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Short title: GmSWEET15 is essential for embryo development.

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Article title: The soybean sugar transporter GmSWEET15 mediates sucrose export from endosperm to early embryo

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One sentence summary:

The sugar transporter GmSWEET15 is essential for soybean embryo development and endosperm breakdown as it mediates sucrose transport from the endosperm to the embryo.

Author contributions

S.W. and H.S. conceived the project. S.W. carried out the designing of transformation constructs, physiological characterization of the mutants and transgenic plants. K.Y. and J-F.M of Okayama University designed and measured sugar transporter. R.G. measured sugar content in seeds. S.W., H.S., J.W., Y.L.R., J.F.M. interpreted results and drafted the publication. All authors reviewed the manuscript.

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Abstract

Soybean [*Glycine max* (L.) Merr.] seed is primarily composed of a mature embryo that provides a major source of protein and oil for humans and other animals. Early in development, the tiny embryos grow rapidly and acquire large quantities of sugars from the liquid endosperm of developing seeds. An insufficient supply of nutrients from the endosperm to the embryo results in severe seed abortion and yield reduction. Hence, an understanding of the molecular basis and regulation of assimilate partitioning involved in early embryo development is important for improving soybean seed yield and quality. Here, we used expression profiling analysis to show that two paralogous sugar transporter genes from the SWEET (Sugars Will Eventually be Exported Transporter) family, *GmSWEET15a* and *GmSWEET15b,* were highly expressed in developing soybean seeds. *In situ* hybridization and RT-qPCR showed that both genes were mainly expressed in the endosperm at the cotyledon stage. GmSWEET15b showed both efflux and influx activities for sucrose in *Xenopus* oocytes. In *Arabidopsis thaliana*, knockout of three *AtSWEET* alleles is required to see a defective, but not lethal, embryo phenotype, whereas knockout of both *GmSWEET15* genes in soybean caused retarded embryo development and endosperm persistence, resulting in severe seed abortion. In addition, the embryo sugar content of the soybean knockout mutants was greatly reduced. These results demonstrate that the plasma membrane sugar transporter, GmSWEET15, is essential for embryo development in soybean by mediating sucrose export from the endosperm to the embryo early in seed development.

Introduction

Soybean is the most widely grown legume globally, providing a major source of proteins and oils for the human and animal diet. Seed development process is the critical period for determining yield and quality, it is important to investigate the molecular mechanism of the soybean seed development (West and Harada, 1993). Seed development is divided into three main stages: seed set (embryogenesis), growth

(filling) and maturation (Ruan et al., 2012). Previous studies on legume seeds have mainly focused on late developmental stages, when storage proteins, starch and oils are synthesized (Rubel et al., 1972; Weber et al., 2005). Early seed development is vital for determining seed number, size and yield potential (Pechan, 1988; Bouttier and Morgan, 1992; Tischner et al., 2003; Weber et al., 2005; Wang and Ruan, 2012), and is more vulnerable to stresses as compared to the late stage of seed development (Ruan et al 2012). During the cotyledon stage, endosperm breakdown to provide sugars to developing embryos is essential for the seed to reach its full maturity (Olsen, 2001; Sun et al., 2010). The importance of embryo-endosperm coordination is evidenced by the fact that the many sucrose transporters and metabolism genes are highly expressed in the Embryo Surrounding Region (ESR) of the endosperm (Bate et al., 2004; Baud et al., 2005). Nevertheless, key sugar transporters that are involved in this process are not identified or characterized in soybean.

Sucrose is the major form of photosynthetic product transported from source to sink tissues (Patrick and Offler, 2001; Ruan, 2014). Sucrose transfers from the maternal tissues to the developing embryos likely occur via membrane-bound sugar transporters as there is a lack of symplastic connections between maternal and filial tissues (Patrick and Offler, 2001). Numerous sugar transporters important for embryo development have been identified and characterized in different plants. For example, *AtSUC5* encodes a sucrose transporter, which was specifically expressed in the endosperm between 4 to 9 days after fertilization and is essential for early Arabidopsis seed development (Baud et al., 2005). The SWEET (Sugars Will Eventually be Exported Transporter) family members, possessing seven transmembrane helices and sugars efflux or influx activity, play important roles in seed development (Chen et al., 2012; Xuan et al., 2013; Yuan and Wang, 2013; Chen et al., 2015b; Eom et al., 2015). For example, sucrose efflux transporters, *AtSWEET11*, *12,* and *15* were found to be required for seed development in Arabidopsis based on the observation that the *sweet11;12;15* triple mutant showed retarded embryo development, reduced seed weight, and reduced starch and lipid content (Chen et al., 2015b). Similarly, mutations in the hexose efflux transporters from maize *(ZmSWEET4c*) or rice (*OsSWEET4* and

OsSWEET11/15) caused defects in grain filling (Sosso et al., 2015; Ma et al., 2017; Yang et al., 2018).

Although *Arabidopsis thaliana* has and continues to be a powerful model system for studying gene functions, it is not a good model for large-seeded plants. Seed development in an important crop, like soybean, needs to be studied directly in order to understand the transporters involved in delivering carbohydrate to the embryo (Li et al., 2006; Le et al. 2007). Soybean presents challenges, as this plant is a polyploid and is difficult to transform or generate knock-out lines using T-DNA insertions. Recent improvements in soybean transformation efficiency and novel gene editing tools, such as CRISPR (clustered regularly interspaced short palindromic repeats) /CAS9 technique, have made it possible to study gene function in soybean (Olhoft et al., 2003; Jacobs et al., 2015; Sun et al., 2015; Cai et al., 2018).

In this study, we identified a pair of sugar transporter genes, *GmSWEET15a* and *GmSWEET15b*, encoding proteins belonging to the SWEET family. Both genes were highly expressed in the endosperm early in seed development. GmSWEET15b was localized to the plasma membrane and mediated sucrose efflux and influx activity. Knockout of both *GmSWEET15a* and *GmSWEET15b* (*gmsweet15*) resulted in reduced sugar content in embryos, retarded embryo development and endosperm persistence, causing high levels of seed abortion. The results demonstrate that *GmSWEET15* is essential for embryo development by mediating sucrose efflux from endosperm to embryo in soybean. Thus two *GmSWEET15* genes play a central role in supplying carbon resources for seed filling of a major oilseed.

Results

GmSWEET15 is specifically expressed in endosperm of early developing seeds

To identify candidate genes involved in soybean seed development, transcriptome analyses were performed on seeds at three early developmental stages. The study found that a group of starch and sucrose metabolism genes are highly expressed during early seed development (Du et al., 2017). In this study, the expression pattern

of sugar metabolism genes was analyzed using publicly available transcriptome datasets of soybean developing seeds, the "Gene Networks in Seed Development" (http://seedgenenetwork.net/soybean), from Robert Goldberg at the University of California at Los Angeles. Fifteen SWEET and six sucrose transporter (SUC), six sucrose synthase (Sus; EC 2.4.1.13) and eleven invertase (INV; EC 3.2.1.26) encoding genes were highly expressed in early seed development (Supplemental Fig. S1). SWEETs and SUCs are different sugar transporters (Baud et al., 2005; Chen et al., 2010; Chen et al., 2015a), and sucrose synthase and invertase are the enzymes that can degrade the sucrose to monosaccharide (Dejardin et al., 1999; Ruan et al., 2010; Ruan, 2014). Among these seed-specific genes, *GmSWEET15a* and *GmSWEET15b* were expressed in the endosperm at heart and cotyledon stages (Fig. 1A, Supplemental Fig. S1).

To verify the above expression pattern of *GmSWEET15*, quantitative RT-PCR (RT-qPCR) was performed on different soybean tissues. Results showed that *GmSWEET15* was highly expressed at the cotyledon stage of developing seed (Fig. 1B). Moreover, the expression is specifically in the endosperm (Fig. 1C). Consistent with this result, fluorescence *in situ* hybridization assay performed on cotyledon stage seed sections using FAM-labeled *GmSWEET15* anti-sense probe showed that *GmSWEET15* transcripts were mainly localized at the degenerating endosperm layers (Fig. 1D). Taken together, the results demonstrated that *GmSWEET15* was specially and highly expressed in endosperm at the cotyledon stage of developing seeds.

GmSWEET15 is a plasma membrane protein belonging to clade Ⅲ of the SWEETs family of sugar transporters.

A protein phylogenetic tree was constructed using the amino acid sequences of soybean, Arabidopsis and rice (*Oryza sativa*) SWEET transporters (Supplemental Fig. S2). We identified at least 37 SWEET members from the soybean genome and named them according to the phylogenetic relationship to Arabidopsis. Consistent with the fact that soybean is an allotetraploid species (Zhu et al., 1994), many SWEET members have a duplicated copy in the genome (Supplemental Fig. S2). Both

GmSWEET15a and GmSWEET15b belong to clade III of SWEETs (Supplemental Fig. S2). GmSWEET15a and GmSWEET15b are predicted to contain seven transmembrane protein domains, as for SWEET sugar transporters in Arabidopsis and rice (Patil et al., 2015; Supplemental Fig. S3).

The coding sequences for GmSWEET15a and GmSWEET15b were fused to eYFP and transiently expressed in leaves of *Nicotiana benthamiana* to determine their sub-cellular localization. Results showed that both GmSWEET15a and GmSWEET15b are localized to the plasma membrane (Supplemental Fig. S4). As GmSWEET15a and GmSWEET15b display 93% amino acid sequence identity, similar spatial expression pattern and sub-cellular localization (Fig. 1 and Supplemental Fig. S4), *GmSWEET15b* was chosen for further expression and transport studies.

GmSWEET15 is a sucrose transporter

To determine transport activity of GmSWEET15b for sucrose and glucose, cRNAs of *GmSWEET15b*, Arabidopsis *SWEET12* (serving as a positive control; Le Hir et al., 2015), or water (serving as a negative control) were injected into *Xenopus* oocytes. Efflux activities were detected by monitoring time-dependent release of ^{14}C-sucrose or ^{14}C-glucose from oocytes after injection of the ^{14}C-labeled sugars and was expressed as % (sugar effluxed/total sugar injected x100) to normalize the variations in injection, oocyte sizes, or cellular status. The result showed that similar to AtSWEET12, GmSWEET15b showed efflux transport activity for sucrose (Fig, 2A). However, GmSWEET15b did not show efflux transport activity for glucose (Fig. 2A).

For influx transport activity, the oocytes were exposed to ^{14}C-labeled sugar (^{14}C-sucrose or ^{14}C-glucose) and the radioactivity in the oocytes was determined. GmSWEET15b also showed influx transport activity for sucrose, but not for glucose (Fig. 2B), which is different from AtSWEET12. Thus these results show that GmSWEET15b mediated both influx and efflux of sucrose (Fig. 2A and 2B).

Effect of GmSWEET15 on seed set

As *GmSWEET15* was highly expressed in the endosperm of early developing seeds, we tested whether *GmSWEET15* affected embryo development and seed set. Two independent mutant lines using the CRISPR/CAS9 system, and three overexpression lines (see Materials and Methods) were produced. Gene-specific primers for *GmSWEET15a* and *GmSWEET15b* were designed and used to detect the sequence changes at the designed CRISPR/CAS9 target region (Supplemental Table S1). In *gmsweet15-1* mutant, results showed that there was an insertion of 'T' in both *GmSWEET15a* and *GmSWEET15b* coding regions. The extra 'T' would cause a frame-shift mutation after Leu59 for GmSWEET15a and Leu58 in GmSWEET15b (Fig. 3A). In *gmsweet15-2*, there is a deletion of 6 bases 'GCTTTG' that result in loss of two residues, Trp60 and Leu61, in *GmSWEET15a*, and an insertion of 'T' in GmSWEET15b, which led to the same frame-shift in *GmSWEET15b* (Fig. 3A). Overexpression lines were obtained by introducing an additional copy of *GmSWEET15*b genomic sequence into the soybean genome. Three independent overexpression lines were generated and designated, OE1, OE2 and OE3. RT-qPCR confirmed that expression of *GmSWEET15* was significantly increased by 2 to 3-fold in the developing seeds of the OE lines (Fig. 3B).

During vegetative stage, no phenotypic difference was observed among the OE, CRISPR mutants in comparison with the wild type (WT) plants grown in the greenhouse. At early seed developmental stage (embryogenesis), although the external appearance of the developing seeds was similar, longitudinal sections showed that embryo development and degradation of endosperm in the seeds of both *gmsweet15-1* and *gmsweet15-2* mutants were substantially delayed compared to those of the WT (Fig. 4A). The delay in seed development process was substantial at the globular, heart and cotyledon stages (Fig. 4A). Moreover, more than 80% of seeds in the *gmsweet15* mutants were aborted (Fig. 4B and 4C). No significant difference was observed in seed abortion rates between the OE and WT (Fig. 4C).

To verify the above phenotype, WT, OE and CRISPR mutant plants were grown in soil plots under field conditions. The increased light intensity in the field

significantly reduced the seed abortion of *gmsweet15* mutants to 52-56%, although it was still significantly higher than that of the WT (Fig. 4D). Plants overexpressing *GmSWEET15* and WT showed similar seed abortion rates under both greenhouse and field conditions (Fig. 4D). Moreover, at R7 stage, when WT plants begin to mature and leaves turn yellow (Fehr et al., 1971), *gmsweet15* mutant plants remained green. Thus, the maturation of *gmsweet15* mutants was delayed for 7-10 days compared to WT (Supplemental Fig. S5).

In order to determine if the seed abortion in *gmsweet15* mutants is maternally or filially controlled, reciprocal crosses between WT and *gmsweet15* mutants were made and the abortion rates of the F_1 crosses were recorded. To minimize the error in hybridization, 60 crosses for each combination were made. As shown in Supplemental Fig. S6, only the cross between *gmsweet15*♀ x *gmsweet 15*♂ produced significantly reduced seed set. When the male parent or female parent were WT, the seed abortion rates were similar to the wild type, indicating that seed abortion of *gmsweet15* mutants was related with the genetic alleles of *GmSWEET15* in the zygote.

Loss of GmSWEET15 function reduced the sugar supply to developing embryos

To determine the transport role of GmSWEET15, the sugar contents of whole seeds, embryos and integuments (including seed coat and endosperm) at the cotyledon stage were analyzed in WT, *gmsweet15* mutants, and the *GmSWEET15* overexpression lines. While overexpression of *GmSWEET15* only resulted in a marginal elevation in sucrose in whole seeds and integument, the sucrose and glucose content were significantly decreased in all seed parts of *gmsweet15* mutants compared to those of WT (Fig. 5). Importantly, the sucrose content in mutant embryos was about 27-28% of that in WT. In contrast, the sucrose contents in mutant integuments also decreased, but to a lesser extent, about 30-40% compared to those of the WT (Fig. 5). Similarly, the glucose content in mutant embryos was less than 10% of the WT (Fig. 5), whereas those in mutant integuments were 30-40% of the WT (Fig. 5). These results suggested that the seed abortion in *gmsweet15* mutants is likely due to insufficient sugar supply for embryo development.

The above results suggested that seed abortion in the *gmsweet-15* mutants might be caused by the shortage of sugars being transported to the embryos. To test the hypothesis, we grew the *gmsweet15* mutants under enhanced light intensity to increase photosynthesis and subsequent sugar production. Under normal light intensity of the growth chamber (4000 LUX), more than 80% seeds aborted in *gmsweet15* mutants, while only 10% seed abortion occurred in WT (Supplemental Fig. S7A). Under a high light intensity of 12000 LUX, the seed abortion rate in *gmsweet15* mutants significantly declined to 20% (Supplemental Fig. S7B). Thus, increasing photosynthesis-derived sucrose supply partially rescued the seed abortion defect. This result supported the hypothesis that seed abortion in *gmsweet15* mutants under normal light intensity was due to limited transport of sugars.

Discussion

Angiosperm seed development requires the coordinated growth of endosperm and embryo, which is critical for seed quality and yield (Berger et al., 2006; Nowack et al., 2006; Ruan et al., 2012). During the early phase of legume seed development, the embryo divides and grows from a globular shape, to a heart shape, then to a mature embryo with two large cotyledons (corresponding to globular stage, heart stage and cotyledon stage, respectively). Concurrently, the relatively large endosperm which provides the embryo with nutrients, decreases in size and degenerates (Hill et al., 2003; Baud et al., 2005). How the transient endosperm delivers sugars to the embryo and in which chemical form the sugars in legume seeds are transported is poorly understood. In this study, we identified that endosperm-specific GmSWEET15 mediated sucrose transport from endosperm to embryo to support seed development in soybean.

We show that GmSWEET15 plays an essential role in soybean seed development by mediating sucrose transport from the endosperm for subsequent uptake by the embryo. This conclusion is supported by: 1) Using *Xenopus* oocytes as a heterologous expression system, we showed that GmSWEET15 mediates the efflux and influx of

sucrose preferentially over glucose (Fig. 2). This was consistent with previous studies that SWEETs were identified as a bidirectional transporter that transports sugar passively along a concentration gradient with low affinity (Chen et al., 2010); 2) *GmSWEET15* was specifically expressed in the endosperm at the cotyledon stage. (Fig. 1 and Supplemental Fig. S1). 3) Knockout of *GmSWEET15* significantly reduced sucrose as well as glucose content in the embryos (Fig. 5). 4) *gmsweet15* mutants had very high seed abortion rates (Fig. 4C and 4D). The abortion is likely caused by the declined sugar concentration in developing embryos, as increasing light intensity to increase photosynthetically generated sucrose can partially rescue the seed abortion phenotype of *gmsweet15* mutants (Fig. 4D and Supplemental Fig. S7). Also, as a decrease in glucose concentration might trigger programmed cell death and inhibit cell division, the programmed cell death may contribute to the high rate of seed abortion observed (Ruan et al., 2012; Liu et al., 2016).

Phylogenetically GmSWEET15 is closely related to its putative ortholog, AtSWEET15, but their biological roles differ in the following aspects: 1) *AtSWEET15* is expressed in roots and leaves as well as developing seeds (Chen et al., 2015b). It is also induced by senescence in leaves (Gamas et al., 1996). In contrast, expression of *GmSWEET15* was not detected in leaves, stems, roots, nodules and flowers (Fig. 1A); 2) Mutation of *AtSWEET15* alone did not affect seed development. Only combined loss of function of AtSWEET11, AtSWEET12, and AtSWEET15 in the *atsweet11;12;15* triple mutant, resulted in altered embryo development, resulting in a "wrinkled" seed phenotype (Chen et al., 2015b), but no seed abortion was found in the *atsweet11;12;15* triple mutant. In contrast, *gmsweet15* showed more serious defects, delayed embryo development and severe seed abortion rate compared to *atsweet15* (Fig. 4). Compared to Arabidopsis seeds, soybean seeds have large amount of storage reserves. Thus, protein and oil-rich soybean seeds should require more sugar than Arabidopsis seeds. It may also represent a case of subfunctionalization, where in soybean *GmSWEET15* is the primary sugar transporter expressed, while in Arabidopsis there may be additional sugar transporters.

During seed development in dicotyledonous plants, the growing embryo invades

and eventually consumes the surrounding endosperm tissue. The *gmsweet15* mutant displayed an endosperm persistence phenotype (Fig. 4A). Previous studies have shown the conversion of sucrose to hexoses during early seed development is important for cell division and cell differentiation (Borisjuk et al., 1998; Weber et al., 2005). In addition to directly providing carbon nutrients to embryos, sugars also function as a signal molecule to regulate development and gene expression in plants (Ruan et al., 2010; Ruan, 2014; Peng et al., 2018). It has been reported that the hexose/sucrose ratio is an important factor for normal seed development (Weschke et al., 2003). In *gmsweet15* mutant seeds, the sugar contents were dramatically lower than those in the WT, both in embryos and integuments. It is possible that the lower sugar contents may affect the sugar signaling pathway and the expression of other genes, and then impede the degradation of endosperm. In addition, studies have shown that the developing embryos can deliver developmental signals to the endosperm (Nowack et al., 2006). In our study, the retardation of embryo development in *gmsweet15* likely resulted from the lower sugar content in the developing seed. The low sugar availability not only reduced sugar supply for embryo development, but also likely affected the signal communication between embryo and endosperm, ultimately leading to endosperm persistence.

Materials and Methods

Plant material and growth conditions

Soybean plants were grown in a greenhouse (16-h-light/8-h-dark, 30℃ day/25℃ night). Cultivar Williams 82 was used as the recipient for Agrobacterium-mediated transformation experiments. For field experiments, soybean plants were grown in Anhui Academy of Agricultural Science, Anhui province, China.

Plasmid construction for transformation

To study the sub-cellular localization of *GmSWEET15a* and *GmSWEET15b*, the cDNAs of *GmSWEET15a* and *GmSWEET15b* were cloned into the pBI121 construct

and fused to N-terminus of eYFP driven by cauliflower mosaic virus (CaMV) 35S promoter. The resulting binary vector, p35S:GmSWEET15-eYFP, was introduced to *N. benthamiana* leaves by agroinfiltration.

To avoid the possible penalty resulting from non-specific expression of the transgene by using a constitutive promoter, we used the *GmSWEET15* endogenous promoter to overexpress *GmSWEET15*. A 4327-bp genomic fragment containing a 2112-bp promoter (upstream ATG of the gene) and 2215-bp coding region sequence of *GmSWEET15b* was cloned into the modified pBI121-eYFP vector. The binary vector was named GmSWEET15-eYFP.

The CRISPR/Cas9 construct was based on the pBlu-gRNA vector and Cas9 MDC123 (Addgene plasmids 59188 and 59184). The 20-bp target sequence (5'-GGCATAGTATAGCCAAAGCA-3') was designed in the conserved region of both *GmSWEET15a* and *GmSWEET15b* in the third exon of the coding sequence. The target sequence was synthesized and cloned into pBlu-gRNA at *Bbs*I site and under the control of U6 promoter. The construct was then digested with *Eco*RI to generate the gRNA cassette and inserted into CAS9 MDC123. The resulting construct was named *gmsweet15*-CAS9.

The above binary vectors were introduced into *Agrobacterium* strain LBA4404. The vectors were transformed into soybean cultivar Williams 82 via *Agrobacterium tumefaciens* as described (Song et al., 2013).

Construction of a Phylogenetic tree

A phylogenetic tree was built based on amino acid sequences of soybean, Arabidopsis and rice SWEET proteins using the Neighbor-Joining method in Mega version 6.0. The accession numbers of the transporters analyzed are listed in Supplemental Table S2.

RNA extraction and quantitative real-time PCR (RT-qPCR)

To determine location of *GmSWEET15* expression, different organs were collected from soybean plants grown in soil pots in greenhouse. Fresh tissues were ground in

liquid nitrogen by a tissue homogenizer (TL2010S, DHS) and extracted for total RNA using a RNA extraction kit (Tiangen Biotech, Beijing, China). cDNA synthesis was performed by the PrimeScript RT reagent kit (Takara Bio, Inc., Dalian, China). The RT-qPCR was conducted using SYBR premix Ex Taq (Takara Bio, Inc., Dalian, China) and the primers were designed at the region with common sequence of *GmSWEET15a* and *GmSWEET15b*. The soybean housekeeping gene cyclophilin 2, *GmCYP2*, was used as an internal control. Transcript levels were calculated relative to *GmCYP2* using the formula $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$. Primers used for RT-qPCR analysis are listed in Supplemental Table S1. All amplification reactions were performed with three biological replicates and two technical replicates.

In situ RNA hybridization

Soybean seeds at 10-14 days (cotyledon stage) after fertilization were collected from plants grown in greenhouse, and immediately fixed in FAA [formaldehyde : glacial acetic acid : ethanol, 5:5:50% (v/v/v)]. The samples were dehydrated through an ethanol series, then embedded in paraffin for sectioning. The 45-bp common sequence of *GmSWEET15a/b*, 5'-GTGATGAACGTGGGTTCCTTCGCCTTGATCTTCCTC GTCACCTAC-3', was used to make sense and antisense RNA probes. *In situ* RNA hybridization was performed as described (Moussu et al., 2017). Briefly, 8-micrometer sections were treated with 0.2mol/L HCl for 15 min at room temperature and washed with RNase-free H_2O. Then the tissue sections were digested with proteinase K (20 mg/mL) for 20 min at 37°C, after that 0.2% (w/v) glycine was used for termination reaction, then fixed with 4% (w/v) paraformaldehyde for 10 min. The samples were treated with 0.25% (w/v) acetic anhydride (pH 8.0) for 5 min and then washed with saline sodium citrate (SSC) for 2 min. The sections were hybridized with 500ng/ml FAM-labeled probes (65℃, 48 h). After hybridization, the sections were successively washed with SSC, the mixture of formamide and SSC (formamide: SSC, 1:1) and finally washed in phosphate-buffered saline (PBS, containing 138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4). The signal was observed under an LSM 710 NLO confocal laser scanning microscope (Zeiss).

Sub-cellular localization

Agrobacterium tumefaciens strain EHA105, carrying the constructs p35S:SWEET15b-eYFP and CD3-1007 (AtPIP2A: mCherry) were infiltrated into leaves of *Nicotiana benthamiana* for transient expression. After infiltration for two days, YFP and mCherry fluorescence signal were observed under an LSM 710 NLO confocal laser scanning microscope (Zeiss). AtPIP2A: mCherry was used as a plasma membrane marker (Nelson et al., 2007).

Soluble sugar analyses

Embryos and whole seeds were collected and weighed before freezing in liquid nitrogen. Samples were ground in liquid nitrogen by a tissue homogenizer. After grinding, 1ml ddH_2O was added into the tubes and samples were boiled for 20min at 100℃. After centrifugation, the supernatant was diluted 9 times with ddH_2O and purified through 0.25μm Millipore filters. The filtrate fraction was analyzed for sugar composition using a Dionex ICS-3000 ion chromatography system (ICS-3000, DIONEX, Germany).

Sugar influx and efflux activity in *Xenopus* oocytes

Transport activity of GmSWEET15b was determined as reported (Chen et al., 2010). cRNA was prepared by T7 polymerase using the mMESSAGE mMACHINE kit. Fifty nanoliter of cRNA or water was injected into each oocyte. After one day, oocytes were tested for influx activity. Six oocytes were transferred into tubes containing 200 ul Na-Ringer buffer (115 mM NaCl, 2mM KCl, 1mM $MgCl_2$, 1.8 mM $CaCl_2$, 10mM HEPES-Tris, pH 7.5) with 100 mg/l gentamycin and 100 μM sucrose (4 μCi/ml ^{14}C sucrose) or glucose (4 μCi/ml ^{14}C glucose). After 2 h at 18^{o}C , cells were washed with cold sucrose in Na-Ringer buffer 4 times. Radioactivity was measured by a liquid scintillation analyzer after the oocytes were extracted by 0.1M HNO_3. Two days after the injection of cRNA, oocytes were injected with 50 nl solution containing 1 mM sucrose (0.18 μCi/μl ^{14}C sucrose) or glucose (0.18 μCi/μl ^{14}C glucose) for measuring the efflux activity. Six oocytes per replicate were transferred into tubes and the cells

were immediately washed with cold Na-Ringer buffer four times. The cells were then incubated in 500 μl Na-Ringer buffer and the external buffer was collected at 0.5 h and 2 h. Radioactivity of the external solution and oocytes was measured by a liquid scintillation analyzer. The efflux activity was expressed as a percentage (radioactivity in external solution/(radioactivity in external solution + radioactivity remaining in the oocytes) x100).

Statistical analyses

For comparisons of treatments in Figures 2, 4 and 5, significance was determined by a one-way analysis of variance (ANOVA) and for differences between groups, with least significant difference (LSD) test.

Accession Numbers

Sequence data from this article can be found in Supplemental Table S2.

Supplemental Data

Supplemental Figure S1. Expression profiles of sugar metabolism-related genes in soybean seeds at early developmental stages.

Supplemental Figure S2. Protein phylogeny of SWEET transporters in *Glycine max* (Gm), *Oryza sativa* (Os) and *Arabidopsis thaliana* (At).

Supplemental Figure S3. Alignment reveals conserved amino acid sequence of SWEETs.

Supplemental Figure S4. Sub-cellular localization of GmSWEET15a and GmSWEET15b by Agroinfiltration in *N. benthamiana* for transient expression.

Supplemental Figure S5. Knock out of *GmSWEET15* caused severe seed abortion and delay of seed development.

Supplemental Figure S6. Abortion rates of the F_1 generation (hybrids) from crosses of parents carrying WT or mutated *GmSWEET15*.

Supplemental Figure S7. Seed abortion rate of *gmsweet15* mutants was reduced under increased light intensity.

Supplemental Table S1. Primers used in this study.

458 **Supplemental Table S2.** The accession numbers of genes in this study.
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461
462 **Supplemental Figure S1.** Expression profiles of sugar metabolism-related genes in
463 soybean seeds at early developmental stages. All GmSWEETs, GmINVs, GmSUSs,
464 GmSUCs expressed in early development seed were listed in heatmap (RPKM>10).
465 The data was downloaded from Gene Networks in Seed Development
466 (http://seedgenenetwork.net/soybean)(Bob Goldberg et al). SUS, sucrose synthase;
467 SUC, sucrose/H+ symport.
468 **Supplemental Figure S2.** Protein phylogeny of SWEET transporters in *Glycine max*
469 (Gm), *Oryza sativa* (Os) and *Arabidopsis thaliana* (At). The inset table listed the
470 numbers of soybean, Arabidopsis and rice SWEET transporters in each of the clades.
471 **Supplemental Figure S3.** Alignment reveals conserved amino acid sequence of
472 SWEETs.
473 **Supplemental Figure S4.** Sub-cellular localization of GmSWEET15a and
474 GmSWEET15b by Agroinfiltration in *N. benthamiana* for transient expression. YFP
475 protein was fused to C terminus of GmSWEET15a and GmSWEET15b and expressed
476 in tobacco leaf epidermal cells using Agrobacterium mediated transformation.
477 Fluorescence signal was detected by a LSM710nlo confocal laser scanning
478 microscope. CD3-1007 was used as a plasma membrane localized marker. Bars=50
479 μm.
480 **Supplemental Figure S5.** Knock out of *GmSWEET15* caused severe seed abortion
481 and delay of seed development.
482 **Supplemental Figure S6.** Abortion rates of the F_1 generation (hybrids) from crosses
483 of parents carrying WT or mutated *GmSWEET15*.
484 **Supplemental Figure S7.** Seed abortion rate of *gmsweet15* mutants was reduced
485 under increased light intensity.
486 **Supplemental Table S1.** Primers used in this study.
487 **Supplemental Table S2.** The accession numbers of genes in this study.
488 **Acknowledgements**

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Figure Legends

Figure 1. Expression of Gm*SWEET15* in developing seeds. (A) Gene expression of GmSWEET15 in endosperm. The expression data was extracted from Gene Networks in Seed Development (http://seedgenenetwork.net/soybean). The square marked (red) in the seed at the cotyledon stage corresponds to Fig. 1D below. EP, Embryo Proper; EPD, Epidermis; ENT, Endothelium; ES, Endosperm; HI, Hilum; II, Inner Integument; OI, Outer Integument; S, Suspensor. RPKM, Reads Per Kilobase of exon model per Million mapped reads. (B) Transcript abundance of Gm*SWEET15* in different soybean organs. Expression was detected by quantitative reverse transcription polymerase chain reaction (RT-qPCR). Transcript levels were calculated relative to soybean cyclophilin 2, *GmCYP2*. Seeds at the cotyledon stage correspond to 10-14 days after fertilization. n.d., not detectable. (C) *GmSWEET15* is highly expressed in endosperms of cotyledon stage seeds. Seed parts were physically separated, and expression was determined by RT-qPCR. Transcript levels were calculated relative to soybean cyclophilin 2, *GmCYP2* (D) Fluorescence *in situ* hybridization of cotyledon stage seed section with FAM-labeled Gm*SWEET15* anti-sense probe. Photos were taken under bright-field (top) or fluorescence microscopy (bottom). Square in red on the left is enlarged in the right-hand panel. E, embryo; ES, endosperm; SC, seed coat. Bars = 100μm. Data shown as the mean ± SD.

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Figure 3. Characterization of CRISPR/CAS9-mediated knock out mutants and over-expressing lines of *GmSWEET15*. (A) Top image, the target site for gene editing. The arrow indicates the target site of the CRISPR/CAS9 system in the common region of the *GmSWEET15a* and *GmSWEET15b* genes; Bottom image, changes highlighted in red in the DNA sequence of the targeted region in the *gmsweet15-1 and 15-2* mutants and amino acid sequence of *GmSWEET15* mutants. (B) Expression of *GmSWEET15* relative to soybean cyclophilin 2, *GmCYP2* in the overexpressing lines. *GmSWEET15* was overexpressed by introducing an additional copy of the *GmSWEET15b* genomic sequence driven by its native promoter. Three independent transgenic lines were generated and designated OE1, OE2 and OE3. RT-qPCR primers were designed at the conserved sequence in *GmSWEET15a* and *GmSWEET15b.* Data are means ± SD (n = 3) *P < 0.05, **P < 0.01 (One-way ANOVA followed by LDS test).

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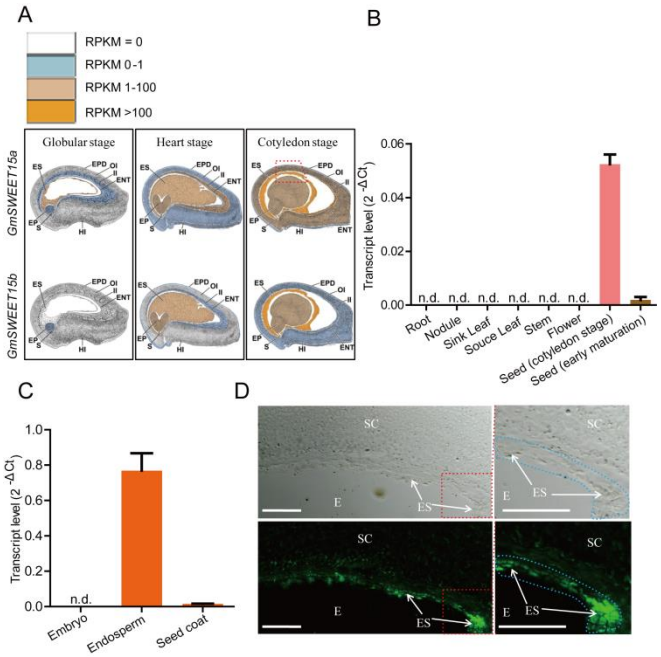

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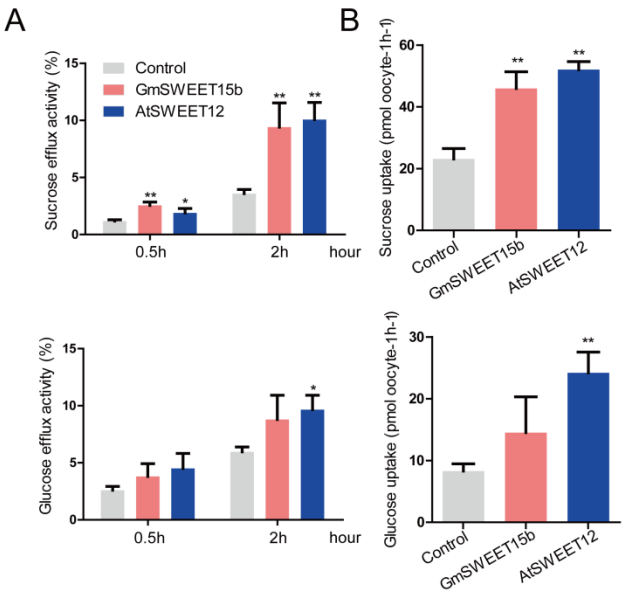

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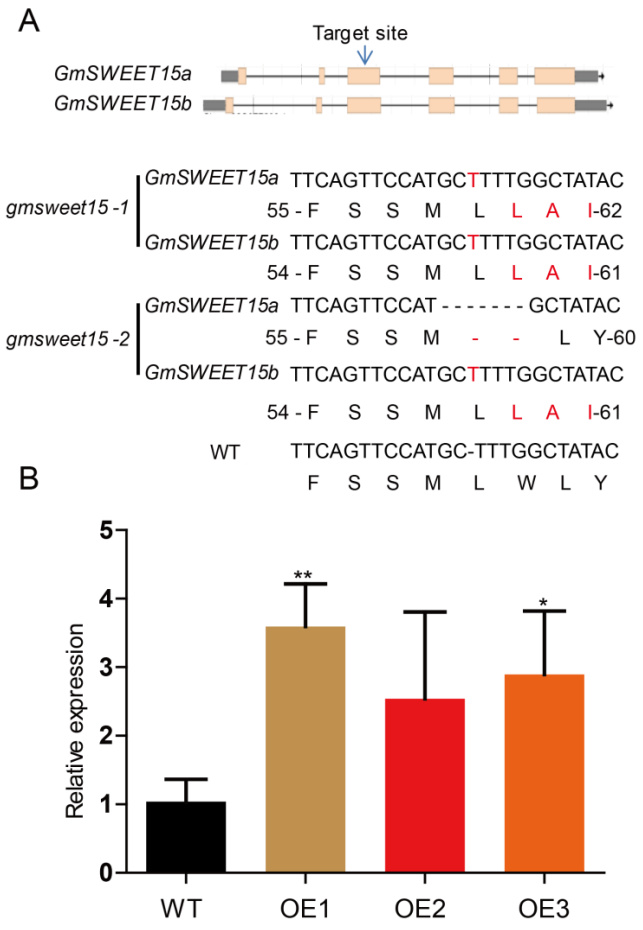

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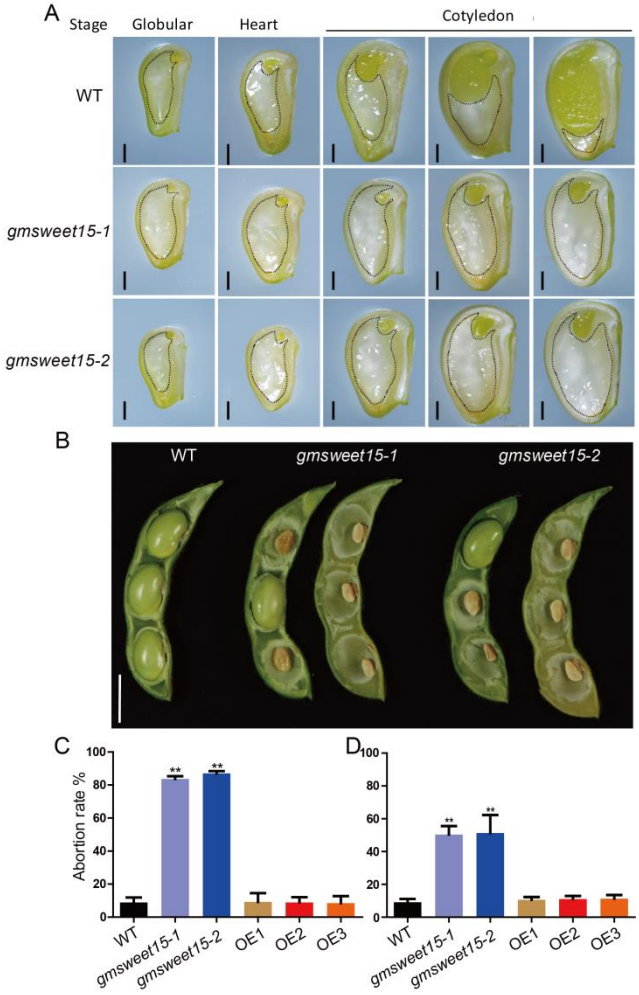

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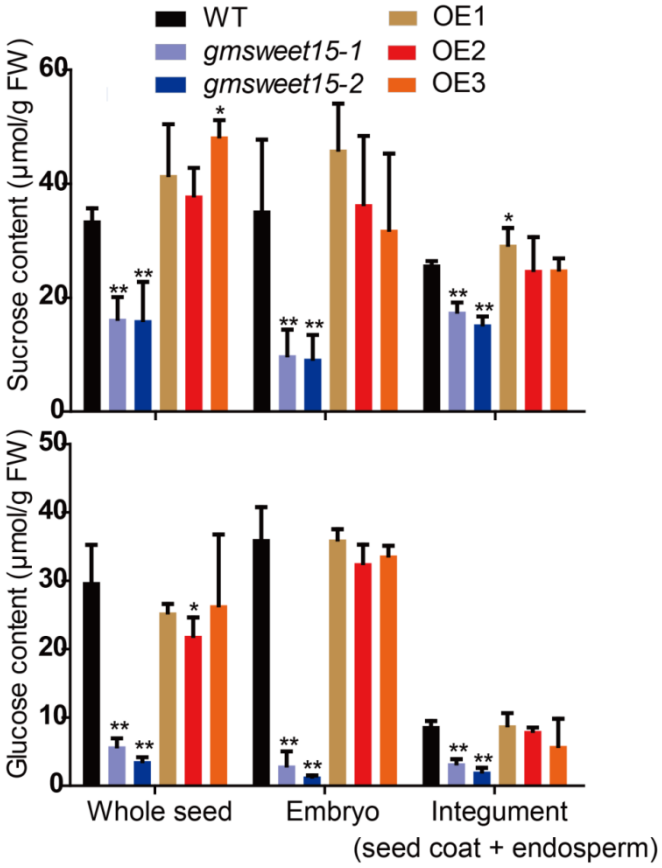

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