Short title: A sugar transporter affects rice seed-setting rate

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Knockout of *OsSWEET15* impaired rice embryo formation and seed-setting

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Abstract: we show that the knockout of a sugar transporter gene *OsSWEET15* led to a significant drop in rice fertility because near half of the knockout mutant spikelets bore blighted or empty grains. The rest spikelets bore fertile grains with slightly reduced weight. Notably, the ovaries in the blighted grains of the *ossweet15* mutants expanded after flowering but terminated development before the endosperm cellularization stage and aborted subsequently. GUS and GFP representing *OsSWEET15* expression showed that the protein was strongly expressed in the embryo surrounding region (ESR) which was supposed to supply nutrients for the embryo development. These results joined with the protein's sucrose transport capacity and plasma membrane localization suggest that *OsSWEET15* plays a prominent role during the caryopsis formation stage probably by releasing sucrose from the ESR to support the embryo development. By contrast, the empty grains were probably caused by the reduced pollen viability of the *ossweet15* mutants. Investigation of the makeup of *ossweet11* mutant grains revealed similar phenotypes that were observed in the *ossweet15* mutants. These results indicate that both *OsSWEET15* and *OsSWEET11* play important and similar roles during rice pollen development, caryopsis formation, and seed-setting in addition to their function in seed-filling that was demonstrated previously.

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Keywords: caryopsis; formation; *OsSWEET15*; sucrose; transporter.

Abbreviations:

CRISPR-Cas9: Clustered Regularly Interspaced Short Palindromic Repeats-Cas9

DAF: days after flowering

DAPI: 4', 6-diamidino-2-phenylindole

ESR: embryo surrounding region

FRET: Förster resonance energy transfer

MCE: micropylar endosperm

67 Pollen development and pollen tube growth are identified to be important to
68 seed-setting rate and product (Higashiyama and Takeuchi, 2015; Arshad et al., 2017;
69 Xu et al., 2017). By contrast, other developmental factors that affect the seed-setting
70 rate and their mechanisms have not been fully addressed. In the seeds of *Arabidopsis*
71 and cereal crops, the embryo is surrounded by the micropylar endosperm (MCE) and
72 the embryo surrounding region (ESR), respectively (Olsen, 2001; 2004; Berger, 2003).
73 Accumulating data show that the embryo is symplastically isolated from the
74 endosperm shortly after the embryo differentiation due to a lack of plasmodesma
75 between the embryo and ESR/MCE in angiosperms (Suzuki et al., 1993; Demanson,
76 1997; Opsahl-Ferstad et al., 1997; Kawashima and Goldberg, 2009; Lafon-Placette
77 and Köhler, 2014). The embryo thus takes in nutrients from the MCE/ESR-embryo
78 interface by itself (Morley-Smith et al., 2008; Lafon-Placette and Köhler, 2014). These
79 nutrients must be first released from the MCE/ESR into the interface (apoplast) before
80 being actively or passively transported into the embryo.

81 Among the nutrients necessary for embryo development, sugar is by far the most
82 abundant component. Previous research shows that sucrose-proton symporters
83 (SUC/T) play important roles in the uptake of sucrose from the apoplast into
84 cytoplasm across the membrane of the cells at the acceptor (e.g., embryo) side
85 (Matsukura et al., 2000; Zhang et al., 2007; Bai et al., 2015; Wang et al., 2021). How
86 sucrose is released from the donor cells (e.g., endosperm) on the other side of the
87 interface remains a question until *SWEET* gene is characterized (Chen et al., 2010;
88 Eom et al., 2015). SWEETs are sugar transporters that translocate mono-/disaccharide
89 sugars across the membrane along their concentration gradients. Some of them are
90 also capable of transporting GA in *Arabidopsis* and rice (Kanno et al., 2016; Morii et
91 al., 2020). Physiological functions of SWEETs in model plants include phloem
92 loading and unloading, nectar secretion, transport sugar between organelles, and
93 providing sugar for symbiont or pathogen propagation (Chen et al., 2012; 2015a,b;
94 Guo et al., 2014; Lin et al., 2014; Sosso et al., 2015; Li et al., 2017; Oliva et al., 2019;
95 Ko et al., 2021). At the molecular level, they play a critical role in the intercellular
96 sugar transport when cells lack plasmodesma connection (Chen et al., 2015b).

97 In rice, besides the interface between the embryo and its surrounding ESR,
98 another interface in caryopsis that lacks plasmodesma connection is the apoplast
99 between the nucellar epidermis and aleurone. By CRISPR-Cas9 or TALEN mediated
100 knockout strategies, Ma et al. (2017) and Yang et al. (2018) demonstrated that the
101 nucellar epidermis-abundantly expressed OsSWEET11 plays an essential role in the
102 early seed-filling stage by acting as a sucrose transporter to release sucrose from the
103 nucellar epidermis; and knockout of the gene impaired grain-filling, reduced

104 seed-setting rate, and prolonged seed maturation time of rice (Ma et al.,2017; Yang et
105 al.,2018; Fei et al., 2021). Moreover, Yang et al. (2018) reported that *OsSWEET15*
106 took part in the seed-filling process, either; since double mutation of *OsSWEET11* and
107 *OsSWEET15* completely sterilized the mutant rice, but knockout of *OsSWEET15*
108 alone did not confer any abnormal phenotype. Despite that these investigations
109 answered how sugar is released from the nucellar epidermis to the maternal-filial
110 interface for rice endosperm development, how sugar is released from the ESR for the
111 embryo development during rice caryopsis formation is still not clear.

112 Here, we report that *OsSWEET15*, a sucrose transporter (Yang et al.,2018), was
113 strongly expressed in rice caryopsis particularly in the ESR after flowering in addition
114 to its expression in the integument, nucellar epidermis, pollen, and other tissues.
115 Knockout of *OsSWEET15* via CRISPR-Cas9 mediated gene editing led to a
116 significant drop in rice seed-setting rate because only about half of the mutant
117 spikelets bore fertile seeds. Inspection of the infertile spikelets revealed that about 40 %
118 of their caryopses terminated development before the endosperm cellularization stage.
119 These caryopses gradually withered and their spikelets developed into blighted grains.
120 About 60 % of the infertile spikelets did not show ovary expansion after flowering
121 and formed empty grains. Moreover, the 1000-grain weight of the fertile grains of the
122 *ossweet15* mutants reduced slightly compared with that of the wild type (WT) control.
123 As GUS and GFP representing gene expression consistently indicate that
124 *OsSWEET15* was expressed in the embryo-surrounding ESR which is supposed to be
125 the nutrient supplier for the embryo development, taking the sucrose transport
126 capacity of *OsSWEET15* and its plasma membrane localization tested in rice
127 protoplast into account, we proposed that the caryopsis abortion of the *ossweet15*
128 mutants is likely due to the deficient supply of sugar during the embryo development.
129 Moreover, the empty grains were likely caused by the reduced pollen viability of the
130 *ossweet15* mutants. Observation of *ossweet11* mutants revealed similar phenotypes.

131 These results imply that both *OsSWEET15* and *OsSWEET11* play important roles
132 in rice pollen function, caryopsis formation and seed-setting, in addition to their
133 function in seed-filling which was reported previously (Ma et al.,2017; Yang et
134 al.,2018; Fei et al., 2021).

135

136 RESULTS

137 Significant Reduction of Seed-Setting Rate of the *OsSWEET15* Knockout 138 Mutants

139 Among the CRISPR-Cas9 edited *OsSWEET15* mutant lines based on rice cultivar
140 Nipponbare (*Oryza sativa* L. ssp. *japonica*), many suffered from marked decreases in
141 their seed-setting rates compared with WT control (Fig 1). Therefore, three
142 independent transgenic lines, *m15-5*, *m15-9*, and *m15-27* were selected for further
143 investigation (*m15-5* and *m15-9* derived from Spacer 2 and *m15-27* from Spacer 1,
144 both spacers targeted the third exon of the gene, spacer sequences were listed in
145 Supplemental Table S1). Both the *m15-5* and *m15-27* contain more than one
146 nucleotide deletion, while the *m15-9* contains a single nucleotide insertion in the
147 target region of *OsSWEET15* (Supplemental Fig. S1). The mutated genes either
148 encode truncated proteins or amino acid-missing protein (Supplemental Fig. S2). The
149 deletions and insertion located in the target DNA regions (Supplemental Fig. S1), and
150 fragment amplification of the mutant lines for verification of the gene-editing was
151 performed using a high-fidelity polymerase (PrimeSTAR, Takana).

152 As Yang et al. (2018) reported they did not observe any abnormal phenotype in
153 their knockout mutants of *OsSWEET15* based on rice cultivar Kitaake (*Oryza sativa* L.
154 ssp. *japonica*), we first checked the specificity of the gene-editing effect in our
155 investigation. Nucleotide sequences partly identical to the spacers particularly at the
156 PAM end from the genome of Japonica rice Nipponbare were retrieved and examined
157 because they might interfere with the specificity of the CRISPR-Cas9 mediated
158 gene-editing (Jinek et al., 2012). The spacer 1 shares 14 bp nucleotides at most with 3
159 additional genomic DNA fragments and it shares 13 bp nucleotides with 4 additional
160 genomic DNA fragments other than its target sequence (Supplemental Fig. S3). All
161 these partly identical sequences locate at the interval regions far from the putative
162 promoter or coding region of the predicted genes they adjoin and only two of them
163 possess the critical PAM (NGG) sequences (Supplemental Fig. S3). The spacer 2
164 shares 13 bp nucleotides at most with additional 133 genomic DNA fragments other
165 than its target sequence. As mentioned above, many lines from the CRISPR-Cas9
166 plasmids-mediated transgenic rice showed consistent decreases in their seed-setting. It
167 is unlikely that both of the spacers confer similar phenotypes even if spacer 2 aims at
168 non-target genes by error. We thus reasoned that the phenotype we observed in the
169 *OsSWEET15* transgenic rice was not an off-targeting effect.

170 To exclude the possibility that the phenotypes we observed in the *ossweet15*

Fig. 1

Figure 1. Comparisons of the phenotypes of *ossweet15*, *ossweet11*, and WT control. The left column shows plants, strains, spikes, grains, and caryopses of *ossweet15* mutants and WT of rice cultivar Nipponbare. The right column shows the images of caryopses, statistical analyses of caryopsis makeups of *ossweet15*, *ossweet11*, and the 1000-grain weight of *ossweet15*.

locus in the rice genome, we introduced the CRISPR-Cas9 gene-editing plasmids into Nipponbare callus for the second time (performed in 2021), and obtained similar results. From the year 2017, we have grown and checked the phenotypes of the *ossweet15* mutants for 6 rounds in 5 years. Particularly, we checked the *ossweet15* mutant's genotypes once again in 2021 and confirmed their identities (data not shown). The *ossweet15* mutants showed consistent phenotypes except that their seed-setting rates fluctuated to some extent due to yearly weather changes. Taking all the facts into account, we conclude that the reduction of seed-setting rate in the transgenic rice that we observed was caused by the knockout of *OsSWEET15*.

Detailed inspection revealed that the *ossweet15* mutant rice lines did not show obvious differences at the vegetative growth stage. At the reproductive growth stage, however, the mutant spikes were lighter than that of the WT rice because near half of their spikelets bore blighted grains or were simply empty (Fig. 1). The seed-setting rate of the WT control was about 82 % based on statistics investigated in 2021 with at least 800 grains in each sample. By contrast, for the three mutant lines of *m15-5*, *m15-9*, and *m15-27*, their seed-setting rates were approximate 53 %, 43 %, and 53 %, respectively (Fig.1). Observation of the spikelets of the three mutant lines showed that averagely about 22 % of them formed blighted grains (Fig. 1); and about 28 % of the spikelets did not show any sign of ovary development after flowering, they thus developed into empty grains. The rest spikelets (about 50 %) seemed normally developed and bore fertile grains (Fig.1). However, the 1000-grain weights of the fertile grains reduced slightly compared with that of the WT control (Fig.1). Notably, the WT rice also bore blighted grains and empty grains, but their makeups were about 7 % and 11 %, respectively.

Previously, Ma et al., (2017) and Yang et al. (2018) demonstrated that knockout of *OsSWEET11* impaired seed-filling of the transgenic rice. Moreover, Ma et al. observed that the seed-setting rate of the *ossweet11* mutants also reduced (Ma et al., 2017). This phenotype was consistent with the investigations of Chu et al. (2006) and Yang et al. (2006) based on RNAi-mediated knockdown rice lines of the gene. As the expressions of *OsSWEET11* and *OsSWEET15* largely keep pace with each other according to RiceXPro (<https://ricexpro.dna.affrc.go.jp/>) and Yang et al. (2018) who showed that *OsSWEET11* and *OsSWEET15* have a very similar expressional pattern in caryopsis, we thus checked the makeup of the *ossweet11* mutant grains and found that similar to the observation that we obtained in the *ossweet15* mutants, the percentages of fertile grain, blighted grain, and empty grains of the *ossweet11* mutants (*11-2*; *11-3*; *11-18*. Ma et al., 2017) were approximately 49 %, 17 %, and 34 %. By contrast, the percentages in the WT rice were 87 %, 5 %, and 8 %, respectively (Fig.1;

209 Supplemental Fig. S4).

210 **Caryopsis Abortion in the Blighted Grains of the *ossweet15* Mutants**

211 To explore the underlying reason for the reduction of seed-setting rate, we first
212 compared floret morphology and structure of the *ossweet15* mutants and the WT
213 control but did not find any obvious difference (Fig. 2). KI-I₂ staining and
214 germination test showed that both the pollen viabilities and pollen germination rates
215 of the *ossweet15* mutants reduced compared with that of the WT control (Fig. 2).
216 These results partly explained that the seed-setting rates of *ossweet15* mutant lines
217 were significantly lower than that of the WT control.

218 Examining the caryopsis development of the *ossweet15* mutants and WT control
219 further revealed their differences (Fig. 3). At 3 days after flowering (DAF), about half
220 of the *ossweet15* mutant's spikelets developed normally and approximately
221 one-quarter of them bore slightly slimmer caryopses compared with that of the WT
222 control. The rest failed to develop because their ovaries did not show any sign of
223 expansion. At 5 DAF, differences in size between the slimmer and normal caryopses
224 became clear since the development of the slimmer caryopses seemed terminated. The
225 spikelets with the unexpanded ovaries remained undeveloped. At 9 DAF, the normally
226 developed caryopses of the mutants and most caryopses of the WT control reached
227 their full sizes. Longitudinal sections of their caryopses showed that their embryos
228 had fully developed and their endosperms had solidified due to starch-formation (Fig.
229 3). By contrast, the development-terminated caryopses of the mutant lines began to
230 wither. Longitudinal sections of them showed that the embryo sacs were still filled
231 with clear sap. Notably, all of the development-terminated caryopses lack the embryo
232 (Fig. 3). At 15 DAF, when WT caryopses and normally developed caryopses of the
233 mutants entered into the dough stage, the development-terminated caryopses of the
234 mutant lines aborted and withered gradually. The spikelets with the aborted caryopses
235 developed into blighted grains. It is worth noting that knockout of *GmSWEET15* and
236 *SISWEET15*, the homolog genes of *OsSWEET15* in soybean and tomato, confer
237 severe seed abortion according to the investigations of Wang et al. (2019) and Ko et al.
238 (2021).

239 **Failure of Embryo Formation at the Endosperm Cellularization Stage in the** 240 **Aborted Caryopsis of the *OsSWEET15* Mutants**

241 In angiosperms, the endosperm development can be divided into the free nuclear
242 stage (coenocyte and alveolar) characterized by nucleus division without cell wall
243 formation, follows by the cellularization stage in which endosperm cell wall

Fig. 2

Figure 2. Comparisons of the *ossweet15* mutant lines (*m15-5*; *m15-9*; *m15-27*) and WT rice. The first lane shows florets, bars=1 mm; the second lane shows pollens stained with KI-I₂ solution; the third lane shows pollen germination on a medium observed under a microscope (Olympus BX 51), bars=200 μm in the second and third lanes. The columns at the bottom of the figure are pollen viabilities and germination rates of *ossweet15* mutant lines and WT rice as control.

Fig. 3

Figure 3. Morphology and structure of the development-terminated caryopses (Withered) of *ossweet15* mutant lines (*m15-5*; *m15-9*; *m15-27*) and WT rice (Nipponbare) at different developing stages. DAF: days after pollination. Bars=1 mm.

Fig. 4

Figure 4. Comparisons of the embryo and endosperm of *ossweet15* mutants and WT rice at the early stage of rice caryopsis development. Upper panel: fluorescence observation of syncytium (multinucleate cell) in the embryo sac of *ossweet15* mutant lines (*m15-5*; *m15-9*; *m15-27*) and WT rice at 4 DAF. Saps that were sucked out from the embryo sacs of the mutants and WT rice were stained with a DAPI solution before being observed under a microscope (Leica DM5000B). Lower panel: transverse sections of the develop-stagnated caryopsis (4 DAF) and the normally developed caryopsis (3 DAF) near the bottom of seeds in the *m15-9* mutant rice line. Bars=10 μ m in the upper panel, bars=200 μ m in the lower panel.

saps were sucked out from the embryo sacs of these caryopses and WT control at 4 DAF and were stained with 4',6-diamidino-2-phenylindole (DAPI) solution. The result showed that the nuclei were still in a free state in the development-terminated caryopses of the blighted grains (Fig. 4, upper panel). By contrast, the nuclei were wrapped with cytoplasm materials (Fig. 4, upper panel) in most of the WT grains and the normally developed mutant grains. Moreover, the peripheral parts of the embryo sac in the normally developed caryopses have cellularized and solidified. These results indicate that the free nuclear stage was normal but the subsequent cellularization stage was impeded in the aborted caryopsis. Traverse sections of *m15-9* caryopses at 4 DAF showed that no embryo emerged in the development-terminated caryopses (Fig. 4, lower panel, left). By contrast, the embryo had established at a similar site in the normally developed caryopsis at 3 DAF (Fig. 4, lower panel, right).

Strong Expression of OsSWEET15 in the Embryo Surrounding Region of Rice Caryopsis

qRT-PCR result (Fig. 5 A) showed that *OsSWEET15* was strongly expressed in anthers and caryopses of rice during the early stages of development after flowering. Moreover, it was weakly expressed in the basal stem or root and leaf. This expression pattern was consistent with the Affymetrix chip-based gene expression profile (<http://ricexpro.dna.affrc.go.jp/>) which shows that *OsSWEET15* was expressed very abundantly in the ovary and endosperm. Histochemical analysis of pOsSWEET15::GUS expression (Fig. 5B, C, F-H) and observation of pOsSWEET15::GFP fluorescence (Fig. 5D, E) in caryopses at 5 DAF from at least 3 independent transgenic lines consistently showed that *OsSWEET15* promoter activity was evident in rice caryopsis, and especially strong in the ESR (Fig. 5B-H) including the nucellar epidermis and aleurone cells adjoining the embryo (Fig. 5F,G). It was also strongly expressed in the cross cell, integument, and embryonic epidermis (Fig. 5G). Moreover, *OsSWEET15* promoter activity can be found in pollen (Fig. 5H), root, leaf blade, sheath, and stem (Supplemental Fig. S5). These results suggest that OsSWEET15 plays multiple roles in rice development besides its important functions in pollen development and caryopsis formation and filling.

Subcellular Localization of OsSWEET15-EGFP Fusion Protein

OsSWEET15 and EGFP fusion protein was used to indicate the subcellular localization of OsSWEET15 in rice protoplast. A membrane protein AtPIP2;1 fused with mCherry was employed as the plasma membrane marker (Breia et al., 2020). Different from OsSWEET11-EGFP fusion protein located on the plasma membrane (Fig. 6; Ma et al., 2017), OsSWEET15-EGFP fusion protein is also expressed in the

Fig. 5

Figure 5. Expressional profiles of *OsSWEET15* in wild type (WT) rice Nipponbare at the transcriptional level and translational level. A: transcriptional levels of *OsSWEET15* in rice tissues examined by real-time qRT-PCR. Caryopsis 2d, 3d, 4d, and 5d show caryopsis samples collected on the 2, 3, 4, 5 DAF. Root, stem, and leaf were collected from 3 week-old rice plants; B, C: longitudinal and transverse sections of rice caryopses expressing pOsSWEET15::GUS at 5 DAF after GUS staining; D, E: GFP fluorescence observation of longitudinal and transverse sections of rice caryopses expressing pOsSWEET15::GFP at 5 DAF; F: a traverse section cross the embryo area of the rice caryopsis expressing pOsSWEET15::GUS at 5 DAF. The image was observed under a microscope (Leica, DM5000B); G: a traverse-sectioned image of the rice caryopsis expressing pOsSWEET15::GUS at 5 DAF to show the detailed tissue organizations. H: rice pollens expressing pOsSWEET15::GUS observed under a microscope (Olympus BX51) after GUS staining. In B-F, En: endosperm; Em: embryo. Red arrowheads in D, E, F indicate the ESR. Bars in B and C =1 mm; in D and E =250 μ m, in F, G, and H =100 μ m.

Fig. 6

Figure 6. Subcellular localization of OsSWEET15-EGFP fusion protein in rice protoplasts. Plasmids consisting of pSAT6A-OsSWEET15-EGFP and CD3-1007 were co-transfected via a PEG-mediated method. Plasmids consisting of pSAT6A-EGFP and CD3-1007 were co-transfected in parallel as a negative control and plasmids consisting of pSAT6A-OsSWEET11-EGFP and CD3-1007 were co-transfected as a positive control. EGFP fluorescence was observed under a Leica SP5 confocal microscope. Bars in the first and the third rows = 10 μ m; bars in the second rows=5 μ m.

286 DISCUSSION

287 Rice caryopsis development can be divided into three consecutive stages
288 including seed (caryopsis) formation, seed filling, and seed maturation. We show that
289 the knockout of *OsSWEET15* led to about 22 % of their caryopses terminated
290 development at the embryo formation stage and eventually forming blighted grains
291 (Fig. 3, 4). In addition, about 28 % of spikelet's ovaries failed to develop and formed
292 empty grains suggesting that the protein functioned in pollen development and
293 probably also in the fertilization process (Fig. 2). Moreover, the 1000-grain weight of
294 the fertile grains of the *ossweet15* mutants decreased slightly (Fig. 1) and double
295 mutation of *OsSWEET11* and *OsSWEET15* led to completely infertile (Yang et al.,
296 2018; Supplemental Fig. S6) implies that *OsSWEET15* participated in the seed-filling
297 process as well.

298 Endosperm cellularization represents an important developmental transition
299 during seed development (Olsen, 2001; Hehenberger et al., 2012). It initiates at the
300 embryo vicinity and follows by the peripheral endosperm region (Engell, 1989; Olsen,
301 2001). Finally, cellularization takes place in the center region of the endosperm
302 (Suzuki et al., 2000; Itoh et al., 2005; Lafon-Placette and Köhler, 2014).
303 Ultrastructural observation in maize, soybean, *Arabidopsis* and *Solanum nigrum*
304 revealed that the embryo is symplastically isolated from the surrounding endosperm
305 shortly after the embryo cell differentiation (Schel et al., 1984; Dute et al. 1989;
306 Mansfield and Briarty, 1991; Briggs, 1996). Analogously, Suzuki et al. (1993)
307 reported that no plasmodesma was observed between the embryo and the endosperm
308 cells in rice caryopsis at 42 hours after pollination. We checked the caryopses of rice
309 cultivar Nipponbare at 5 DAF and confirmed Suzuki's observation although there are
310 many plasmodesmata between the cells within the embryo or endosperm (Fig. 7).
311 These facts imply that nutrients in or out of the embryo after ESR cellularization have
312 to be transported across the plasma membranes via transporters.

313 GUS and GFP representing protein expression showed that *OsSWEET15* was
314 strongly expressed in the ESR region (Fig. 5). This expression pattern is similar to the
315 reports that *AtSWEET15* and *GmSWEET15* were expressed in the micropylar
316 endosperms of *Arabidopsis* and soybean seeds (Chen et al., 2015b; Wang et al., 2019).
317 It is also consistent with the report that *SISWEET15* was expressed in the tomato seed
318 coat encompassing the embryo (Ko et al., 2021).

319 MCE/ESR is supposed to be the tissue responsible for nutrient supply for embryo
320 development (Olsen, 2001; 2004; Dumas and Rogowsky, 2008). Taking the sucrose
321 transport capacity (Yang et al., 2018) and plasma membrane localization of

Fig. 7

Figure 7. Observation of a longitudinal section of the embryo and endosperm in WT rice caryopsis at 5 DAF under a transmission electron microscope (Hitachi H-7650). Yellow asterisks indicate the interface of the embryo and endosperm. Red arrowheads indicate plasmodesmata. Em: embryo cell; En: endosperm cell. Bar=2 μ m. Magnification rate=3000.

lacked the embryos and they aborted at the endosperm cellularization stage (Fig. 4) into account, we propose that OsSWEET15 played an important role in the seed formation stage likely by providing sucrose for the embryo development (Fig 5F, G). Therefore, the knockout of *OsSWEET15* led to the termination of embryo development since the ESR is essential for embryo-endosperm communication (Becraft, 2001; Olsen et al., 2004; Zhou et al., 2013).

Previously, both Ma et al (2017) and Yang et al. (2018) reported that the knockout of *OsSWEET11* affected the seed-filling of rice. In addition, Ma et al. (2017) reported that the seed-setting rate of *ossweet11* mutants reduced and Yang et al. (2018) reported that the maturation of *ossweet11* seeds was prolonged. We scrutinized the *ossweet11* mutants based on rice cultivar Nipponbare (in 2021) and confirmed these phenotypes. However, Yang et al. (2018) reported that mutation of *OsSWEET15* alone did not confer any abnormal phenotype whereas our data showed clearly that OsSWEET15 exerted significant impacts on rice seed-setting rate and pollen viability (Fig.1-4). Notably, both Wang et al. (2019) and Ko et al. (2021) demonstrated that the CRISPR-Cas9 mediated knockout of *GmSWEET15* and *SisSWEET15*, the homolog genes of *OsSWEET15* in soybean and tomato, led to serious defects in seed-filling and embryo development. As a result, more than 80 % of soybean seeds and most tomato seeds were aborted in the genes mutants. These phenotypes are very similar but more severe than what we observed in our *ossweet15* mutants. Wang et al. (2019) and Ko et al. (2021) concluded that GmSWEET15 and SisSWEET15 played critical roles in sucrose unloading from the endosperm or seed coat to support the developing embryos. These conclusions support our proposal that OsSWEET15 releases sucrose from the ESR including nucellar epidermis and aleurone to nurture the embryo's development in rice.

It should be mentioned that both Yang et al. (2018) and our results (Fig. 5F) showed that OsSWEET15 was strongly expressed in the nucellar epidermis of rice caryopsis. However, we also showed that the protein was strongly expressed in the ESR region as we traverse-sectioned rice caryopsis cross the embryo proper. One possible reason for the discrepancy between Yang et al. (2018) and our results (Fig. 1-4) is that different gene-editing methods (Yang et al. (2018) used TALEN while we used CRISPR-CAs9) or/and different rice cultivars (they use Kitaake while we used Nipponbare) were used for the investigations. Actually, the impairment in the seed-filling of the *ossweet11* mutants reported by Yang et al. (2018) is much milder than that we found in our *ossweet11* mutants (2017) judged from the images and descriptions in their article.

Consistent with the results of Chu et al. (2006) and Yang et al. (2006) based on

their RNAi knockdown rice lines, our results showed that knockout of *OsSWEET11* reduced rice seed-setting rate (Ma et al., 2017; Fig.1, Supplemental Fig. S4). As *OsSWEET11* and *OsSWEET15* have a similar expression pattern in the developing caryopsis (RiceXPro <https://ricexpro.dna.affrc.go.jp/>; Fig. 5F, Ma et al., 2017; Yang et al., 2018), it is not surprising that the makeups of fertile grain, blighted grain, and empty grain of *ossweet11* and *ossweet15* mutant were similar (Fig.1; Supplemental Fig. S4). Moreover, knockout of *OsSWEET11* and *OsSWEET15* that strongly expressed in the nucellar epidermis (Fig. 5F, Ma et al., 2017; Yang et al., 2018) reduced rice grain weight similarly but in varying degrees (Ma et al., 2017; Fig.1). Double mutation of *OsSWEET15* and *OsSWEET11* sterilized the transgenic rice (Supplemental Fig. S6; Yang et al., 2018). These results indicate that both of them play important roles in rice seed-filling.

Interestingly, our results showed that OsSWEET15-EGFP was also expressed in the cytoplasm of rice protoplast in addition to the plasma membrane. Moreover, the fluorescence of the OsSWEET15-EGFP fusion protein was weaker than that of the OsSWEET11-EGFP fusion protein under the same cultivation condition. Future investigation may reveal the underlying reason.

MATERIALS AND METHODS

Gene Cloning and Plasmid Preparation

The coding sequence of *OsSWEET15* (LOC_Os02g30910) was amplified using rice inflorescence cDNA template and P1/P2 primers (sequences listed in Supp. Table 1) based on a full-length cDNA clone (accession No. AK103266). The amplified fragment was then cloned into a pEASY-Blunt vector (Transgene Biotech) and sequenced. A putative promoter of *OsSWEET15* (genomic sequence of *OsSWEET15* from the translation start code ATG to upstream 2.262 Kb) was amplified with rice Nipponbare genomic DNA template and primers P3/P4 with restriction sites *Pst*I and *Bam*HI added, respectively. It was integrated into a modified pCAMBIA1300 vector (contains a GUS-coding sequence) or pCAMBIA1302 to drive GUS or GFP expression (pOsSWEET15::GUS or pOsSWEET15::GFP). Open reading frame (ORF) sequence of *OsSWEET15* was amplified with P5/P6 (with restriction sites *Hind*III and *Bam*HI) and P7/P8 primers (with *Hind*III and *Bam*HI). They were integrated into pSAT6A-EGFP-N1 and pSAT6A-EGFP-C1 (Chung et al. 2005), respectively, in-frame with the EGFP-coding sequence to create fusion proteins after digestion. Subsequently, the plasmids were digested with restriction endonuclease *PI*-PspI (NEB), and the fused EGFP expression cassettes from the digested plasmids were integrated into the pRCS2-ocs-nptII vector for transformation (Chung et al. 2005).

For the CRISPR–Cas9-mediated knockout of *OsSWEET15*, two pairs of specific spacers (sequences listed in Supp. Table 1) of *OsSWEET15* were synthesized according to the rice CRISPR database (provided by Professor Lijia Qu) with additional *BsaI* restriction site sequences at either end. The annealed double-stranded spacers were inserted into the *BsaI*-digested pOs-sgRNA vector. A Gateway Cloning LR reaction (Invitrogen) of the resulting pOs-sgRNA constructs was performed with the destination vector pH-Ubi-Cas9-7 (Miao et al. 2013). All destination plasmids were confirmed by sequencing.

Transient Expression of OsSWEET15–EGFP Fusion Protein in Rice Protoplast

Protoplasts were isolated from culms of etiolated rice seedlings and were subsequently transfected with plasmids pSAT6A-EGFP-OsSWEET15+CD3-1007 using a PEG mediated transfection method (Miao and Jiang, 2007; Ying et al., 2017). The plasmid CD3-1007 (Nelson et al., 2007) containing the plasma membrane protein AtPIP2;1 marked with mCherry (AtPIP2;1-mCherry fusion) were used to indicate the plasma membrane (Breia et al., 2020). pSAT6A-EGFP-N1+CD3-1007 were co-transfected in parallel as the negative control; and pSAT6A-EGFP-OsSWEET11 + CD3-1007 were co-transfected in parallel as the positive control. Detection of the fluorescence signal in the rice protoplasts was performed with a confocal laser scanning microscope (Leica SP5) after incubation of the protoplasts in the dark at room temperature for 12-15h. The excitation/emission wavelength of GFP is 488/506 to 538 nm, and the excitation/emission wavelength of mCherry fluorescent protein is 561/575 to 630 nm.

Transforming and Screening Positive Transgenic Rice

Plasmids harboring pOsSWEET15::GUS, pOsSWEET15::GFP cassettes, and constructs for CRISPR-Cas9 gene editing were introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation, and positive agrobacteria were used to infect rice Nipponbare callus. The pOsSWEET15::GUS, pOsSWEET15::GFP, and CRISPR-Cas9 edited transgenic lines were initially screened based on hygromycin resistance. They were subsequently evaluated by PCR amplification with gene-specific primers P9/P10 (Supp. Table 1) and fragments were sequenced. Seedlings about 15d after germination of transgenic and WT rice as control were transplanted into a rice field in Nanjing (N:32°01', E:118°51', altitude: 18m.), China, in late April each year and grow in natural conditions with a light/dark regime around 14h/10h in early May.

Quantitative Real-Time PCR Analyses of the Expression of *OsSWEET15* in Different Tissues of Rice

432 Total RNAs were extracted from rice tissues using the TRIzol reagent
433 (Invitrogen), following the manufacturer's protocol. Root, stem, and leaf samples
434 were collected from 3-week-old hydroponic rice seedlings. Anthers were collected
435 from rice plants approaching flowering. Caryopses were collected from spikelets 2, 3,
436 4, and 5 days after flowering (DAF). Quantitative real-time reverse transcription-PCR
437 (qRT-PCR) amplification was performed with primers P11/P12 for the *OsSWEET15*
438 fragment. P13/P14 for amplification of *OsActin* (LOC_Os03g50890) cDNA fragment
439 (AB047313) and P15/16 for amplification of α -Tubulin (LOC_Os07g38730) cDNA
440 fragment (AK104900) as housekeeping genes (Li et al., 2010). PCR products were
441 confirmed by sequencing. Expression levels of *OsSWEET15* were normalized based
442 on the expression levels of Actin or α -Tubulin with three biological replicates
443 according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). All the primer
444 sequences for amplification are listed in Supp. Table 1.

445 **Tissue Localization of OsSWEET15 Analyzed through Histochemical Staining** 446 **and GFP Fluorescence Observation**

447 Rice tissues from four different transgenic rice lines containing the
448 pOsSWEET15 (2262 bp)::GUS expression cassette were collected and submerged
449 into a GUS reaction mix, and were incubated at 37 °C overnight as described by Ma et
450 al. (2017). Caryopses were collected at 5 DAF. Pollens were collected from flowering
451 inflorescences. Root, leaf blade, and sheath samples were collected from 3-week-old
452 rice seedlings. GUS-stained samples were observed under a stereomicroscope
453 (Olympus MVX10) or a microscope (Olympus BX51). To obtain detailed
454 expressional information of the protein, the GUS-stained tissues were rinsed and fixed
455 in a FAA solution (formalin: glacial acetic acid: 50 % ethanol=1:1:18) for 24 h, and
456 were then embedded in Epon812 (Sigma) as described by Coulter (1967). Fresh
457 sections of pOsSWEET15::GFP expressing caryopses were observed using a confocal
458 microscope (SP5; Leica).

459 **Transmission Electron Microscopy**

460 The upper parts of caryopses of WT rice at 5 DAF were removed and the bottom
461 parts were fixed in 2.5 % glutaraldehyde solution. They were further fixed in 1 %
462 Osmium tetroxide after being rinsed with 0.1M PBS solution. After gradient ethanol
463 dehydration, the samples were embedded in Epon812 and sliced longitudinally and
464 transversely. The sliced samples were double-stained with uranyl acetate and lead
465 citrate before being observed using a transmission electron microscope (Hitachi
466 H-7650) at an accelerating voltage of 80 kV.

467 **Test of Pollen Germination**

468 Pollens in stamens of *ossweet15* mutant lines and wild-type rice were scattered on
469 Petri dishes containing a medium consisting of 5 % potato starch, 5g /L H₃BO₃, 15 %
470 sucrose. They were incubated at 28 °C for one hour before being observed and
471 photographed under a microscope (Olympus BX51).

472 **Observation of Embryo and Endosperm Development**

473 Nucleate in the developing embryo sac of *ossweet15* mutant lines and WT control
474 were sucked out by a pipette with 10 µl tips, they were placed on slides and stained
475 with a DAPI solution in the dark at room temperature for 10 mins. DAPI fluorescence
476 was observed with a Leica microscope (DM5000B).

477 **Supplemental Data**

478 **Supplemental Figure S1.** OsSWEET15 expression in different tissues of rice
479 (Nipponbare) indicated by GUS staining. OsSWEET15 was expressed in root, leaf
480 blade, sheath, and stem. Samples were collected from 3-week-old rice seedlings. Bars
481 in a =250 µm; in b =1 mm; in c=3 mm.

482 **Supplemental Figure S2.** Confirmation of CRISPR-Cas9 edited *OsSWEET15* mutant
483 rice lines. DNA fragments of *OsSWEET15* in mutant lines were amplified with a
484 high-fidelity polymerase and sequenced. Sequences and diagrams under the first-row
485 sequence of each panel are CRISPR-Cas9 edited sequences.

486 **Supplemental Figure S3.** Comparison of *ossweet11* mutant rice lines and WT
487 control (Nipponbare) grew in the year 2021 and statistical analyses of their 1000-grain
488 weights.

489 **Supplemental Figure S4.** Comparison of predicted OsSWEET15 protein structures
490 (right) of the mutants and WT control. Transmembrane helix prediction of
491 *OsSWEET15* mutant and WT proteins was performed using TMHMM
492 (<http://www.cbs.dtu.dk/services/TMHMM/>).

493 **Supplemental Figure S5.** Spike and caryopsis comparisons of
494 *OsSWEET11/OsSWEET15* double gene mutant lines and WT rice control
495 (Nipponbare). Upper panel: spikes of 3 double-gene mutant lines of (m11/15-15,
496 m11/15-16, m11/15-21) (upper left) and WT (upper right). Lower panel: caryopses of
497 the double gene mutants and WT. The spikelets of the double gene mutants either bore
498 withered grains or were simply empty. Bar=0.3mm in the lower panel.

499 **Supplemental Table S1.** PCR primer pairs for cloning, checking and expression
500 analyses or knockout of *OsSWEET15* in rice.

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503

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