

A Cascade of Sequentially Expressed Sucrose Transporters in the Seed Coat and Endosperm Provides Nutrition for the Arabidopsis Embryo^{OPEN}

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Developing plant embryos depend on nutrition from maternal tissues via the seed coat and endosperm, but the mechanisms that supply nutrients to plant embryos have remained elusive. Sucrose, the major transport form of carbohydrate in plants, is delivered via the phloem to the maternal seed coat and then secreted from the seed coat to feed the embryo. Here, we show that seed filling in *Arabidopsis thaliana* requires the three sucrose transporters SWEET11, 12, and 15. SWEET11, 12, and 15 exhibit specific spatiotemporal expression patterns in developing seeds, but only a *sweet11;12;15* triple mutant showed severe seed defects, which include retarded embryo development, reduced seed weight, and reduced starch and lipid content, causing a “wrinkled” seed phenotype. In *sweet11;12;15* triple mutants, starch accumulated in the seed coat but not the embryo, implicating SWEET-mediated sucrose efflux in the transfer of sugars from seed coat to embryo. This cascade of sequentially expressed SWEETs provides the feeding pathway for the plant embryo, an important feature for yield potential.

INTRODUCTION

Developing embryos of Metazoa and plants have to be nurtured by maternal tissues: the placenta and umbilical cord in mammals and the seed coat and endosperm in plants. Glucose transporters of the GLUT (SLC2) and SGLT (SLC5) families are likely involved in supplying glucose to mammalian embryos (Illsley, 2000; Baumann et al., 2002; Kevorkova et al., 2007), although direct evidence, e.g., from the analysis of mutants, is lacking. The mechanisms for nutrition of plant embryos have also remained elusive. Although seeds can undergo greening, embryo development depends on the supply of photoassimilates from maternal tissues, particularly photosynthetic source leaves. Sucrose is the major long-distance transport form of sugars delivered from photosynthetic tissues to the growth and storage organs, including seeds of many plants; green silique walls may also contribute to some extent. Importantly, sucrose creates the driving force for long-distance translocation of all other compounds in the phloem. Sucrose is imported into the developing embryo by plasma membrane SUT sucrose/proton cotransporters (Patrick and Offler, 1995; Baud et al., 2005; Zhang et al., 2007).

In *Arabidopsis thaliana*, sugars are delivered to the maternal seed coat via the funicular phloem, which is symplasmically connected to the outer integument (Stadler et al., 2005). Sugars are released from the outer integument to the inner integument,

and potentially the suspensor, followed by secretion into the seed apoplast by yet unknown membrane transport mechanisms. How sucrose is released from maternal tissues (seed coat) to support filial tissues (embryo) also remains unclear, except for the contribution of a subset of transporters of the SUT sucrose/H⁺ cotransporter family. Evidence from studies of pea (*Pisum sativum*) and bean (*Phaseolus vulgaris*) seed coats implicated SUF transporters, which appear to have lost proton coupling to act as uniporters, in sucrose efflux from seed coat (Ritchie et al., 2003; Zhou et al., 2007). These uncoupled SUFs appear to have evolved from recent gene duplications and likely represent a specific adaptation in legumes to sustain the large seeds in some of these legumes, yet they have not been found in other plants, such as *Arabidopsis* or maize (*Zea mays*) (Zhou et al., 2007).

The recently identified SWEET sugar transporters of eukaryotes have seven transmembrane domains and function predominantly in cellular efflux (Chen et al., 2010, 2012; Xu et al., 2014). The SWEET family is divided into four clades. Clade III SWEETs appear to transport sucrose in a pH-independent manner and are typically involved in cellular efflux processes (Chen et al., 2010, 2012; Lin et al., 2014). Two members of this clade, SWEET11 and 12, appear to localize to the plasma membrane of phloem parenchyma cells and export sucrose from these cells into the phloem's apoplast in preparation for phloem loading. A third member, the nectary-specific SWEET9, was shown to be essential for nectar secretion in angiosperms (Lin et al., 2014). Rice SWEET11, 13, and 14 and cassava (*Manihot esculenta*) SWEET10a play important roles in pathogen susceptibility, possibly supplying nutrients to pathogens (Chen et al., 2012; Streubel et al., 2013; Cohn et al., 2014). Analysis of microarrays generated by laser capture microdissection of tissues in the seed of *Arabidopsis* indicated that several Clade III SWEETs were expressed in developing seeds

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(Figure 1A; Supplemental Figure 1) (Dean et al., 2011; Belmonte et al., 2013). We thus hypothesized that one or several Clade III SWEETs may serve as the elusive sucrose carriers for efflux from the integument into the apoplast, as well as from the endosperm to support growth and development of the embryo.

Here, we show that SWEET11, 12, and 15 are expressed in particular tissues of the seeds during development. We demonstrate that a *sweet11;12;15* triple mutant shows retarded embryo development, reduced seed weight and lipid content,

and “wrinkled” seeds. The seed coat of *sweet11;12;15* mutants accumulated more starch, while the embryos had reduced starch content compared with the wild type. These findings, together with results from reciprocal crosses showing that the phenotypes of *sweet11;12;15* are mainly maternally controlled, indicate that SWEETs are responsible for sugar efflux from the maternal seed coat. Differential expression in seed coat and endosperm indicates that the path of sugar from the phloem embedded in the funiculus to the embryo involves a developmentally controlled multistep

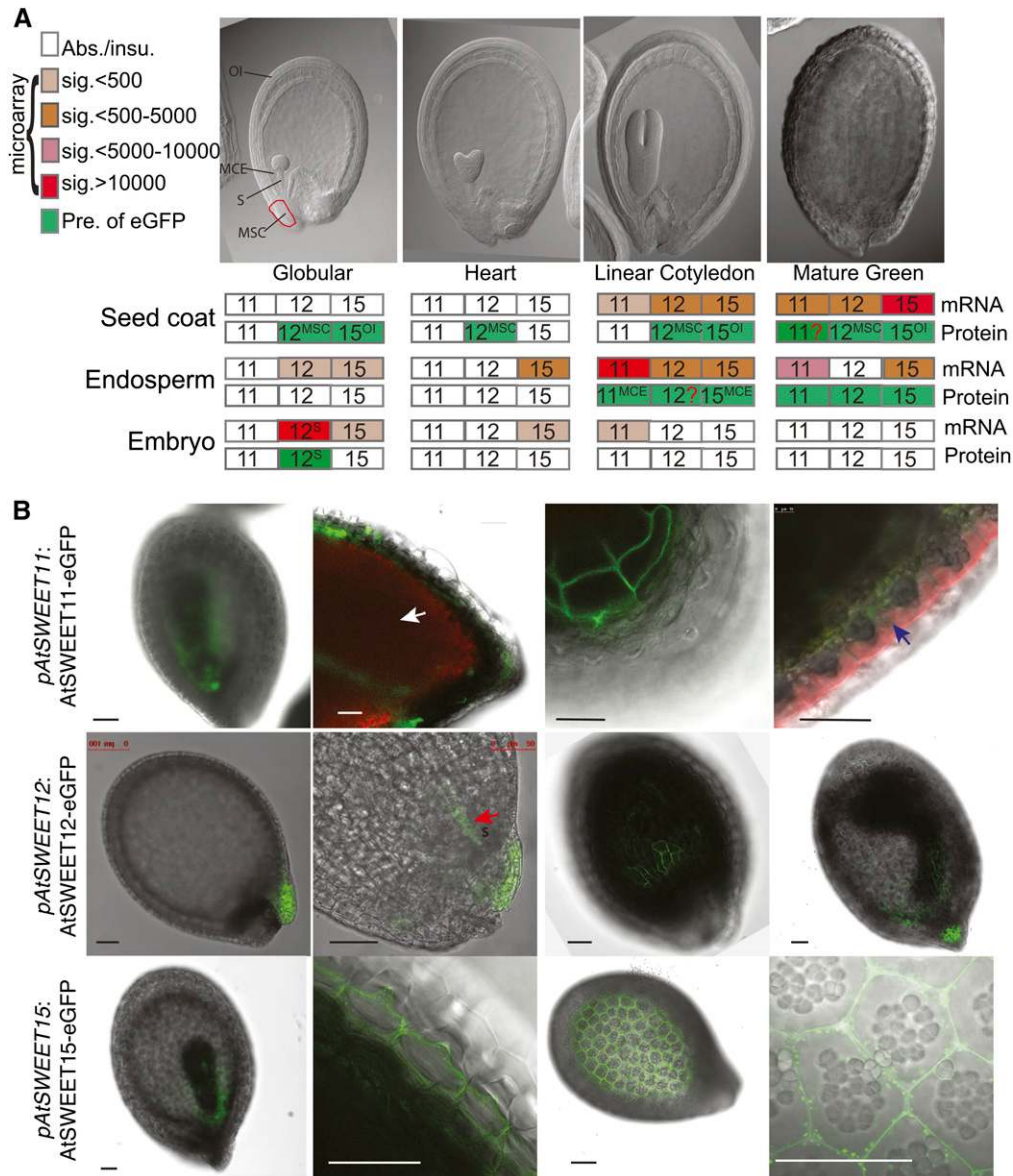


Figure 1. Expression and Localization of SWEET11, 12, and 15 in Seeds.

(A) Tissue-specific *SWEET* gene expression from microarray analysis (Dean et al., 2011) and protein accumulation as assessed by translational SWEET-GFP fusions during seed development. Representative images of developing seeds are shown above the panels. MSC, micropylar end of seed coat; MCE, micropylar endosperm; OI, outer integument; S, suspensor.

(B) Confocal images of eGFP fluorescence in transgenic Arabidopsis seeds expressing translational SWEET11-, 12-, or 15-eGFP fusions under control of their native promoters. The white arrow points to red autofluorescence of the cotyledon. The blue arrow points to red propidium iodide staining of cell walls. The red arrow points to the suspensor. Bar = 50 μm.

process with several apoplasmic transport steps mediated by SWEET11, 12, and 15.

RESULTS

Expression of SWEET11, 12, and 15 in Seeds

SWEETs are prime candidates to play roles in sugar secretion from maternal tissues during seed development. To explore whether SWEETs may function in supplying the developing embryo with sucrose, we analyzed the expression of SWEET genes in Arabidopsis seeds from two sets of microarray data with cell-type or tissue-specific resolution (Dean et al., 2011; Belmonte et al., 2013). Microarray data analyses indicate that SWEET11, 12, and 15 may be candidates for sucrose efflux from seed coat, as well as efflux from the endosperm to ultimately supply the developing embryo with phloem-derived sugar (Figure 1A; Supplemental Figure 1) (Chen et al., 2010; Dean et al., 2011; Belmonte et al., 2013). SWEET11 transcripts accumulated primarily in the endosperm and seed coat during the linear cotyledon stage and the maturation green stage. SWEET12 transcripts were most abundant in the seed coat at the same stages (i.e., linear cotyledon and maturation green stage) and appeared in the suspensor and at the micropylar end of the seed coat at the globular stage. SWEET15 transcripts were detected in the endosperm during the globular and maturation green stages. Only weak expression of SWEET15 was found in seeds at the preglobular stage, but SWEET15 expression in seed coat became dominant during the linear cotyledon and maturation stages. Despite partial overlap of SWEET11, 12, and 15 expression in several subregions during multiple stages, each gene appeared to be regulated differentially during development (Figure 1A; Supplemental Figure 1A), implying a complex, multistep feeding pattern.

To explore the tissue and cellular distribution of SWEET11, 12, and 15 at the protein level, Arabidopsis seeds from plants expressing native promoter-driven translational SWEET11-, 12-, or 15-eGFP (enhanced green fluorescent protein) fusions were examined using confocal microscopy. Strong SWEET11-eGFP fluorescence was observed in the endosperm at the linear cotyledon stage, while weaker fluorescence was also detected in the chalazal seed coat (Figure 1B; Supplemental Figure 2 and Supplemental Movies 1 and 2). Transmission electron microscopy (TEM) immunocytochemistry also supported plasma membrane localization of SWEET11 (Supplemental Figure 2) (Chen et al., 2012). Confocal microscopy of SWEET12-eGFP-expressing seeds detected strong fluorescence at the micropylar end of the seed coat from the preglobular stage throughout the maturation stage and in the suspensor at the globular stage (Figures 1A and 1B). Accumulation of SWEET15-eGFP was detected in the epidermal cells of the seed coat at the preglobular stage, which then faded from globular stage to heart stage, but appeared bright again from the linear cotyledon stage throughout the maturation stage, as well as in the micropylar endosperm layer closest to the embryo at the linear cotyledon stage (Figure 1B; Supplemental Figure 2). In seed coat cells, SWEET15-eGFP was detectable both at the plasma membrane and in intracellular puncta, which likely are Golgi bodies, as indicated by TEM (Figure 1B; Supplemental Figure 2). The localization pattern of the three transporters did not perfectly match the microarray expression profiles, potentially implying posttranscriptional regulation.

Overall, each SWEET appears to exhibit a specific spatiotemporal expression and distribution pattern in the developing seed, especially in the micropylar end of the seed coat, the micropylar endosperm, and the suspensor, intimating diversified roles in different cell types over time, as well as partially redundant roles during seed development.

Tissue-Specific Expression of SWEET15

In addition to their presence in seed tissues, we found SWEET11 and 12 proteins in the vascular tissue of rosette leaves (Chen et al., 2012). SWEET15, also named SAG29 (SENESCENCE-ASSOCIATED GENE29), was localized at the plasma membrane of Arabidopsis protoplasts (Seo et al., 2011). SWEET15/SAG29 expression was used as a senescence marker because the transcript had been found to increase during senescence (Quirino et al., 1999; Seo et al., 2011). To evaluate the temporal and spatial expression pattern of SWEET15 at the protein level, a histochemical analysis using a translational SWEET15-GUS (β -glucuronidase) fusion driven from its native promoter was performed. In young plants (before bolting), GUS activity was below the detection level except for very weak staining in the petioles (Figure 2A). The 38-d-old plants (2 d after bolting) and 44-d-old plants (8 d after bolting) showed high GUS activity in young buds of the inflorescence, which declined during maturation. However, only low GUS activity was observed in senescent leaves (Figures 2B to 2E). Consistent with the SWEET15-eGFP data shown above, GUS activity was also detected in the seed (Figure 2F). Thus, while SWEET15 gene expression appears to be highly induced during senescence, there is little evidence for SWEET15 protein accumulation in leaves at the stages used here for analyzing the role of SWEETs in seed filling, intimating that SWEET15 is not likely to make a major contribution to phloem export of sucrose for a major part of the seed filling phase.

SWEET11, 12, and 15 Transport Sucrose in *Xenopus laevis* Oocytes

To test whether SWEET15 functions as a sucrose transporter, we expressed SWEET15 in *X. laevis* oocytes and measured [14 C]-sucrose uptake. As one might expect from its phylogenetic proximity to the sucrose transporting SWEET11 and 12 (Chen et al., 2012), SWEET15 also functions as a sucrose transporter (Supplemental Figure 3).

Delayed Embryo Development in a *sweet11;12;15* Mutant

We hypothesized that if less sugar was released from maternal tissues of *sweet* mutants, less sugar would be available to filial tissues (embryo), possibly leading to defects in embryo growth and development. Therefore, we tested embryo growth of single *sweet11*, 12, and 15 mutants. All three single *sweet* mutants appeared normal; only embryos of *sweet12* were slightly smaller compared with the wild type (Figure 3A). Similarly, the double mutants *sweet11;12*, *sweet11;15*, and *sweet12;15* were characterized by a more severe reduction in embryo size relative to the *sweet12* single mutant (Figure 3B). By contrast, the triple mutant of *sweet11;12;15* showed a significant delay in embryo development compared with the double mutants, at least during the period between 3 and 13 d postanthesis (DPA) (Figures 3C and 4).

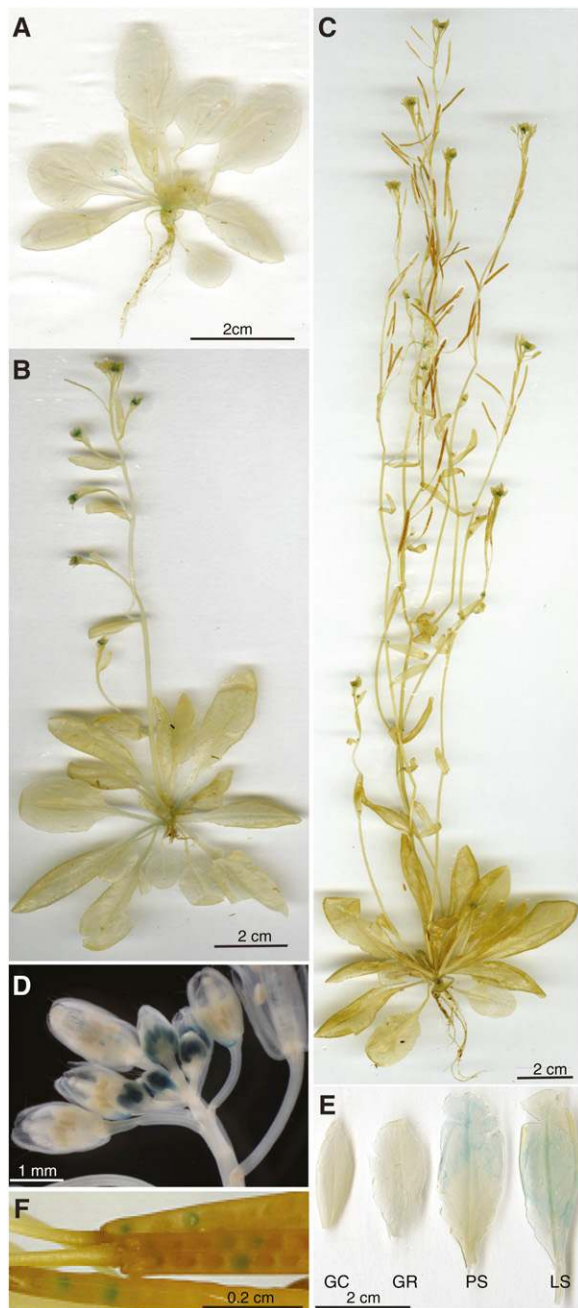


Figure 2. Spatial and Temporal Expression of SWEET15.

(A) to (C) Distribution of GUS activity in whole plants at different ages: (A) 25 d old, (B) 38 d old, and (C) 44 d old. (D) High GUS activities detected in the inflorescence. (E) and (F) GUS activity (E) in leaves at different developmental stages and in seed (F). Two individual transgenic lines were tested. One of the lines giving stronger GUS activity is shown here. GC, green cauline leaf; GR, green rosette leaf and seed; PS, partially senescent leaf; LS, late stage senescent leaf.

Although the difference in embryo size between the wild type and the triple mutant gradually diminished after 8 DPA, in terms of developmental milestones, e.g., greening (Figures 3C [at 13 DPA] and 4), they remained smaller in size even at the mature stage. The defect in embryo size was rescued by complementation with any one of SWEET11, 12, or 15 (as translational eGFP fusions) expressed from their own native promoters (Supplemental Figure 4), strongly supporting the hypothesis that in the triple mutant, retarded embryo growth is caused by loss of SWEET function. The result also demonstrates that the SWEET-GFP fusions were functional in planta. It is worth noting that SWEET11 and 12 are key for phloem loading (Chen et al., 2012) and may thus affect delivery of sucrose from the leaves, thereby further limiting seed filling. However, SWEET15, which is unlikely to make a major contribution to phloem loading, can mostly rescue the delayed growth of *sweet11;12;15* triple mutant embryos.

To analyze how embryogenesis is affected in the triple *sweet11;12;15* mutant, we compared embryo development at different stages by differential interference contrast (DIC) microscopy of cleared seeds (Figures 4A and 4B) and quantified embryo size (Figure 4C). Differences between embryos of *sweet11;12;15* and the wild type became apparent at the transition from globular to heart stage (4 DPA) in young siliques (Figures 4A and 4B). The maximal difference was observed around 8 DPA, at a time when SWEET11, 12, and 15 proteins accumulated to high levels (Figure 1; Supplemental Figure 2). The delay in development was clearly identifiable when comparing mutant to the wild type; i.e., at 6 DPA, the wild type reached the linear cotyledon stage, while the triple mutant had reached the heart stage. Transporter-mediated sucrose export from the endosperm is supported by the observation that fluorescence derived from SWEET fusions accumulated in the endosperm, a finding in line with published microarray data (Belmonte et al., 2013) (Figure 1B; Supplemental Figure 2).

To test whether the delay in embryo growth in *sweet11;12;15* is maternally controlled, we performed reciprocal crosses between the wild type and *sweet11;12;15*. A dramatic reduction in *sweet11;12;15* embryo size was observed only when *sweet11;12;15* was used as the maternal parent (Figure 4D). Inheritance of integuments is maternally controlled and inheritance of endosperm is maternal in part (Berger et al., 2006); thus, the sequential expression of SWEETs in the seed coat and endosperm is in agreement with the results of reciprocal crosses. In conclusion, SWEET11, 12, and 15 play key roles on the maternal side and are necessary for securing efficient sugar supply for seed development.

Role of SWEETs in Determining Yield

To characterize the effect of changes in SWEET activity on yield, we measured the dry seed weight in triple mutants. In four independent experiments, the yield (seed weight per plant) of the *sweet11;12;15* mutant was reduced by ~43%, while that of *sweet11;12* was reduced by ~23% (Figure 5A). Reduced seed weight could be caused by smaller seeds, fewer seeds, or fewer siliques per plant. To differentiate between these possibilities, we measured the dry seed weight from a defined number of siliques and found a decrease in seed weight of *sweet11;12* by 17% and *sweet11;12;15* by 34% (Figure 5B). The lower seed weight was

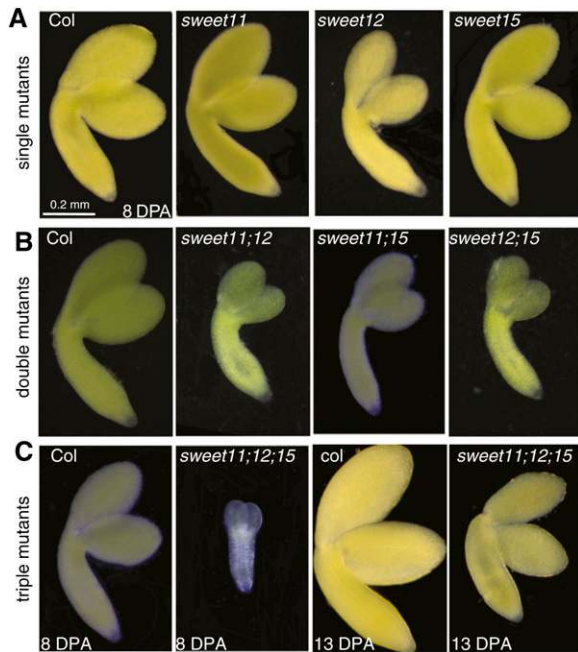


Figure 3. SWEET11, 12, and 15 Are Required for Embryo Development. Embryo phenotype of *sweet* single mutants at 8 DPA (A), double mutants at 8 DPA (B), and triple mutants at 8 and 13 DPA (C). Bars = 0.2 mm.

not caused by fewer seeds per silique, since there was no difference in the number of seeds per silique when comparing *sweet11;12* and *sweet11;12;15* with Columbia-0 (Col-0) (Figure 5C). Accordingly, it was deduced that the number of siliques was reduced on average by ~7% in *sweet11;12* and 14% in *sweet11;12;15*.

Oil and protein are the major components of dry Arabidopsis seeds, each contributing ~40% to the seed mass (Baud et al., 2002). Seed dry mass closely follows the increase in oil during development (Baud et al., 2003). In seeds, sucrose is the primary source of acetyl-CoA, which serves as the precursor for the lipid biosynthesis (Rawsthorne, 2002). As one may have expected, seed lipid content (fatty acid methyl esters [FAMES]) was reduced by 34% in *sweet11;12* and 71% in *sweet11;12;15*.

Scanning electron microscopy was used to ascertain possible morphological alterations in the seed. Seeds of the triple mutant had a deformed surface, phenotypically similar to the wrinkled *wri1* mutants, which are defective in an APETALA2/ethylene-responsive element binding protein transcription factor involved in seed storage metabolism (Focks and Benning, 1998; Cernac and Benning, 2004). The wrinkled seed phenotype was not simply the result of lower polysaccharide levels in the seed epidermis because seeds hydrated in Ruthenium Red did not show a mucilage extrusion defect (Supplemental Figure 5B). The wrinkled phenotype in *sweet11;12;15* is consistent with incompletely filled seeds containing smaller embryos (Figures 3A to 3C).

Rescue of Impaired *sweet11;12;15* Seedling and Embryo Growth by Sucrose

Early postgerminative growth critically depends on sufficient amounts of nutrients stored in the seed. In the case of Arabidopsis,

nutrients are transiently stored as starch, which in later development is converted to oil (Kelly et al., 2011; Theodoulou and Eastmond, 2012). To determine whether early postgerminative growth of *sweet11;12;15* is impaired, we analyzed root growth. When germinated on sugar-free media, *sweet11;12;15* exhibited reduced root length that could be ameliorated by external sucrose addition (Figure 6A). Reduced root growth, although less severe, was observed in the *sweet11;12* double mutant (Figure 6A) (Chen et al., 2012).

Sucrose is used as a carbon and energy source for in vitro growth of immature embryos (Raghavan, 2003). To test whether exogenously supplied sucrose can promote embryo growth of the *sweet11;12;15* mutant, in vitro embryo culture was performed. Embryos dissected from *sweet11;12;15* mutants and Col-0 at 3.5 to ~4 DPA failed to grow in vitro in the absence of sucrose when cultured for additional 5 to ~5.5 days (9 DPA; Figure 6B). Supply of sucrose accelerated embryo growth of *sweet11;12;15* mutants and Col-0, and the area of *sweet11;12;15* embryos reached 48% that of Col-0 embryos. However, in vivo, the area of embryos dissected from *sweet11;12;15* plants was only 24% that of embryos from Col-0 plants (Figures 6B and 6C). The reason why *sweet11;12;15* embryos are still smaller than Col-0 embryos when cultured in vitro could be due to the differences already present at the time of culture initiation (3.5 to ~4 DPA). The difference is shown in Figure 3C: The embryo area of *sweet11;12;15* was 65 and 45% of that of Col-0 at 3 and 4 DPA, respectively. Thus, embryos of the triple mutant grew faster in vitro when supplied with sucrose compared with in vivo, directly supporting the notion that the observed embryonic phenotype in the triple mutant is caused by reduced release of sucrose from seed coat and endosperm.

Relative Contribution of SWEET11, 12, and 15 to Seed Filling in Seeds versus Leaves

Reduced carbohydrate export from leaves is expected to negatively impact embryo development (Andriotis et al., 2012). SWEET11 and 12 are not only expressed in seeds, but also in leaves, as shown previously (Chen et al., 2012). To determine whether leaf export contributes in a major way to the mutant phenotype, we supplied sugar to wild-type and mutant flowers cultured in vitro (Figure 7). Similarly sized flowers of *sweet11;12;15* and Col-0 right after anthesis were cultured vertically with the pedicel inserted into Murashige and Skoog (MS)-agar medium with or without 3% sucrose. After 9 d of culture in a light/dark cycle, siliques developed from wild-type flowers cultured in the absence of sugars produced a few seeds, some of which contained a normal sized embryo (Figures 7A and 7B). Siliques from triple mutant flowers produced only a few seeds, and those that were produced did not develop beyond the heart stage (Figures 7C and 7D). In contrast, siliques cultured in sucrose-supplemented medium were able to produce seed from both wild-type and mutant flowers, although a significant number of aborted seeds was observed. Importantly, embryos from wild-type siliques were on average much larger compared with the ones from mutant siliques, in spite of the large variation of the embryos in size (Figures 7E to 7I). These results, which eliminate potential effects of the mutations of phloem delivery from leaves, are consistent with the results from intact plants, strongly

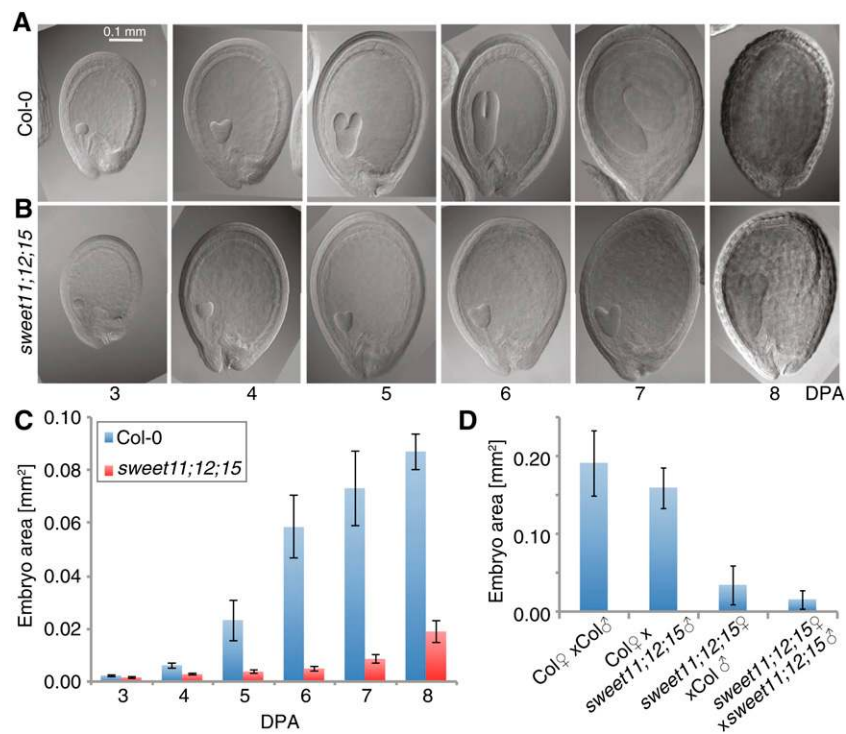


Figure 4. Comparison of the Developmental Stages of Embryos in Col and *sweet11;12;15* and Determination of the Maternal Contribution to Embryo Growth. **(A)** and **(B)** Images of cleared seeds taken by DIC microscopy. Col-0 **(A)** and *sweet11;12;15* **(B)** seeds at similar stages of embryogenesis. Bar = 0.1 mm. **(C)** Embryo area measured at the corresponding stages from images in **(A)** and **(B)** (mean \pm sd, 3 DPA, $n \geq 167$; 4 DPA, $n \geq 61$; 5 to 8 DPA, $n \geq 18$). **(D)** Embryo area measured at 9 d after pollination from the indicated reciprocal crosses (mean \pm sd, $n \geq 13$).

supporting a role of SWEET11, 12, and 15 in embryo sustenance within the seed itself (Figure 3C).

Changes in Starch Accumulation in Seed Coats and Embryos of the Triple Mutant

To investigate the role of the three SWEETs in sugar release from maternal tissues of the seed itself, we tested the effect of the mutations on starch accumulation. We hypothesized that if reduced efflux from maternal leaves was the primary cause for delayed embryo development, one would predict reduced sugar levels in the seed coat and embryo, whereas if reduced efflux from the maternal seed coat was the primary cause, sugar might accumulate in the seed coat. Starch accumulation was analyzed in *sweet11;12;15* seeds and embryos using Lugol's iodine, which stains starch blue/black. Starch was barely detectable in *sweet11;12;15* embryos and dramatically reduced relative to wild-type seeds at 9, 10, and 11 DPA, indicative of reduced delivery of sucrose to mutant embryos (Figure 8). Interestingly, starch in the mutant stained blue (Figures 8C and 8D). Consistent with the optical analysis of the starch staining, enzymatic quantitation of starch showed that the seed coat of *sweet11;12;15* mutants accumulated up to 5 times more starch compared with Col-0. Embryos of *sweet11;12;15* mutants accumulated up to 19 times less starch relative to Col-0 at 10 DPA (Figure 8E; Supplemental Figure 6). The accumulation of starch in the seed coat implicates

a block in sugar release from the seed coat as a key step in seed filling. Consistent with the elevated abundance of carbohydrates in mutant seed coats, mucilage extrusion did not appear to be affected negatively in the mutant, as one might have expected if sugar supply from the phloem was the main cause for the seed phenotype (Supplemental Figure 5).

DISCUSSION

Embryo development is fully dependent on an adequate supply of nutrients from maternal tissues. Significant progress has been made in understanding the translocation from source tissues toward the various sinks (Lalonde et al., 2004; Chen et al., 2012); however, the mechanism of sucrose efflux from the maternal seed coat and the endosperm (partially maternal and partially paternal) and the exact path have remained elusive. Here, we identified SWEET15, as well as SWEET11 and 12, as key players in seed coat efflux.

Studies of developing bean seeds suggested that ~50% of sucrose efflux from the seed coat is mediated by sucrose/proton antiporters driven by the proton-motive force (Fieuw and Patrick, 1993; Walker et al., 1995), while the remaining activity was attributed to mechanisms independent of the proton gradient (Walker et al., 1995). In legume seeds, results from inhibitor studies led to the proposal that nonselective passive pores are responsible for sucrose efflux (van Dongen et al., 2001; Ritchie et al., 2003). Neither the predicted antiporters nor the nonselective pores have been

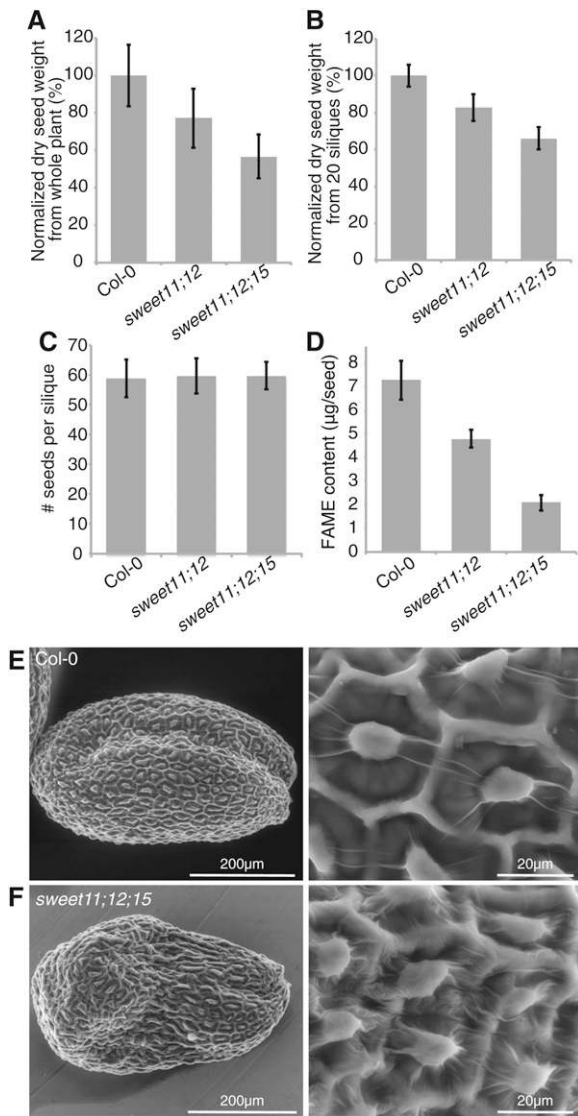


Figure 5. Characterization of Other Seed Development-Related Phenotypes of *sweet11;12;15*.

(A) Comparison of dry seed weight per plant from Col-0, *sweet11;12*, and *sweet11;12;15* (mean \pm SD, $n \geq 14$ plants, from four independent experiments). **(B)** Comparison of dry seed weights of 20 siliques from Col-0, *sweet11;12*, and *sweet11;12;15* (mean \pm SD, $n \geq 13$ plants, from four independent experiments). **(C)** Comparison of number of seeds from each silique from Col-0, *sweet11;12*, and *sweet11;12;15* (mean \pm SD, $n = 30$, from three plants). Two biological repeats were done. **(D)** Total fatty acid content (measured as FAME) of dry seeds from Col-0, *sweet11;12*, and *sweet11;12;15* (mean \pm SD, $n = 14$ plants, from four independent experiments). **(E)** and **(F)** Scanning electron microscopy images of dry seeds from Col-0 **(E)** and from *sweet11;12;15* **(F)**.

identified at the molecular level to date. Uniporters play critical roles in intercellular transport in both yeast and human; it is therefore conceivable that plants also use a uniport mechanism for sucrose efflux from the seed coat. Members of SWEET family were characterized as proton-independent sugar transporters. Interestingly,

the human SWEET1 (SLC50A1) is found in decidual cells of the placenta (Chen et al., 2010) (Human Protein Atlas, <http://www.proteinatlas.org/ENSG00000169241-SLC50A1/tissue>). Decidual cells accumulate glycogen and are thought to be involved in embryo nutrition, therefore potentially implicating these sugar efflux transporters in human embryo development. Here, we identify SWEET11, 12, and 15 as being key to sucrose efflux from maternal tissues of Arabidopsis seeds and as necessary for filial tissue growth.

Developing Arabidopsis seeds have at least three apoplasmic borders that require membrane transporters: (1) between the outer integument (a symplasmic extension of the phloem) and the inner integument; (2) between the inner integument and the endosperm; and (3) between the endosperm and the embryo (Stadler et al., 2005). Based on expression patterns, all three SWEETs contribute in cascade-like patterns to distinct steps in seed filling. Moreover, we identified localization patterns that may implicate new domains involved in seed filling, particularly the micropylar end of seed coat, the micropylar endosperm, and the suspensor.

The Role of SWEETs in Specific Expression Regions

The specific spatiotemporal distribution of SWEET11, 12, and 15 proteins at the micropylar end of the seed coat, the micropylar endosperm, and the suspensor intimates specific roles for SWEET11, 12, and 15, in addition to their redundant functions during seed development.

SWEET15, which accumulated in the outer integument, is likely responsible for mediating sucrose efflux from the outer integument into the apoplasm. Interestingly, strong SWEET12-GFP fluorescence was observed specifically at the micropylar end. This is exactly the location where symplasmic GFP movement was reduced or absent relative to the rest of the seed coat (Stadler et al., 2005), indicating that symplasmic connectivity of the outer integument is reduced or interrupted at the micropylar end. It is therefore proposed that sucrose carriers are responsible for sucrose transport at the micropylar end, which based on the high accumulation of SWEET12 in this region is likely mediated by SWEET12.

The suspensor is thought to be a site where, during the early stages of embryo development, nutrients and growth factors are transferred to the embryo proper (Kawashima and Goldberg, 2010). However, nutrient transfer across the suspensor has not been demonstrated experimentally. The suspensor is symplasmically connected to the embryo during the globular stage (Stadler et al., 2005). However, the symplasmic connectivity between suspensor and embryo hypophysis is reduced during the transition from the globular stage to the heart stage at which the suspensor starts to degenerate (Stadler et al., 2005). The initiation of SWEET12 expression in the suspensor at the globular stage may coincide with the time when symplasmic connections between suspensor and embryo are blocked. The endosperm, which is divided into three distinct domains, micropylar, peripheral, and chalazal endosperm, is an essential part of the seed that sustains embryo development and stores reserves. Experiments in which [14 C]-sucrose was supplied to intact oilseed rape (*Brassica napus*) siliques support the hypothesis that import of sugars into developing embryos occurs via the micropylar rather than the chalazal endosperm (Morley-Smith et al., 2008). The localization of

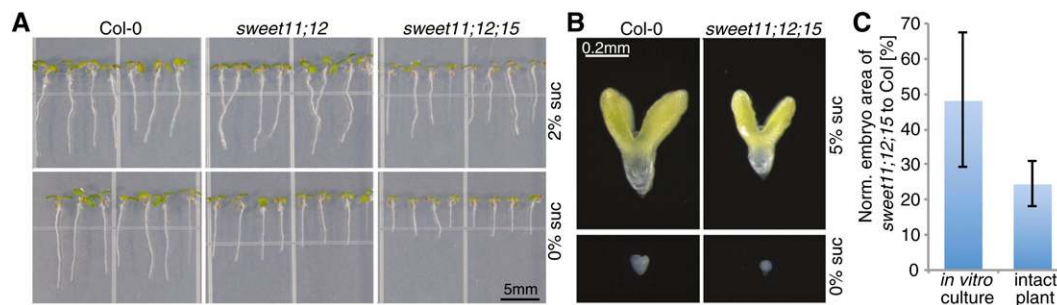


Figure 6. Effect of Sucrose on Early Root Growth in Seedlings and on in Vitro-Grown Embryos.

(A) Comparison of root growth of Col-0, *sweet11;12*, and *sweet11;12;15* seedlings grown on sugar-free and sugar-supplied media.

(B) Embryos dissected from seeds at 3.5 DPA grown in vitro for 5.5 d with and without supply of sucrose.

(C) Embryo size as approximated by area measurements of embryo from in vitro culture with 5% sucrose and dissected seed of intact plant at 9 DPA. Area of *sweet11;12;15* is normalized to Col-0 (mean \pm SD, $n \geq 20$, from three independent experiments).

SWEET11 and 15 in the micropylar endosperm indicates roles in sucrose transfer from the endosperm.

Overall, *SWEET11*, *12*, and *15* coexpress in several subregions and in several stages; nevertheless, each of them also showed distinct expression patterns during development. The temporal and spatial profiles observed at both the transcriptional and translational levels indicate a cascade of sucrose efflux steps that begins at the outer integument, passes first through the inner integument, then through the endosperm, and involves a number of particular steps at the micropylar end, the micropylar endosperm, and the suspensor, before it reaches the embryo proper. The expression patterns of *SWEET11*, *12*, and *15* overlap partially and show very

specific patterns (e.g., in the suspensor and different domains of seed coat and endosperm) that change during development (Figures 2 and 9; Supplemental Figures 1 and 2), indicating the existence of several alternate routes for sucrose import, which shift during seed development.

The Seed Phenotype in the *sweet11;12;15* Mutant Is Predominantly Caused by Defects in Seed Import

Carbohydrate imported from leaves is critical for the growth of young reproductive tissues, especially at night, as shown in elegant studies using the high-starch mutant *sex1* (*starch excess1*)

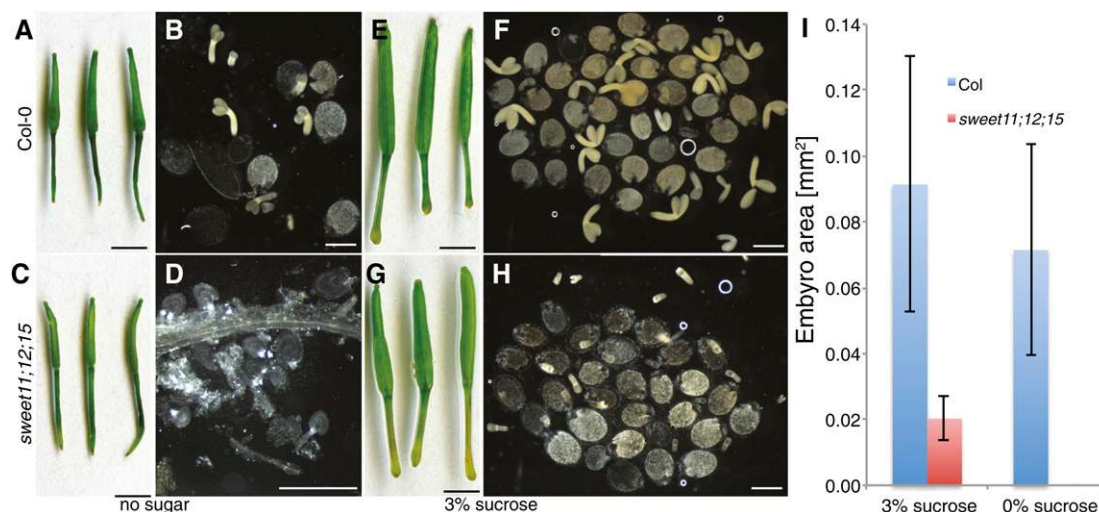


Figure 7. Analysis of Silique and Embryo Growth for in Vitro-Cultured Flowers in Medium with or without Sucrose.

(A) to (D) Comparison of silique development ((A) and (C)) and embryo growth ((B) and (D)) from Col-0 ((A) and (B)) and *sweet11;12;15* ((C) and (D)) cultured for 9 d in sugar-free medium.

(E) to (H) Comparison of silique development ((E) and (G)) and embryo growth ((F) and (H)) from Col-0 ((E) and (F)) and *sweet11;12;15* ((G) and (H)) cultured for 9 d in medium with 3% sucrose.

(B), (D), (F), and (H) Embryos were dissected from three siliques cleared with 0.2 NaOH and 1% SDS solution by gently pressing cover slide. Yellow coloration was observed in Col-0, while *sweet11;12;15* showed reduced coloration. Black bar = 5 mm and white bar = 0.5 mm.

(I) Embryo area was measured for data shown in (B), (D), (F), and (H) (mean \pm SD, $n = 7$ [B], $n = 24$ [F], and $n = 21$ [H]). None of embryos of *sweet11;12;15* cultured in medium without sucrose could be measured due to abortion. The data shown are from a single experiment. Two independent experiments showed similar results.

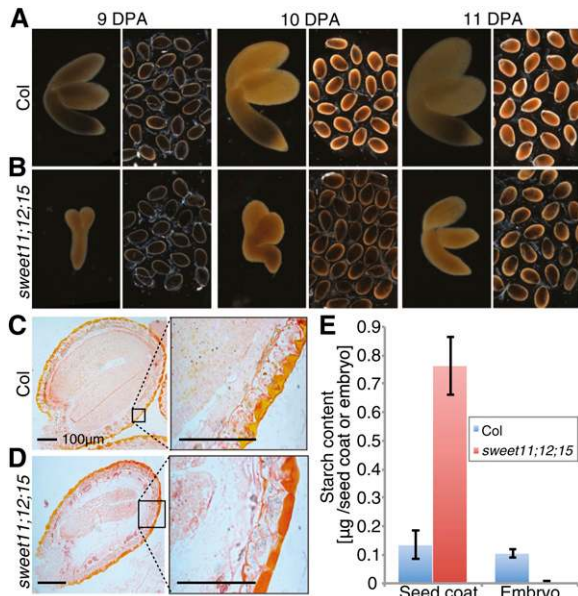


Figure 8. Analysis of Starch Accumulation in Embryos and Seed Coats.

(A) and (B) Comparison of starch accumulation in *sweet11;12;15* and Col-0 at 9, 10, and 11 DPA.

(C) and (D) Cross section of seeds stained with Lugol's iodine solution at 9 DPA.

(E) Enzymatic quantification of starch content of seed coats and embryos from Col-0 or *sweet11;12;15* at 10 DPA (mean \pm SD, $n = 4$).

(Andriotis et al., 2012). Similar to the *sweet11;12;15* triple mutant, embryo development is significantly delayed in *sex1*. The delay in *sex1* growth is caused by low carbohydrate availability during the night (Andriotis et al., 2012). SWEET11 and 12 were found to be expressed both in leaves (Chen et al., 2012) and in seeds; thus, the observed seed phenotype is likely due to a combination of reduced supply from leaves and reduced import into filial tissues. Similarly, one could hypothesize that SWEET15 could contribute to phloem loading particularly during senescence, although analysis of translational GUS fusions indicates that SWEET15 levels are not elevated at the seed filling stage at least in the early phase. Here, we decoupled the contribution of SWEETs in leaves from their role in seed nutrition by culturing flowers axenically (Figure 7). We found that seeds were able to develop to maturation stages in wild-type siliques when sucrose was supplied in the medium to mimic the phloem supply. However, embryos from *sweet11;12;15* remained much smaller compared with the wild type, while developed in the same conditions. These data strongly support the notion that the mutant effect on embryo growth is mainly due to limited availability of sucrose to the embryo proper. Comparison of siliques grown in sugar-free medium further indicates that photosynthesis of silique walls is insufficient to support seed growth and that normal seed development, as one may have expected, relies to a large extent on supply from leaves. The fact that only a few embryos developed beyond the bent cotyledon stage further supports the assumption that sucrose produced in the siliques is just enough to feed a few seeds when sucrose export from the seed coat is functional, as in Col-0, in contrast to *sweet11;12;15*.

Further evidence for a major contribution of the three SWEETs to seed filling stems from the observation that starch accumulated more specifically in the seed coat of the *sweet11;12;15* mutant (Figure 8). If leaf supply would be the dominant factor, one would have expected the opposite, i.e., reduced starch levels in both seed coat and embryo.

Functional Model of SWEETs in Sucrose Translocation in Seeds

Based on the results presented here, we propose a functional model for sucrose translocation from the maternal phloem toward the developing embryo (Figure 9). During early developmental stages, sucrose is unloaded into the postphloem unloading domain via the vascular bundle of the funiculus. Then, sucrose moves toward the micropylar end of the seed coat within the symplasmically connected outer integument. Subsequently, sucrose appears to be released into the apoplasm by the action of SWEET15. Within the micropylar end, symplasmic loading switches to apoplasmic loading with the appearance of SWEET12

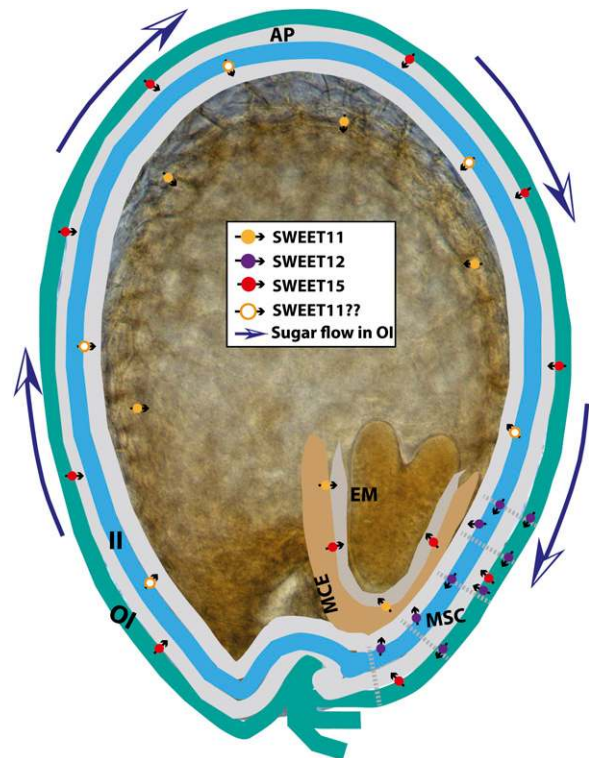


Figure 9. Model for Multistep Sequential Apoplasmic Transport Steps during Seed Development Initiated by SWEET Sucrose Transporters.

Sucrose, which arrives in the seed coat via the funicular phloem, can enter the outer integument of the seed coat likely through plasmodesmata. SWEET15, expressed in the outer integument (OI), exports sucrose into the apoplasm (AP). Possibly, SWEET11 is involved in the release of sucrose from the inner integument (II). SWEET12 appears to play a role in transport of sucrose out of cells at the micropylar end of the seed coat (MSC) and SWEET11 and 15 from the micropylar endosperm (MCE) to supply the embryo proper (EM).

at the globular stage (Werner et al., 2011). The presence of SWEET12 protein in the micropylar end of seed coat implies a role for SWEET12 in mediating sucrose transport to and within this subregion of the seed coat. Unloaded sucrose is used for starch accumulation in the seed coat and cell wall biosynthesis in the endosperm (Fallahi et al., 2008). Some of the sucrose may be directly delivered to the embryo from the seed coat via the suspensor, a hypothesis compatible with SWEET12 expression in the suspensor during early seed development. However, cellular hexose import may dominate over sucrose import during the early stages of seed development (Baud et al., 2002), consistent with high acid invertase activity (Hill et al., 2003). High hexose accumulation is also in line with detected expression of glucose/proton symporters in the seed coat during early seed development (Supplemental Figure 7A). It is worth noting that most of the sugars in the central vacuole in the endosperm of oilseed rape are hexoses, and the bulk of the sugar pool may, at early developmental stages, not be exchangeable with that of phloem-derived sugars (Morley-Smith et al., 2008). How sucrose is exported into the apoplast during the stages when SWEETs expression is hardly detected in seed coat remains an open question. Yet to be identified sucrose/proton antiporters may be responsible for this function (Fieuw and Patrick, 1993; Walker et al., 1995). The existence of additional transport mechanisms could be one of the reasons why *sweet11;12;15* triple mutants are not lethal. During later developmental stages, sucrose is mainly exported from the outer integument by SWEET15 and likely by SWEET11 from the inner integument. SUC5 may be responsible for uptake of sucrose into the endosperm from the apoplast between the seed coat and the endosperm (Baud et al., 2005). SUC5 could also act at the interface between the endosperm and embryo, since it is expressed in the endosperm and specifically the epidermis of the outer surface of the cotyledons (Supplemental Figure 7B) (Pommerrenig et al., 2013). Data from a SUC9 translational fusion to GUS indicated that SUC9 protein was also present in the embryo (Sivitz et al., 2007), although no transcriptional GUS activity was detected in seeds (Sauer et al., 2004). All these reports are in agreement with the observation that *in vitro* dissected embryos from the globular stage can reach the mature stage when grown in media supplemented with sucrose.

Together, our data demonstrate a critical role of SWEETs in sucrose release from the seed coat and multiple sites in the seed to supply the developing embryo. It will be interesting to determine whether human SWEET1 plays an orthologous function in the placenta. It also will be interesting to explore the parallels and potential convergent evolution of embryo nutrition in plants and animals, particularly the role of SWEETs in these processes.

METHODS

Plasmid Constructs

Constructs for Expression in *Xenopus laevis* Oocytes

Oocyte expression construct for SWEET12 has been described previously (Chen et al., 2010). The open reading frame of *Arabidopsis thaliana* SWEET15 (with stop codon) in vector pDONR221-f1 was transferred to the oocyte expression vector pOO2-GW as described previously for other SWEETs (Chen et al., 2010).

eGFP Fusion Constructs under Native Promoters

For analyzing the expression of SWEET12 and 15, GFP fusions were constructed. The fragment of SWEET12 comprising a 1887-bp SWEET12 promoter sequence and 1858 bp of the coding region up to but not including the stop codon was amplified with the forward primer AtSWT12KpnIF containing a *KpnI* restriction site and the reverse primer AtSWT12PstIR containing a *PstI* restriction site and subcloned into the eGFP fusion vector pGTKan3 (Kasaras and Kunze, 2010) via *KpnI* and *PstI* restriction sites. A similar strategy was applied for SWEET15. The 3784-bp fragment of SWEET15 was amplified by primers AtSWT15KpnIF and AtSWT15PstIR and ligated into pGTKan3 by *KpnI* and *PstI* restriction sites. The translational GUS fusion construct was generated by amplifying the identical sequence used for GFP fusion construct using primers AtSWT15attB1 and AtSWT15attB2 and then cloning into donor vector pDONR221-f1 followed by recombination reaction into Gateway vector pGWB3 (Nakagawa et al., 2007) by LR reaction as described before (Chen et al., 2012). Construction of *pSWEET11:SWEET11-eGFP* has been described previously (Chen et al., 2012). Primer sequences are provided in Supplemental Table 1.

Plasmids for Complementation of Mutants

For complementation of the *sweet11;12;15* triple mutant, the *Agrobacterium tumefaciens* GV3101 transformants carrying constructs *pSWEET11:SWEET11-eGFP*, *pSWEET12:SWEET12-eGFP*, and *pSWEET15:SWEET15-eGFP* were used to transform *sweet11;12;15* triple mutants.

Tracer Uptake in *X. laevis* Oocytes

Linearization of the plasmids in pOO2 vector, capped cRNA synthesis, *X. laevis* oocyte isolation and cRNA injection, [¹⁴C]-labeled sugar uptake, and efflux were performed as described before (Chen et al., 2010).

Plant Material and Growth Conditions

Plants were grown under light (150 to 170 $\mu\text{E m}^{-2} \text{s}^{-1}$ with 16 h light/8 h dark) photoperiod conditions. Only primary shoots and the first secondary shoots were used. To collect siliques of desired developmental stages, individual flowers were tagged with colored strings on the day of flowering (referred to as 0 DPA) between 11 AM and 1 PM. Tagging plants started within the first week of plant bolting.

Seeds of transposon line SM_3_14944 for SWEET15 were ordered from ABRC. In order to get triple mutant, *sweet15* was crossed with *sweet11;12*. Homozygous *sweet11;12;15* was selected by genotyping.

For seedling growth analysis, seeds were sown on half-strength MS medium with or without sucrose (as indicated) and then kept at 4°C for 3 d before being transferred to a growth chamber and positioned vertically (16-h-light period). At indicated days after transfer, seedlings were digitally photographed.

Arabidopsis wild-type Col-0 and *sweet11;12;15* triple mutants were transformed by the floral dip method (Davis et al., 2009). Transgenic seedlings were selected on media with kanamycin for *pSWEET11:SWEET11-eGFP*, *pSWEET12:SWEET12-eGFP*, and *pSWEET15:SWEET15-eGFP*.

Genotyping and Transcript Analysis of T-DNA Mutants

Genomic DNA was extracted from *Arabidopsis* Col-0 and F3 segregating lines after crossing *sweet15* (SM_3_14944) with *sweet11;12* (*Salk_073269* and *Salk_031696*) and was used as template for genotyping. Primers used for *sweet11* and *sweet12* genotyping were as described (Chen et al., 2012). Primers specific to SWEET15 sequence flanking the transposon element were AtSWT15LP and AtSWT15RP. The left border primer of transposon element was Spm32. PCR was performed as described on the website <http://signal.salk.edu/database/T-DNA/SM.435.pdf>.

Total RNA was extracted from leaves of *Arabidopsis* from Col-0, controls, and insertion lines using a Spectrum plant total RNA kit (Sigma-Aldrich; #120M6117). First-strand cDNA was synthesized using oligo(dT) and M-MuLV reverse transcriptase following the instructions of the supplier (Fermentas). Primers for the full-length open reading frame of *SWEET15* (AtSWT15attB1 and AtSWT15attB2) were used for RT-PCR to determine the expression levels. *ACTIN2* (primers AtACT2F and AtACT2R) served as reference gene (Chen et al., 2010). Primer sequences are provided in Supplemental Table 1.

Starch Staining

After being harvested in the late afternoon, siliques were placed in 80% (v/v) ethanol plus 5% (v/v) formic acid and incubated in a 60°C oven overnight, then washed once with 70% ethanol. Then, siliques were stained in Lugol's iodine solution for 5 min and washed twice in water.

GUS Histochemical Analysis

GUS staining was performed immediately after samples were collected at the different ages, following the procedures described previously (Chen et al., 2012). To avoid overlooking the expression of *SWEET15* in other tissues, staining was extended to 24 h. A flatbed scanner was used to document the results.

Lipid Analysis

Lipid content of dry seeds was analyzed following the published procedures with minor changes (Focks and Benning, 1998). Seeds from mutants and Col-0 plants, carefully planted and managed together, were harvested on the same day. Dry seeds were counted and prepared in 8-mL glass reaction tubes. Three seeds from each background were used for the first biological repeat and 20 seeds for the next two biological repeats. At least four technical repeats were included in each experiment. One hundred microliters of 50 µg/mL pentadecanoic acid (C15:0) was used as an internal standard. Samples were incubated in 1 mL of 1 N HCl in methanol at 80°C for 90 min. Tubes were allowed to cool to room temperature. FAMES were extracted in 1 mL hexane following the addition of 1 mL 0.9% (w/v) NaCl. The hexane phase was transferred to a gas chromatography vial after vigorous vortexing and centrifuged for 3 min at 3000 rpm. Samples were analyzed using a gas chromatography-flame ionization detector.

Seed Coat and Embryo Starch Measurement

Starch content was measured enzymatically following published procedures with some changes (Andriotis et al., 2010). Thirty embryos or seed coats were pooled from one plant for one analysis. In detail, 10 embryos or seed coats were rapidly dissected from seeds of each freshly harvested siliques and transferred to 300 µL iced-cold 0.7 M perchloric acid. Three siliques were collected from each individual plant. In total, four individual *sweet11;12;15* or Col-0 plants were used. Sixteen samples were pooled, homogenized, and centrifuged at 11,000g for 45 min at 4°C. Sediments were washed once in 1 mL distilled water and twice in 1 mL 80% (v:v) ethanol, then vacuum dried. Dried sediments were resuspended in 40 µL distilled water by boiling for 6 min twice. A starch measurement kit (R-Biopharm) was used to quantify starch content. Briefly, starch was hydrolyzed to D-glucose by amyloglucosidase. D-glucose was enzymatically assayed in the presence of hexokinase and glucose 6-phosphate dehydrogenase by determining the amount of NADPH (absorbance at 340 nm). We note that the seed dissection cannot fully separate embryo, endosperm, and seed coat; thus, we cannot exclude cross-contamination.

In Vitro Embryo Culture

The procedure was slightly modified based on the published protocols (Sauer and Friml, 2008). Embryos were dissected gently from 3.5 to 4 DPA siliques using sterile forceps. Half of undamaged embryos from one silique

were immediately transferred to in vitro culture medium plates with 5% sucrose under 70% ethanol cleaned stereo microscope. The other half from the same silique was transferred to a plate without sucrose. Embryos were dissected carefully from seeds and then the rest of the debris from the seeds was removed from the plates. Five to 10 siliques were used for each experiment. Plates were placed in a 22°C growth chamber with 150 to 170 µE m⁻² s⁻¹ light and 16-h-light/8-h-dark diurnal cycle. Two sheets of plain white paper were used to shield the excess light. After 5 d of culture, embryos were imaged using a Leica MZ125 stereomicroscope.

In Vitro Flower Culture

On the day of anthesis, similarly sized flowers were cut and immediately transferred to 0.025% (v/v) sodium hypochlorite for 8 min. The pedicel was inserted in half-strength MS medium with 0.3% (w/v) Phytigel, 4 mM L-Gln, 1 mM L-Asn, and 3% (w/v) sucrose or without sugar. Flowers were cultured for 9 d in a 22°C growth chamber with 130 to 150 µE m⁻² s⁻¹ light and 16-h-light/8-h-dark photoperiod conditions.

Microscopy

For all experiments, siliques were freshly harvested.

Phenotype Observation and Documentation

For phenotype observation, siliques were opened on a microscope slide right after collection. Seeds were mounted in water, and gentle pressure was applied to the cover slip on the seeds to release the embryos. Afterwards, nondamaged embryos were documented under a Leica MZ125 stereomicroscope.

Clearing Seeds and Imaging

For determination of embryo developmental stages, siliques were fixed in ethanol:acetic acid (9:1) overnight and washed with 90 and 70% ethanol. Siliques were cleared with chloral hydrate:glycerol:water (8:1:2, w:v:v) solution up to overnight (Yadegari et al., 1994). After being dissected from siliques, seeds were mounted in clearing solution under a microscope with DIC optics. Afterwards, embryo area was measured using Fiji software as needed.

Scanning Electron Microscopy

Dry seeds from Col-0 and *sweet11;12;15* were prepared. Scanning electron microscopy was performed using FBI Quanta 200 at an accelerating voltage of 30 kV.

Cryofixation and Immuno-TEM

Seeds were staged according to DPA (Western et al., 2000). The 7 and 10 DPA seeds were high-pressure frozen in 1-hexadecene in B-type sample holders (Ted Pella) using a Leica HPM-100. Samples were freeze substituted in 0.1% uranyl acetate, 0.25% glutaraldehyde, and 8% dimethoxypropane in acetone for 5 d and then brought to room temperature and infiltrated with LR white resin (London Resin Company) over 4 d. Immunolabeling was performed as described before (McFarlane et al., 2008) using 1/100 anti-GFP (Invitrogen A6455) and 1/100 goat-anti-rabbit conjugated to 10-nm gold (Ted Pella). Samples were viewed using a Hitachi 7600 transmission electron microscope at 80-kV accelerating voltage with an AMT Advantage CCD camera (Hamamatsu ORCA).

Confocal Laser Scanning Microscopy

Fluorescence imaging of seed was performed on a Leica TCS SP5 microscope. eGFP was visualized by standard procedures as described before (Chen et al., 2012). For cell wall staining, fresh siliques were incubated with 0.5% propidium iodide in 1.5-mL centrifuge tubes for 10 min at room temperature and were washed twice with water. eGFP was

excited by a 488-nm argon laser and detected from 497 to 526 nm. Propidium iodide was excited by 488-nm light and detected from 595 to 640 nm (Stadler et al., 2005). A water-corrected 20× or glycerol-corrected 63× objective was used. Image analysis was performed using Fiji software.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: SWEET11 (At3g48740), SWEET12 (At5g23660), SWEET15 (At5g13170), SUC1 (At1g71880), SUC2 (At1g22710), SUC3 (At2g02860), SUC4 (At1g09960), SUC5 (At1g71890), SUC9 (At5g06170), STP1 (At1g11260), STP4 (At3g19930), STP5 (St1g34580), STP7 (At4g02050), STP8 (At5g26250), STP12 (At4g21480), STP14 (At1g77210), and human SWEET1 (ENSG00000169241).

Supplemental Data

Supplemental Figure 1. Tissue-specific expression of SWEET transcripts in developing seeds of Arabidopsis.

Supplemental Figure 2. Localization of SWEETs indicated by eGFP fluorescence and by immunogold particles.

Supplemental Figure 3. SWEET15 functions as a sucrose transporter.

Supplemental Figure 4. Complementation of the triple *sweet11;12;15* mutant by individual *SWEET* genes.

Supplemental Figure 5. Phenotypes of *sweet11;12;15* mutant seeds.

Supplemental Figure 6. Starch content measurement.

Supplemental Figure 7. STP and SUT/SUC sugar transporter expression profiles in developing seed.

Supplemental Table 1. Primer sequences.

Supplemental Movie 1. Localization of SWEET11 during late seed development (mature green stage).

Supplemental Movie 2. Localization of SWEET11 during late seed development (mature green stage).

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AUTHOR CONTRIBUTIONS

W.B.F., L.-Q.C., and A.L.S. conceived and designed the experiments. L.-Q.C., I.W.L., X.-Q.Q., D.S., H.E.M., and A.L. performed the experiments. W.B.F. and L.-Q.C. analyzed the data. L.-Q.C. and W.B.F. wrote the article.

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