NEB), then cloned into vector pE3150 (Lee and Gelvin, 2014). Vector pE3275 encoding mCherry with a nuclear localization signal (NLS) was used for nuclear localization.

Constructs for Bimolecular Fluorescent Complementation (BiFC)

The CDS of *KRP4* and *KRP5* were cloned into pE2913 (Lee and Gelvin, 2014) for construct *KRP4-nEYFP or KRP5-nEYFP*, and the CDS of *CDKB1*, *KRP4*, and *FB3* into pE2914 (Lee and Gelvin, 2014) for *CDKB1-cEYFP, KRP4-cEYFP* or *FB3-cEYFP*; the rice promoter *KRP5* ($KRP5_{promoter}$), maize promoter *Ubiquitin* ($Ubiq_{promoter}$), and rice promoter *CDKB1* ($CDKB1_{promoter}$) were added to 5' termini of the construct *KRP5-nEYFP, KRP4-nEYFP, KRP4-cEYFP,* and *CDKB1-cEYFP*, respectively. To test the interaction of CDKB1 and KRP4 or KRP5, the construct $CDKB1_{promoter}$-*CDKB1-cEYFP* was linked to $Ubi_{promoter}$-*KRP4-nEYFP* and $KRP5_{promoter}$-*KRP5-nEYFP*, separately, using Gibson Assembly kit (NEB). To test the interaction of KRP4 and KRP5, the construct $Ubi_{promoter}$-*KRP4-cEYFP* was linked to $KRP5_{promoter}$-*KRP5-nEYFP* via Gibson Assembly. Primers are listed in Table S5.

Constructs for Multi-Color BiFC (mcBiFC)

The CDS of *KRP4* was cloned into pE3233 (Lee and Gelvin, 2014) for construct *KRP4-nVenus*, CDS of *KRP5* into pE3247 (Lee and Gelvin, 2014) for *KRP5-nCerulean*, and CDS of *CDKB1* into pE3449 (Lee and Gelvin, 2014) for *CDKB1-cCFP*; then promoters (maize $Ubiquitin_{promoter}$, rice $KRP5_{promoter}$, and rice $CDKB1_{promoter}$) were added to 5' termini of construct *KRP4-nVenus, KRP5-nCerulean* and *CDKB1-cCFP*, respectively. The three cassettes were linked in the same vector by Gibson Assembly with primers (Supplemental Table S5).

Suspension culture to express FB3 protein in rice protoplasts

Two methods were used to express FB3 in protoplasts. One introduced HA-tagged FB3 CDS into pE3449 (Lee and Gelvin, 2014), then transfected rice leaf protoplasts with FB3-HA (Wang et al., 2013) for protein extraction. The other used a ß-estradiol inducible FB3 expression system. For this, the CDS of *FB3* was linked to GFP in pUH-GFP2 (Sreekala et al., 2005) and transfected into leaf protoplasts. The transfected cells were cultured for 6-9 days (26°C, dark with shaking at 80 rpm) in Chu's medium (3.99 g/l, Caisson Cat# CHP03) with Hygromycin (35 mg/l), Ampicillin (100 mg/l) and ß-estradiol (inducer, 20 µM) plus supplementals (Chen et al., 2017; Chen et al., 2006; Kim et al., 2008): 2,4-D (2 mg/l), casamino acids (300 mg/l), L-glutamine (500~1000 mg/l), L-proline (1000 mg/l), pyridoxine (1.0 mg/l), kinetin (0.2 mg/l), biotin (0.01 mg/l) and retinol (0.01 mg/l) and Sucrose (30 g/l.). Fresh sterile Chu's medium was used to expand the culture (1:3 in volume) every 3 days.

Protein extraction from rice ovary (for 26S proteasomes with SCF complexes)

About 200 ovaries were homogenized on ice in 0.5 ml protein degradation buffer [25 mM Tris pH7.4, 150 mM NaCl, 1 mM EDTA, 1% NP40, and 5% glycerol with freshly added plant protease inhibitor cocktail (Sigma) and 1 mM PMSF]. Cellular debris was pelleted at 3000 ***g***, 4°C for 5 min, and the supernatant was aliquoted and stored at -20°C.

Protein degradation assay

The purified proteins of KRP4 and KRP5 (tagged with c-Myc) and FB3 protein (tagged with HA or GFP) were added to a degradation assay based on Kim et al (2008). Briefly, ovary extract (100 µl) was pretreated with the proteasome inhibitor, MG132 (10 µM), to slow protein degradation. Extracts were then mixed with purified KRP4 or KRP5 and FB3 proteins (1 µg each); in the control, MG132 was replaced by DMSO (1%) for normal protein degradation. The two mixtures were incubated at 4°C and sampled (16 µl) every 30 minutes. KRP4 or KRP5 was detected with anti-HA or anti-Myc antibody in western blot, and Actin was used as the loading control.

552 **Genomic DNA isolation and genotyping**

553 Leaf tissue was collected from young plants (~200 mg, 2-4 weeks) and frozen in liquid nitrogen followed
554 by grinding in Geno Grinder (SPEX SamplePrep 2010) at 1450 rpm for 60 seconds. The sample was
555 mixed with 200 µl of Extraction Buffer (100 mM Tris-Cl, pH 8.0, 1 M KCl and 10 mM EDTA), heated
556 (60°C, 30 min) and extracted with 600 µl of dH_2O (14,000 ***g***, 10 min). Supernatant (1 µl) was used for
557 genotyping with primers (Supplemental Table S5).

558

559 **Seed-setting assay**

560 Rice seeds (1000) were harvested from 3-5 plants of each wildtype or mutant line and counted for the
561 number of filled or aborted seeds. The seed-set rate was calculated as (number of filled seeds / total
562 number of seeds) x 100%.

563

564 **Cross-pollination**

565 Crossing was conducted following the McCouch Lab procedure
566 (http://ricelab.plbr.cornell.edu/cross_pollinating_rice). To ensure pollination, the glassine bags covering
567 panicles for pollination were tapped every half hour from 11 am to 2 pm for 3 days. The seeds were
568 counted in 1 week and harvested in 4 weeks.

569

570 **Light microscopy of rice ovary**

571 Based on previous description (Skepper and Powell, 2008). 3-5 rice ovaries were dissected from mature
572 florets without pollination (day 0) or 1 or 3 days after pollination, and fixed with 2% paraformaldehyde
573 (PFA) and 2% glutaraldehyde (GA) in 50 mM PIPES (pH 6.8), with 1 mM $MgSO_4$, 5 mM EGTA. After
574 dehydration, samples were infiltrated with LR White resin (Sigma), then transferred to gelatin capsules
575 containing fresh resin with the appropriate orientation and polymerized at 60°C for 24-48 hours in a fume
576 hood. After sectioning with a microtome, sections (2 µm) were stained with Toluidine Blue O (0.5% in
577 2% Sodium Borate) for microscopy.

578

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580

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592

593 ## Author Contributions

594

595 H.X. designed and performed all experiments, analyzed data, and drafted the manuscript. L.B. organized
596 and managed analysis of multiple rice mutants, assisted with qPCR performance and participated in
597 manuscript preparation. M.L. provided vectors and participated in molecular cloning and manuscript
598 preparation. V.S. coordinated and supported the entire research project and provided rice mutant lines,
599 RNA-seq data and instructive suggestions. H.F. managed rice plant growth and participated in the routine
600 cell isolation. S.R. supervised and supported the entire research project, managed rice cell isolation, and
601 participated in manuscript preparation.
602

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Figures and Table

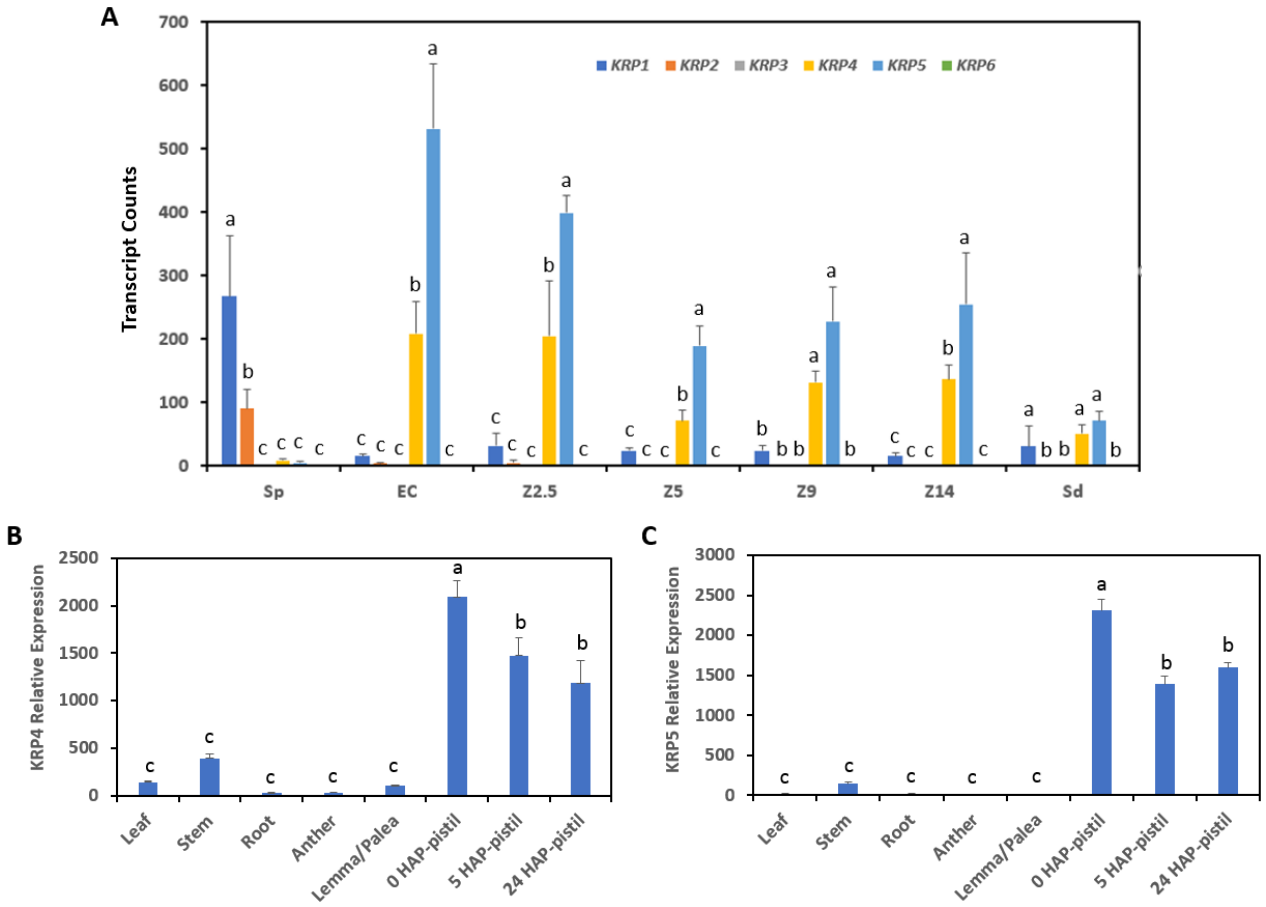


Figure 1 *KRP4* and *KRP5* genes are preferentially expressed in rice egg cells and zygotes. A, Transcription of 6 rice *KRP* genes in rice egg cells and zygotes, based on RNAseq data (Anderson et al., 2013; Anderson et al., 2017). Sp, sperm cell; EC, egg cell; Z2.5, zygote at 2.5 hours after pollination (HAP); Z5, zygote of 5 HAP; Z9, zygote of 9 HAP; Z14, zygote of 14 HAP; Sd, seedling. B, RT-qPCR expression profiles of *KRP4* in different organs. C, RT-qPCR expression profiles of *KRP5* in different organs. Data are means of 3 biological replicates and error bars represent the standard deviation. Significantly different groups are indicted with different letters determined by one-way ANOVA (with Tukey's post-hoc test, $P < 0.05$).

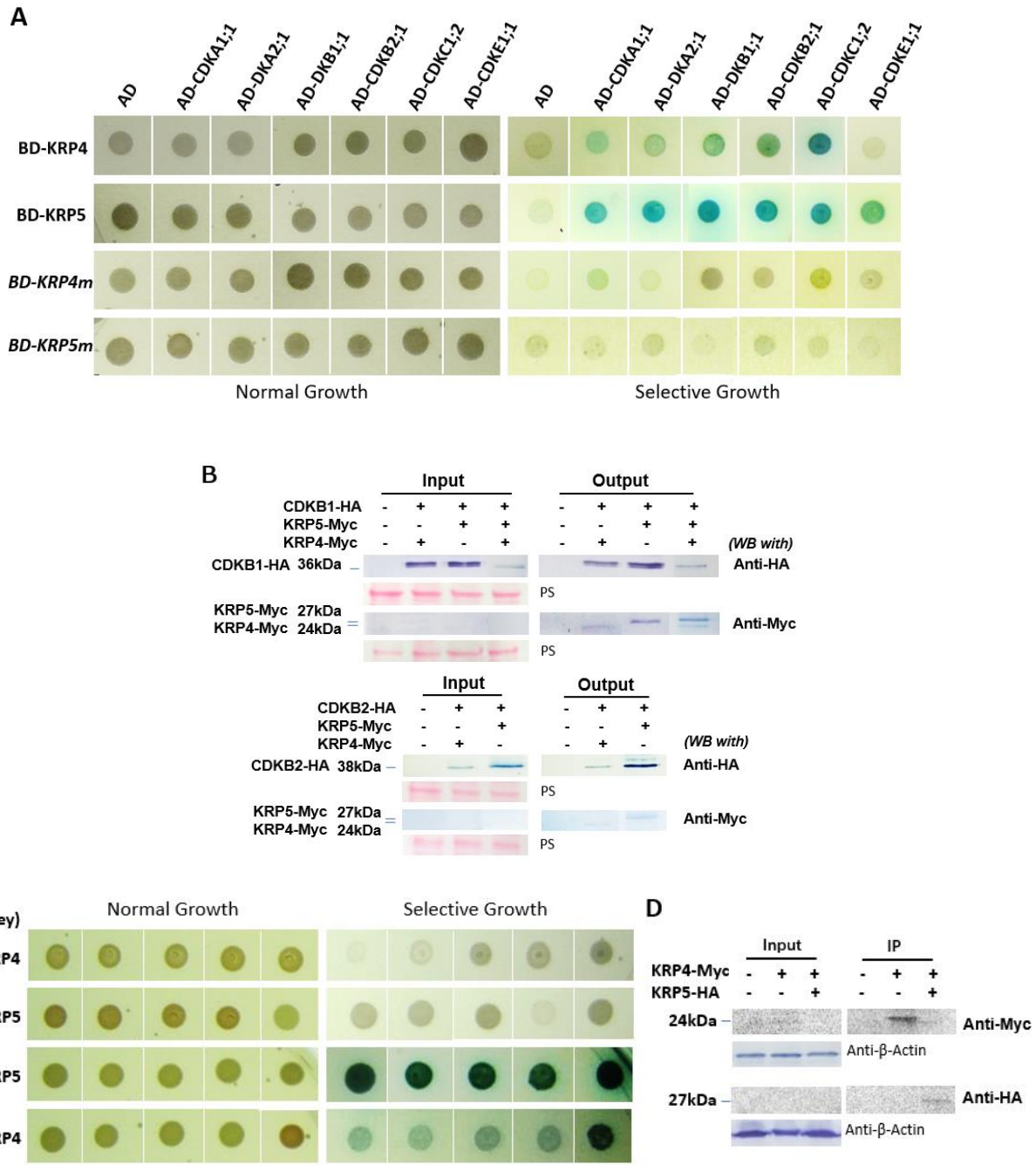
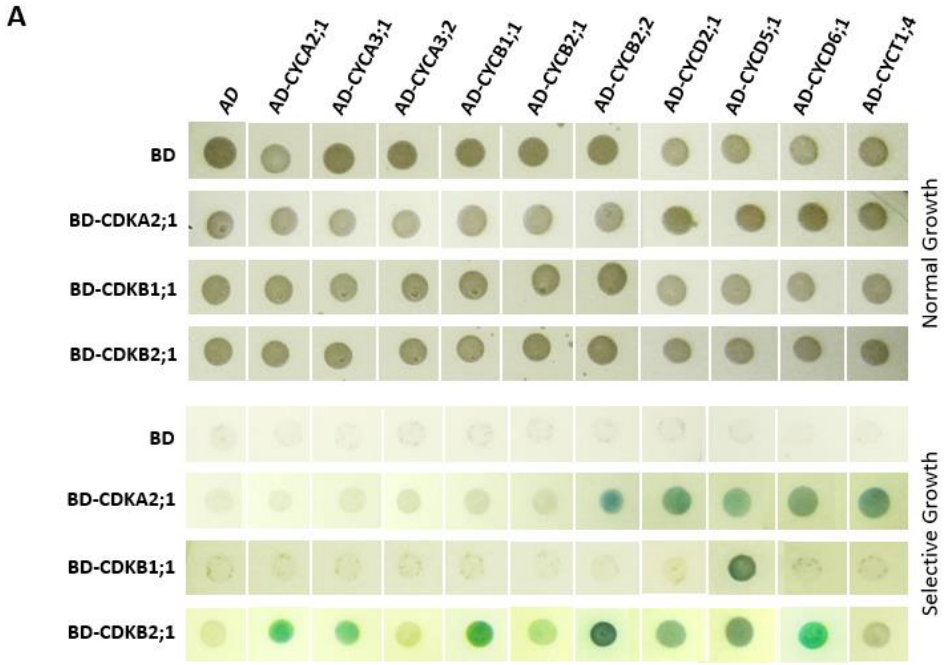


Figure 2 Interactions among rice cell cycle proteins expressed in yeast (I). A, Interactions of KRP4 and KRP5 with CDKs. The blue spots on the selective medium indicate interactions of KRP4 or KRP5 with rice CDKs. The prey vector pGADT7 (AD), and both truncated KRP4 and KRP5 (i.e., C-terminal truncations KRP4m and KRP5m) as baits were used as negative controls. Similar results were observed in three independent assays. In A and C, The growth control is on DDO (SD-Trp-Leu) and the selective growth is on QDO (SD-Trp-Leu-His-Ade) with X-α-gal and Aureobasidin A (AbA), at 30°C for 3 days. B, Co-immunoprecipitation (Co-IP) for interactions of KRP4 and KRP5 with CDKB1 or CDKB2 was

conducted with Anti-c-Myc antibody using nuclear proteins extracted from the yeast cells harboring CDKB1-HA with KRP4-c-Myc and KRP5-c-Myc (the upper panel) or harboring CDKB2-HA with KRP4-c-Myc or KRP5-c-Myc (the lower panel). Proteins on Western blots were detected with Anti-HA and anti-c-Myc antibodies. Equal loading of protein samples is indicated by Ponceau staining (PS). C, Y2H was used to detect the interaction (indicated by blue spots) of KRP4 and KRP5. KRP1 was used as the bait for the control. Similar results were obtained from two independent experiments. D, Co-IP (lower panel) was conducted with Anti-c-Myc for the interaction between KRP4 and KRP5; the Western blots were probed with Anti-HA and Anti-c-Myc and visualized in a chemiluminescent system. Actin detected with Anti-ß-Actin was used as a loading control.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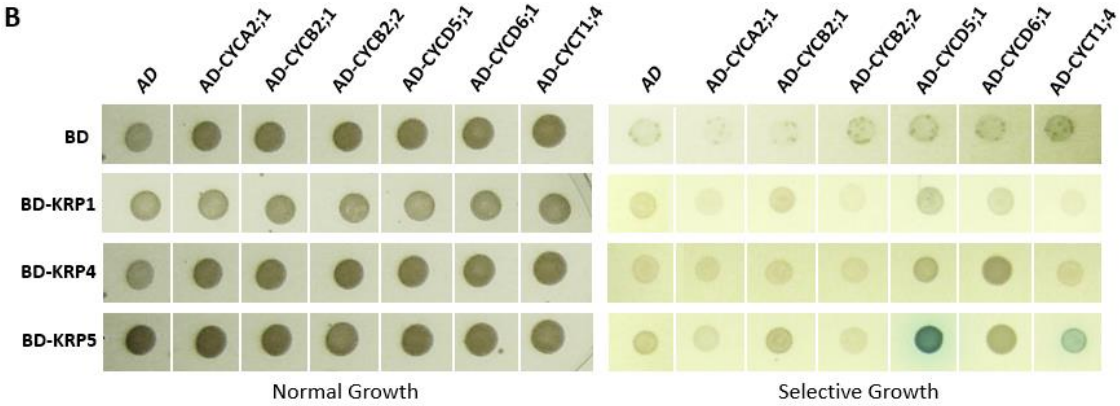
AD
AD-CYCA2;1
AD-CYCA3;1
AD-CYCA3;2
AD-CYCB1;1
AD-CYCB2;1
AD-CYCB2;2
AD-CYCD2;1
AD-CYCD5;1
AD-CYCD6;1
AD-CYCT1;4
BD
BD-CDKA2;1
BD-CDKB1;1
BD-CDKB2;1
Normal Growth
BD
BD-CDKA2;1
BD-CDKB1;1
BD-CDKB2;1
Selective Growth

62


B
AD
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AD-CYCA2;1
AD-CYCB2;1
AD-CYCB2;2
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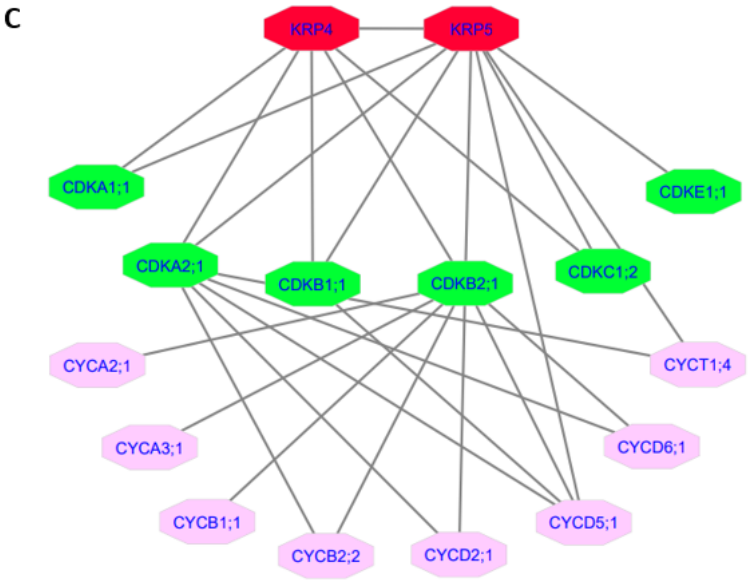
BD
BD-KRP1
BD-KRP4
BD-KRP5
Normal Growth
Selective Growth

63


C
KRP4
KRP5
CDKA1;1
CDKE1;1
CDKA2;1
CDKB1;1
CDKB2;1
CDKC1;2
CYCA2;1
CYCT1;4
CYCA3;1
CYCD6;1
CYCB1;1
CYCD5;1
CYCB2;2
CYCD2;1

64

Figure 3 Interactions among rice cell cycle proteins expressed in yeast (II). A, Interactions of CDKA or CDKB with different rice CYCs (blue spots). CDKB1 interacted only with CYCD5. Three independent assays were performed with similar observations. B, Interactions of KRPs with different rice CYCs (blue spots). KRP5 interacts with CYCD5 and CYCT1:4. A similar outcome was obtained in three separate assays. In A and B, both bait vector (BD) and prey vector (AD) were used as negative controls. The growth control is on DDO (SD-Trp-Leu) and the selective growth is on QDO (SD-Trp-Leu-His-Ade) with X-α-gal and Aureobasidin A (AbA), at 30°C for 3 days. C, Scheme representing Y2H-based interactome of cell cycle core complex components in rice egg cells and zygotes displayed with Cytoscape 3.8.2.

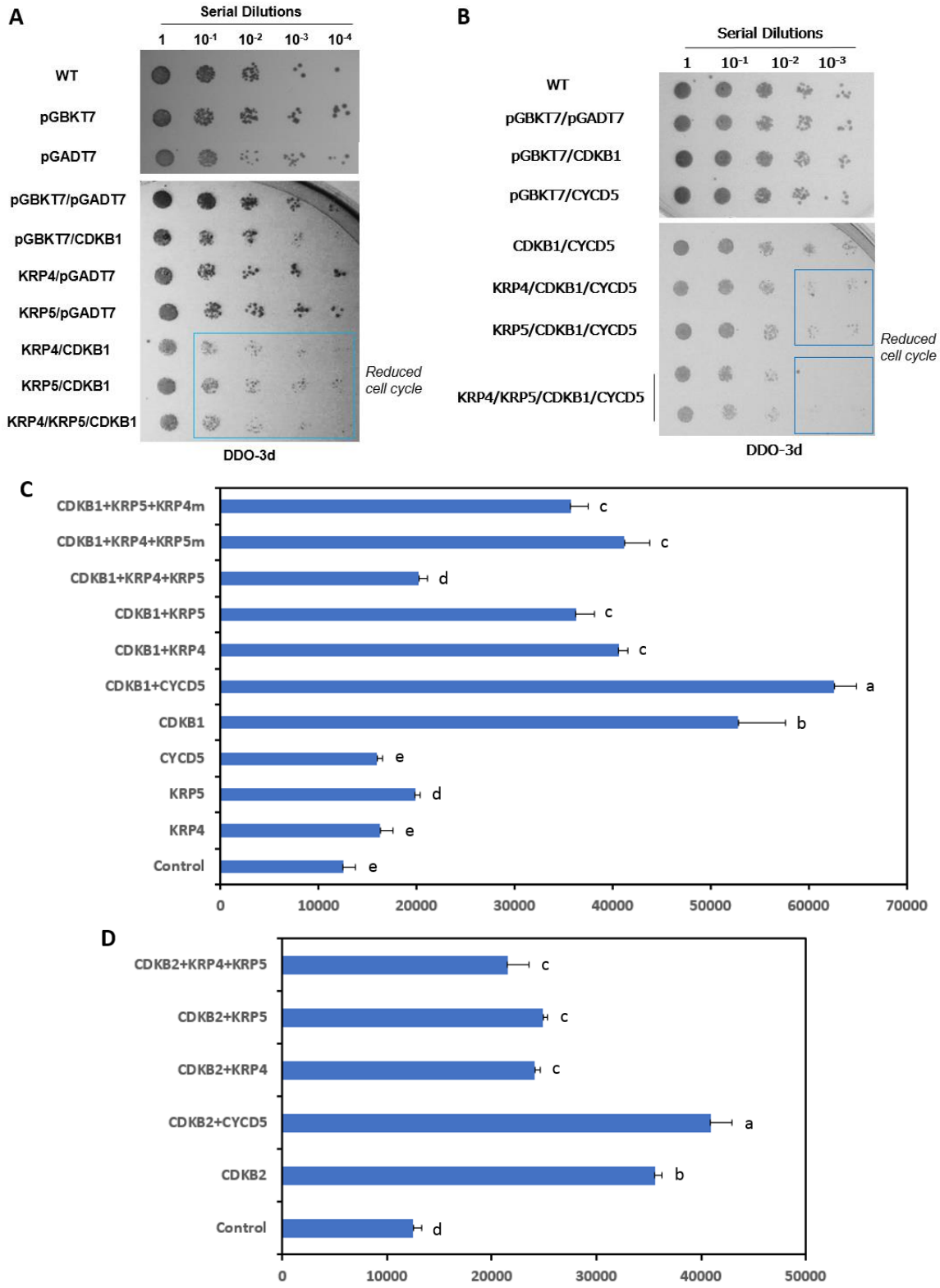


Figure 4 KRP4 and KRP5 coordinately interact with CDKB. A, Growth of serial dilutions of yeast cells transfected with rice core cell cycle genes indicating the interactions between rice CDKB1 with KRP4

and KRP5. B, Growth of serial dilutions of yeast cells transfected with core rice cell cycle genes indicating interactions of rice CDKB1-CYCD5 with KRP4 and KRP5. In A and B, BD represents the bait vector, pGBKT7; AD, the prey vector pGADT7. DDO-3d, growth on the medium SD-Trp-Leu for 3 days. Blue boxes demark the inhibition of yeast growth due to the presence of cell cycle inhibitory complexes from rice. Similar results were observed in 3 independent experiments. C, ADP Sensor^{TM} kinase activity assay showing coordinated inhibitory effect of KRP4 and KRP5 on Kinase Activity of CDKB1. D, Kinase activity assay showing coordinated inhibitory effect of KRP4 and KRP5 on kinase activity of CDKB2. In C and D, Kinase activity was measured in relative fluorescent units (RFU). – Control: reaction containing no recombinant protein eluted in Co-IP using yeast cells harboring only the empty pGBKT7 and pGADT7 vectors. CYCD5, rice Cyclin D5;1. The activities of CDKB1 (C) and CDKB2 (D) are increased by CYCD5 but maximally decreased by both KRP4 and KRP5. KRP4m, the C-terminally truncated protein encoded of KRP4 (N-terminal -105 amino acids); KRP5m, C-terminally truncated protein of KRP5 (N-terminal -177 amino acids). Values in C and D are mean ± standard deviation, N=3 biological replicates. Letters indicate significant differences among group means determined by one-way ANOVA analysis (with Tukey's post-hoc test, $P < 0.05$).

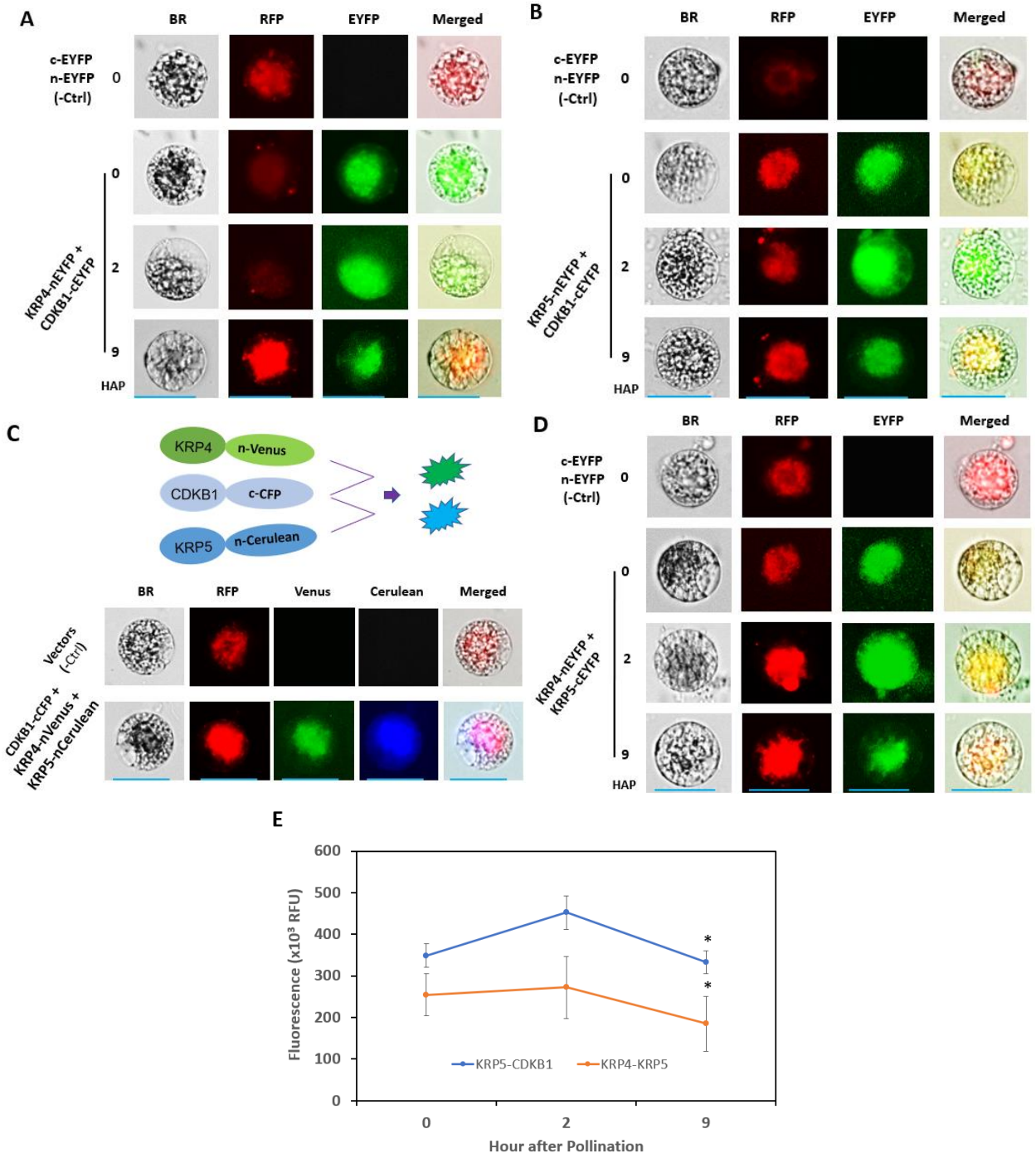


Figure 5 Bimolecular Fluorescent Complementation (BiFC) demonstrates interactions of core cell cycle complex components in rice egg cells and zygotes. A, Representative images of BiFC for the interactions of KRP4 with CDKB1 in the egg cell and zygote (harvested at 2 and 9 HAP) transfected with the construct *Ubi*$_{pro}$*-KRP4-nEYFP + CDKB1*$_{pro}$*-CDKB1-cEYFP*. The observation had 2 biological replicates (N = 3 cells in each). B, Representative images of BiFC shows the interactions of KRP5 with CDKB1 in the egg cell (0

HAP) and zygotes (2 and 9 HAP) transfected with the construct *KRP5*$_{pro}$*-KRP5-nEYFP* + *CDKB1*$_{prom}$*-CDKB1-cEYFP*. HAP, hours after pollination. The observation had 3 biological replicates (N = 3-6 cells in each). In A and B, empty vectors containing *nEYFP* and *cEYFP* were used for the negative control (-Ctrl); BR show cell images under bright field illumination; RFP shows the localization of mCherry$_{NLS}$, where NLS stands for nuclear localization signal; EYFP shows the interaction of the two partner proteins; the merged image is compiled from all three images. C, Representative images of multi-color BiFC (mcBiFC) for the interactions of both KRP4 and KRP5 with CDKB1 in rice egg cells transfected with the constructs for CDKB1-cCFP + KRP4-nVenus + KRP5-nCerulean under native promoters. Empty vectors (with cCFP, nVenus and nCerulean) were used as the negative control; BR shows cell images in gray under bright field illumination; RFP represents mCherry$_{NLS}$, used for nuclear localization; Venus indicates the interaction of KRP4 with CDKB1, and Cerulean the interaction between KRP5 and CDKB1; Merged is created from overlapping other three images. The observation had 2 biological replicates (N = 3 cells in each). D, Representative images of BiFC for the interaction of KRP4 and KRP5 in rice egg cells and zygotes (2 and 9 HAP at harvest) transfected with the construct *Ubi*$_{pro}$*-KRP4-nEYFP* + *KRP5*$_{pro}$*-KRP5-cEYFP*. For each assay (A-D), scale bars: 50 µm. E, Mean fluorescence from cellular images of BiFC between KRP4 and KRP5 (D) plus those between KRP5 and CDKB1 (B). Each observation had 3 biological replicates (N = 3-6 cells in each). The error bars denote the standard deviation. The * indicates the significant difference of apparent protein interaction levels at 9 HAP vs 2 HAP via one-way ANOVA test ($P < 0.05$).

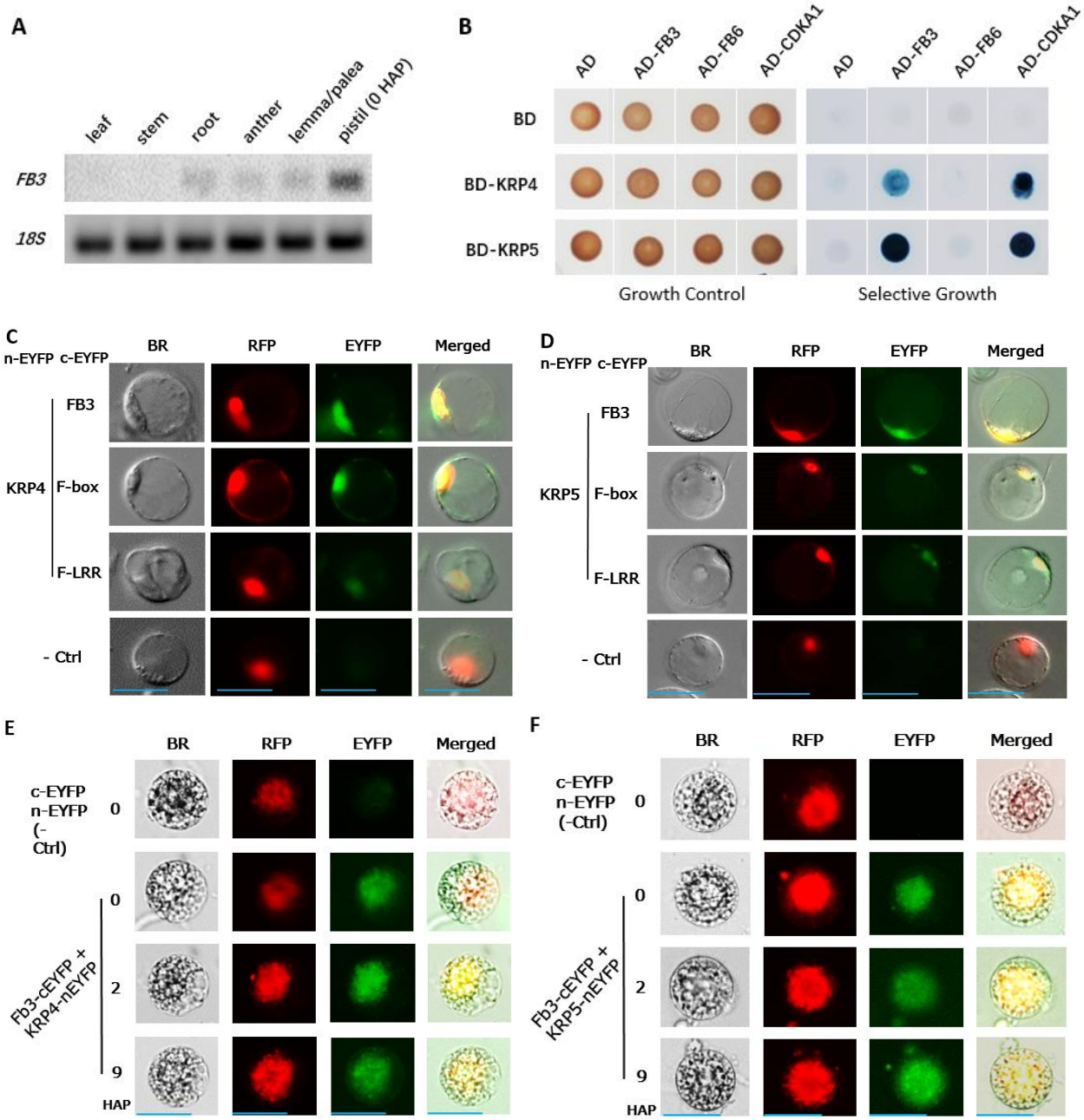


Figure 6 The rice FB3 protein interacts with CDK inhibitors, KRP 4 and KRP5. A, Gel image of RT-PCR products showing expression of FB3 in six different rice organs collected from flowering rice plants (HAP, hours after pollination). 18S rRNA was used as the loading control. B, Interactions between rice F-box proteins with rice CDK inhibitors, KRP4 or KRP5, detected in Y2H (blue spots). The preys include CDKA1 (+ control), two F-box proteins, FB3 and FB6, and the empty prey vector (AD); the baits are KRP4, KRP5, and the empty bait vector (BD). Yeast cells were grown on SD-Trp-Leu and the selective medium containing X-Gal and AbA. C, Representative images of Bimolecular Complementation (BiFC) indicating the interaction of FB3 with KRP4 in rice leaf protoplast. The protoplasts were co-transfected

with *35Spro-FB3-cEYFP*, *35Spro-FB3*$_{F\text{-}box}$*-cEYFP* or *35Spro-FB3*$_{LRR}$*-cEYFP* with *35Spro-KRP4-nEYFP* and *35Spro-RFP (mCherry*$_{NLS}$*)*. D, Representative images of BiFC indicating the interaction of FB3 with KRP5. Leaf protoplasts were co-transfected by *FB3-cEYFP*, *FB3*$_{F\text{-}box}$*-cEYFP* or *FB3*$_{LRR}$*-cEYFP* with *KRP5-nEYFP and RFP (mCherry*$_{NLS}$*)*. In C and D, transfection with *nEYFP* + *cEYFP* was used as -Ctrl. BR indicates bright-field images. EYFP indicates the YFP signal from BiFC protein-protein interactions. RFP represents mCherry linked with Nuclear Localization Sequence (NLS) as the nuclear marker. Merged refers to the overlaid image from the other three in each transfection. Scale bars, 30 µm. E, Representative images of BiFC to detect interactions of FB3 with KRP4 in egg cells and zygotes (2 and 9 HAP). Cells were transfected with the construct *FB3*$_{pro}$*-FB3-cEYFP+Ubi*$_{pro}$*-KRP4-nEYFP*. F, Representative images of BiFC to detect interactions of FB3 with KRP5 in egg cells and zygotes (2 and 9 HAP). The cells were transfected with the construct *FB3*$_{pro}$*-FB3-cEYFP+KRP5*$_{pro}$*-KRP5-nEYFP*. In E and F, both *nEYFC* and *cEYFC* were used as - Ctrl; BR, cells under the bright field; mCherry$_{NLS}$, indicates the location of the nucleus; Merged, the result of overlapping the other three images. Each combination (C-F) was repeated in three independent experiments with N = 3-6 cells per experiment. Scale bars 50 µm.

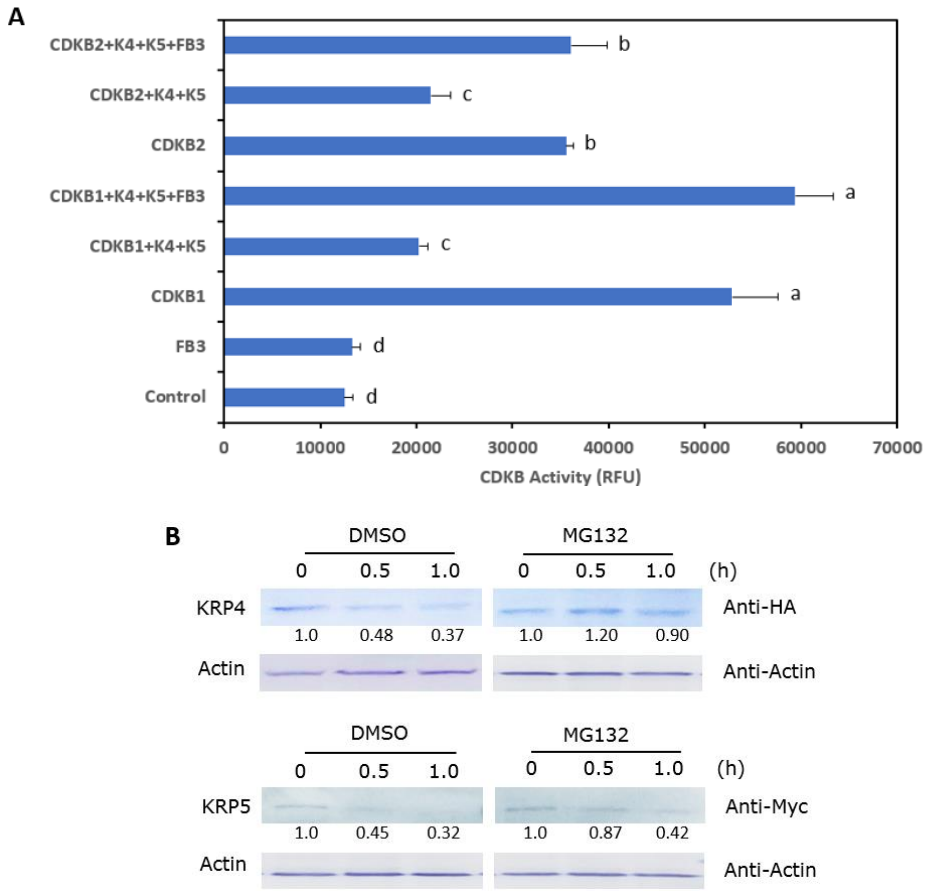


Figure 7 FB3 and the proteasome regulates KRP4 and KRP5. A, Rice FB3 protein counteracts the inhibitory effect of KRP4 and KRP5 on kinase activities of CDKB1 and CDKB2. Kinase activity was measured in relative fluorescent units. The Control is with the "mock" Co-IP from extracts of yeast that express only empty vectors, i.e., the bait vector pGBKT7 and the prey vector pGADT7. Data are the mean ± standard deviation (N = 3 biological replicates). Values in A are the mean ± standard deviation, N = 3 biological repeats. Different letters indicate significantly different groups determined by one-way ANOWA analysis ($P < 0.05$). B, The 26S proteasome-based degradation of KRP4 (the upper) or KRP5 (the bottom). Purified FB3 and KRP4 or KRP5 were mixed with rice ovary extract containing the protease inhibitor cocktail and treated with the specific proteasome inhibitor (MG132) or DMSO (control), incubated at 4ºC for 0, 0.5, or 1 hour (h), and detected via western blot with anti-c-Myc and anti-HA. Quantification of band intensity is shown in numbers normalized to KRP4 OR KRP5 at 0h, and Anti-actin antibody was used to show equal loading.

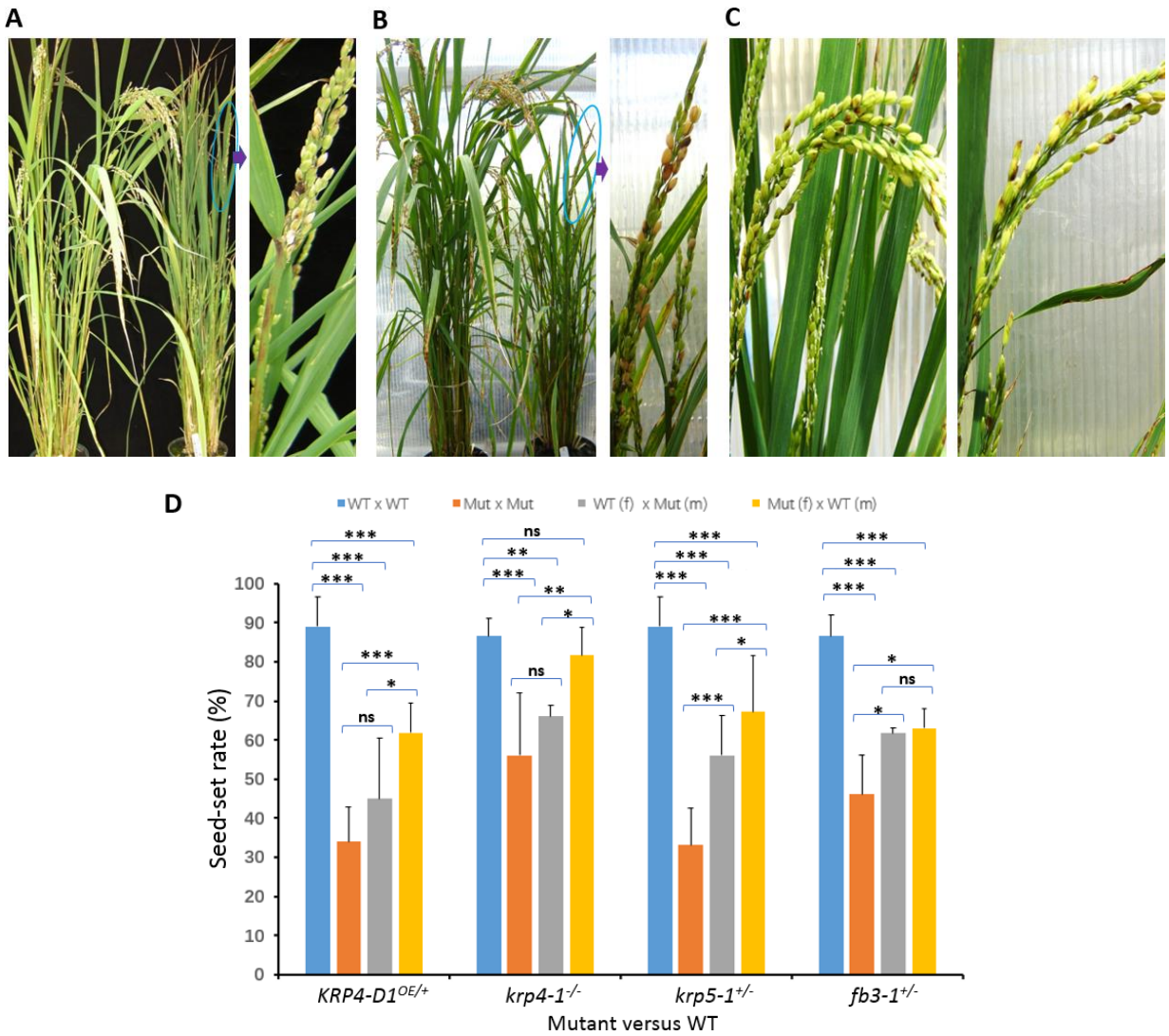


Figure 8 Mutations of KRP5, KRP5, and FB3 genes significantly reduce rice seed-set rate. A, Representative WT (left) and KRP4-over-expression mutant *KRP4-D1*$^{OE/+}$ (right) plants. B, Representative WT (left) and the heterozygous KRP5 knockout mutant *krp5-1*$^{+/-}$ (right) plants. C, Representative rice panicles of WT (left) and the heterozygous FB3 knockout mutant *fb3-1*$^{+/-}$ (right). D, The reduced seed-set rate in transgenic mutant lines in the T_2 generation (n = 1000 seeds counted from 4 rice plants per line) and the partially restored seed-set rate from reciprocal pollination between rice transgenic mutants and WT in the F_o generation (n = 100 emasculated florets in multiple plants for each reciprocal pollination). Compared with the pollination using WT as female, pollination using WT as male significantly restored the seed-setting rates which were reduced in mutants of *krp4-1* $^{-/-}$, *KRP4-D1*$^{OE/+}$, *krp5-1*$^{+/-}$ *and fb3-1*$^{+/-}$. Each cross is written such that the first parent listed is female (f) and the second listed parent is male (m). Columns are mean values and error bars are the standard deviation. One-way ANOVA analysis was performed to determine the significant differences among the data of different

genotypes: *** indicates the difference at $P < 0.001$, ** at $P < 0.01$, and * at $P < 0.05$.

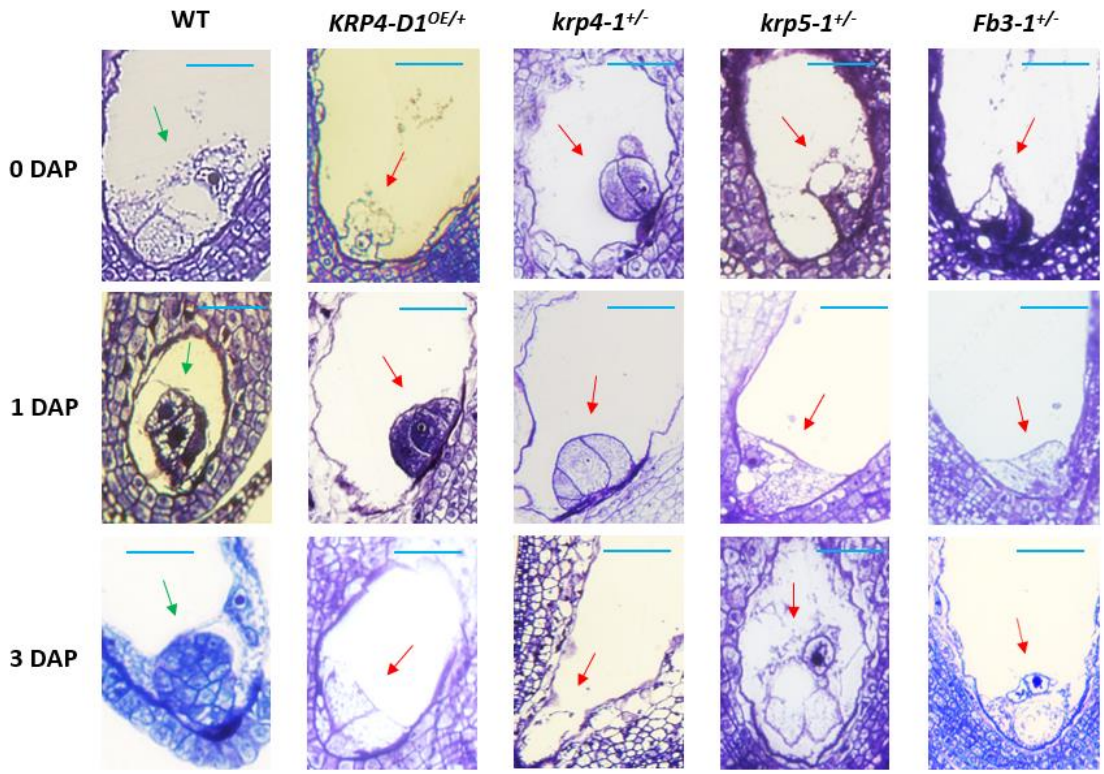


Figure 9 Micrographs of rice ovaries in WT and transgenic mutants at different stages stained with Toluidine Blue O shows abnormal cellular structure of female germ units (egg cell, synergids and central cell) and embryos in mutant ovaries (red arrows) and compares with normal, wild type (WT) in female germ units (green arrows) at different stages. The dark purple spots represent nuclei. Scale bars, 50 µm. DAP, days after pollination; 0, 1 and 3 DAP: 0, 24, 72 and 144 hours after pollination. WT 1 DAP is to show the two-celled pre-embryo; WT 3 DAP, a globular like embryo; WT 6 DAP, both embryo and endosperm.

Table 1 Results of cross-pollination between selected rice mutant lines

Purpose	Cross[a]	Hand-Pollinated-Florets (#)	Seeds harvested (#)	Progeny survival (%)	Seed-set (%)
Control	WT (Nip) x WT (DJ)	100	93 filled	95	90
Obtain double mutant	*krp4-1*$^{-/-}$ x *krp5-1*$^{+/-}$	200	1 filled + 5 half-filled	0	0
KRP4 complementation	*krp4-1*$^{-/-}$ x *KRP4-D1*$^{OE/+}$	100	3 filled + 5 half-filled	38[b]	74

[a] *Nip, Nipponbare; DJ, Dongjing*

[b] *The survived progeny was genotyped: all carried the OE alllele.*

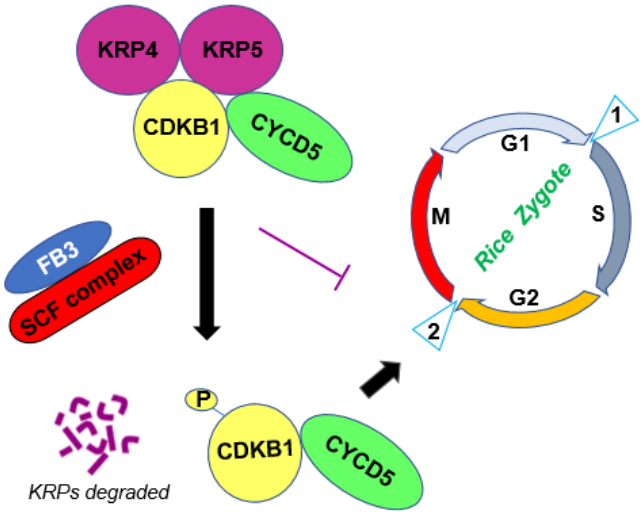


Figure 10 Model of the core complex for rice zygotic cell cycle control at checkpoint 2, Gap 2 to Mitosis (G2-M). CYCD5 dependent CDKB1 is coordinately inhibited by KRP4 and KRP5, and the two inhibitors are degraded via the FB3-mediated proteasome pathway, which then permits the cell cycle to proceed.

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