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RESEARCH PAPER

Localization of sucrose synthase in developing seed and siliques of *Arabidopsis thaliana* reveals diverse roles for SUS during development

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Abstract

This study investigated the roles of sucrose synthase (SUS) in developing seeds and siliques of *Arabidopsis thaliana*. Enzyme activity assays showed that SUS activity was highest in developing whole siliques and young rosette leaves compared with other tissues including mature leaves, stems, and flowers. Surprisingly, quantitative PCR analyses revealed little correlation between SUS activity and transcript expression, which indicated the importance of examining the role of SUS at the protein level. Therefore, immunolocalization was performed over a developmental time course to determine the previously unreported cellular localization of SUS in *Arabidopsis* seed and silique tissues. At 3 d and 10 d after flowering (daf), SUS protein localized to the silique wall, seed coat, funiculus, and endosperm. By 13 daf, SUS protein was detected in the embryo and aleurone layer, but was absent from the seed coat and funiculus. Starch grains were also present in the seed coat at 3 and 10 daf, but were absent at 13 daf. Co-localization of SUS protein and starch grains in the seed coat at 3 and 10 daf indicates that SUS may be involved in temporary starch deposition during the early stages of seed development, whilst in the later stages SUS metabolizes sucrose in the embryo and cotyledon. Within the silique wall, SUS localized specifically to the companion cells, indicating that SUS activity may be required to provide energy for phloem transport activities in the silique wall. The results highlight the diverse roles that SUS may play during the development of silique and seed in *Arabidopsis*.

Key words: *Arabidopsis*, companion cells, immunolocalization, nectary, seed development, silique wall, sucrose synthase.

Introduction

Sucrose, produced in photosynthetic source tissues, is transported to various vegetative and/or reproductive sink tissues where it is used for active growth processes or may be utilized for biosynthesis of cellulose and of storage carbohydrate such as starch, fructans, or lipids. Cleavage of the glycosidic bond is a prerequisite step for sucrose metabolism (Fu *et al*., 1995). Two enzymes, sucrose synthase (SUS; EC 2.4.1.13) and invertase (EC 3.2.1.26), catalyse this cleavage reaction *in vivo*. The regulation of these reactions and its consequences has therefore become a central issue in plant carbon metabolism (Koch, 2004). In several plants, SUS activity is the main route for entry of sucrose into cellular metabolism. Previous studies have shown that a reduction in SUS activity reduced the availability of assimilate for storage and normal growth (Craig *et al*., 1999). For example, inhibition of SUS activity in tomato decreased the fruit setting and sucrose unloading capacity (D'Aoust *et al*., 1999), and in transgenic potato tubers reduction in the level of SUS activity decreased starch biosynthesis (Zrenner *et al*., 1995). SUS

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Abbreviations: CC, companion cell; daf, days after flowering; DAPI, 4′,6-diamidino-2-phenylindole; RT-PCR, reverse transcription-PCR; SUS, sucrose synthase.

has also been localized in the companion cells (CCs) in maize leaves and citrus fruits (Nolte and Koch, 1993), where it has been proposed to play a role in providing energy for loading and unloading in the phloem (Lerchl *et al*., 1995). In addition, SUS is believed to be involved in cell wall biosynthesis by providing UDP-glucose directly to cellulose synthase (King *et al*., 1997; Ruan and Chourey, 1998; Haigler *et al*., 2001). Ruan *et al*. (2003) reported that a 70% reduction in SUS activity suppressed cell initiation in cotton fibre, probably through inhibition of cell wall biosynthesis.

A small multigene family has been found to encode several SUS isoforms in many plant species examined to date, including maize (Carlson *et al*., 2002), pea (Barratt *et al*., 2001), rice (Wang *et al*., 1992; Huang *et al*., 1996; Harada *et al*., 2005; Hirose *et al*., 2008), *Lotus* (Horst *et al*., 2007), and *Arabidopsis* (Barratt *et al*., 2001). Studies of the predicted amino acid sequences and gene structure have shown that the *AtSUS* family consists of six SUS genes, distributed in three separate groups. *AtSUS1* and *AtSUS4* can be classified into the dicot SUS1 group; *AtSUS2* and *AtSUS3* belong to the SUSA group; while *AtSUS5* and *AtSUS6* make a separate group on their own (Baud *et al*., 2004). Each of these isoforms may have a specific role *in planta* as revealed through mutant and transgenic plant analysis (Bieniawska *et al*., 2007). In order to examine the role of the six SUS isoforms in *Arabidopsis*, comparative analysis was carried out with the SUS gene families of other species (Barratt *et al*., 2001; Baud *et al*., 2004; Harada *et al*., 2005; Bieniawska *et al*., 2007). Measurement of the changes in the transcript level of the SUS isoforms in response to different treatments (including anoxia, dehydration, cold treatment, and sugar feeding) revealed that some isoforms respond to biotic and non-biotic stresses. For example, under anaerobic conditions, the transcript levels of *AtSUS1* and *AtSUS4* were found to increase in rosette leaves. This finding was further confirmed by abnormal growth of a *sus1*/*sus4* double mutant under hypoxia (Bieniawska *et al*., 2007). The roles of *AtSUS5* and *AtSUS6* remain unclear as their expression levels exhibited no response to treatment conditions (Baud *et al*., 2004), and a *sus5*/*sus6* double mutant exhibited normal growth when compared with the wild type (Bieniawska *et al*., 2007). However, it has been shown that the promoter sequences of both of these isoforms are active in the vascular system of the flower and cotyledon (Bieniawska *et al*., 2007). It is not known, however, whether changes in the expression level of the transcript of SUS isoforms would result in similar changes in the level of SUS activity.

The involvement of SUS in seed development has been investigated in several studies (Doehlert *et al*., 1988; Xu *et al*., 1989; Counce and Gravois, 2006). A shrunken seed phenotype in maize was found to be related to a mutation in one of the SUS genes, *Sh1* (Chourey *et al*., 1998). SUS transcript has been shown to be induced during seed development in the legume plant *Vicia faba* L. (Heim *et al*., 1993). King *et al*. (1997) reported that SUS activity increased during development of canola seeds. Further evidence is provided by localization of SUS in developing seeds of cotton (Ruan and Chourey, 1998) and maize kernels (Wittich and Vreugdenhil, 1998). In addition, microarray analysis performed on developing *Arabidopsis* seeds also showed an increase in SUS expression level during seed development (Ruuska *et al*., 2002). These results were confirmed by reverse transcription-PCR (RT-PCR) which clearly showed that during the early stages of seed development there is an increase in *AtSUS2* transcript level, whilst the amount of *AtSUS3* transcript increased during the late stages of seed maturation (Bieniawska *et al*., 2007). Since the transcript level of *AtSUS2* was low in vegetative tissues, *AtSUS2* was considered to be a 'seed maturation marker' (Baud *et al*., 2004). However, in the dicot model plant *Arabidopsis thaliana*, knowledge of SUS expression during seed development is limited to transcript level measurements, and there is no direct information on changes in temporal and/or spatial expression patterns of SUS protein during seed and silique development. As a model for oilseed plants, which accumulate reserves in the form of carbohydrate during early stages of seed development and convert it to lipids during the later stages (Focks and Benning, 1998), it is important to know where and how SUS protein expression changes in the developing siliques and seed of *Arabidopsis*.

In this study, a combination of SUS activity assays, transcript expression analysis, and immunolocalization techniques was used to investigate the potential roles of SUS during the early, mid, and late stages of seed development in *Arabidopsis*. The presence of SUS protein in various tissue and cellular locations at these stages of seed development is discussed in the context of phloem loading and unloading, starch remobilization, and endosperm and embryo development

Materials and methods

Plants growth and conditions

Arabidopsis thaliana seeds, ecotype Columbia (Col-0), were surface sterilized by incubation in 0.1% (w/v) mercuric chloride followed by several washes in sterile distilled water. The seeds were germinated on a half-strength MS medium, containing 1% sucrose, in a growth room at 23 °C with a 12 h photoperiod. Seedlings were transferred to soil 1 week after germination and were grown at 23 °C under a 14 h light regime. Seedling samples were harvested at the fourth true leaf stage. Samples of stem, cauline leaves, and mature rosette leaves after flowering were harvested at 5 weeks after germination. Individual flowers were tagged at the day of flowering, and developing siliques were collected at 3, 10, and 13 daf.

Protein extraction and SUS activity measurement

Soluble protein samples were extracted by grinding the tissues (250 mg) in liquid nitrogen, before adding 1 ml of a cold extraction buffer containing 25 mM HEPES-KOH (pH 7.3), 5 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% polyvinyl pyrrolidone (PVP, M_r 40 000), 1 mM phenylmethylsulfonyl flouride (PMSF), and 0.01 mM leupeptin. The supernatants were collected by centrifugation at 13 000 *g* for 5 min at 4 °C. A 500 µl aliquot of the supernatant was passed through a Sephadex G-25 column (Amersham Biosciences) and collected in 1 ml of elution buffer (50 mM HEPES pH 7.3, 5 mM EDTA). Protein concentrations were determined using the Coomassie Plus™ protein assay kit (Pierce) using bovine serum albumin (BSA; fraction V Sigma) as the standard. SUS activity assays, in the direction of sucrose breakdown, were carried out as described by Ruan *et al*. (2003), and the resultant UDP-glucose was measured using the method of Lunn and Ap Rees (1990).

RNA extraction and cDNA synthesis

Total RNA samples were extracted using the RNeasy Plant Kit (Qiagen). Briefly, 200 mg of plant tissue was ground to a fine powder in liquid nitrogen. An 850 µl aliquot of RLT buffer containing 0.143 M β-mercaptoethanol was added to the sample. All other steps were performed following the manufacturer's protocol. After treatment of RNA samples with Turbo DNA-free™ (Ambion), 1 µg of RNA was subjected to first-strand cDNA synthesis using a Thermoscript™ RT-PCR system (Invitrogen) in a total volume of 10 µl.

Quantitative RT-PCR

Specific primers, as used by Bieniawska *et al.* (2007), were synthesized for all six isoforms of the AtSUS gene family (Table 1). Real-time PCR was performed in order to determine the transcript expression level of each gene. The reactions were carried out on a RotorGene (Corbett research) using SYBR® Green Jumpstart™ (Sigma) following the manufacturer's protocol. For an internal control, a cyclophylin primer set was used for calibration of the data (Millar *et al*., 2006). To verify the specificity of the PCR amplification, a heat dissociation protocol at 55–99 °C was performed. Rotor-Gene6 software was used to estimate relative gene expression via the ΔΔCT method (Livak and Schmittgen, 2001).

Immunoblotting

Protein samples, prepared as described for SUS activity measurements, were separated using SDS–PAGE [10% (w/v) acrylamide] and transferred to nitrocellulose membranes (Amersham Biosciences) by western blotting. The membranes were blocked with 5% skimmed milk protein in TTBS buffer (10 mM TRIS-HCl pH 7.5, 0.5 M NaCl, 0.1% Tween-20). The membranes were probed with an antibody raised against a rice SUS2 peptide (T. Hirose, NARC Japan) used at a dilution of 1:10 000 in TTBS buffer for 16 h at 4 °C. Following washing, the membranes were incubated in secondary antibody, anti-rabbit IgG (FC)–alkaline phosphatase conjugate (Promega) at a 1:10 000 dilution in TTBS buffer. The membranes were subsequently washed, and labelled proteins detected by development with FAST™ BCIP/NBT (Sigma).

Fixation and sectioning of tissues

Samples of flowers harvested at the day of flowering and siliques harvested at 3, 10, and 13 daf were fixed in formaldehyde solution in phosphate-buffered saline (PBS) as described in Scofield *et al*. (2007). Dehydration was carried out by passing the samples through a graded ethanol series, which was followed by an ethanol:Histoclear series. The samples were infiltrated and embedded in paraffin wax (Paraplast plus, TYCO Healthcare). Longitudinal sections of 12.5 µm thickness were cut using a microtome (Microm HM350) and collected onto drops of distilled water on polysine-coated glass slides. The slides were incubated on a hot plate at 42 °C for 24 h and stored at 4 °C.

Immunolocalization and starch localisation

Sections were de-waxed using Histoclear and were re-hydrated through a graded ethanol series and incubated in DIG buffer I (100 mM TRIS-HCl, pH 7.5, 150 mM NaCl). Immunolocalization was carried out as previously described (Scofield *et al*., 2007). The sections were probed with the anti-rice SUS2 antibody at a dilution of 1 in 200 in blocking solution for 20 h at 4 °C. For the control slides, a pre-immune serum at the same dilution was used. An anti-rabbit IgG (FC)–alkaline phosphatase conjugate antibody (Promega) at a 1 in 1250 dilution was used as the secondary antibody. The signal was developed by incubating the slides in a substrate solution containing 0.25 mM nitrotetrazolium blue chloride (NBT, Sigma) and 0.25 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma). The reaction was stopped by washing the slides in several changes of distilled water.

Starch localization was performed by incubating de-waxed sections in a solution of 0.5% (w/v) iodine in 5% aqueous potassium iodide for 15 min. To identify nuclei, de-waxed sections were incubated in 200 µl of a solution of 0.25 µg ml^{-1} of 4′,6-diamidino-2-phenylindole (DAPI, Sigma) in distilled water. After

Table 1. *Sequence of primers used in quantitative PCR experiments to measure the transcript level of the* Arabidopsis *sucrose synthase isoforms*

Isoforms	Primers
AtSUS1 (At5g20830)	5′-GAACGTCTCGAGAGAGTTTATGATTCTG-3′
	5′-GTATCAGCAGCCTGATCACCATGGTAAG-3′
AtSUS2 (At5g49190)	5′-TAGTGGTACAGAACACGCACACATTCTG-3′
	5′-GTCTCAAAGAAGCTGACCAAGGTAGCTG-3′
AtSUS3 (At4g02280)	5′-CAGCGGAACAGAGCATACTCATATTCTC-3′
	5′-ATCAGCCATTATGTTACCCGCTTGCTCA-3′
AtSUS4 (At3g43190)	5′-GCTGCTTCCTGACGCAGCAGGAACCACTTGT-3′
	5′-CTCTCAGCTGCCTTGTCACCATGATATGGA-3′
AtSUS5 (At5g37180)	5′-ATGCAACCAAGAGTTGGAACCTATTTTC-3′
	5′-CAATAATCAGGATCCATACCGGATTTCTC-3′
AtSUS6 (At1g73370)	5′-GAACTAGAAGCCATTGAAGGAACCAAGC-3′
	5′-ATACAAACCATCTGAACGGCATTTGCTG-3′
Cyclophilin (At2g29960)	5′-TGGACCAGGTGTACTTTCAATGG-3′
	5′-CCACTGTCTGCAATTACGACTTTG-3′

45 min incubation at room temperature in the dark, the slides were washed in water for 5 min. Sections were examined using a Leica DMR microscope with brightfield and fluorescence illumination, respectively. Digital images were recorded using a Leica DC500 digital camera.

Sugar extraction and measurement

Water-soluble hexose sugars were extracted from developing siliques (20 mg) harvested at various time points. The samples were ground in liquid nitrogen, and 500 µl of 80% (v/v) ethanol added to each tube and incubated at 80 °C for 60 min. The pellets were collected following centrifugation at 13 000 *g* for 5 min, and were extracted twice more with 500 µl of 80% ethanol in the same conditions. The supernatants of all three extractions were pooled together and dried to completeness under vacuum. The residue was dissolved in 300 µl of distilled water. The glucose content of the samples was quantified by adding 25 µl of the extracts to a 175 µl volume of hexose determination buffer (0.1 M TRIS-HCl pH 8, 5 mM $MgCl_2$) containing freshly prepared 0.5 mM NADP and 1 mM ATP. The production of NADPH was monitored at 340 nm after adding 1.75 U of glucose-6-phosphate dehydrogenase (Roche) and 1.5 U of hexokinase (Roche). The fructose content of the samples was determined by adding 1.5 U of phosphoglucoisomerase (Roche) and measurement of subsequent NADPH production. Sucrose was converted to glucose and fructose using sucrose phosphorylase (Roche), and the resultant content of hexoses was determined enzymatically as described above.

Results

Expression of the SUS gene family in Arabidopsis

Due to some noted discrepancies in transcript expression data for the six AtSUS isoforms in previously published work (Baud *et al*., 2004; Bieniawska *et al*., 2007), it was decided to repeat transcript expression analysis in a range of tissues from plants cultured in the growth conditions used here. In addition, measurement of SUS activity was performed in the same range of tissues.

To investigate the expression patterns of all six isoforms of SUS in *Arabidopsis*, transcript levels of each gene were determined in a range of tissues. cDNA samples prepared from seedling, young immature rosette and mature rosette leaves, cauline leaves, stem, flower, and developing siliques at 3 and 10 daf were subjected to quantitative PCR, using gene-specific primers. The cyclophilin transcript level was used for calibration of data, as cyclophilin was found to be expressed at a similar level in all tissues examined (data not shown). The *AtSUS1* transcript was present in all tissues examined (Fig. 1A). Cauline leaves and stem contained the lowest amounts of *AtSUS1* mRNA compared with other tissues, while flowers contained the highest level of *AtSUS1* expression. The transcript levels of *AtSUS2* and *AtSUS3* were most highly expressed in developing siliques at 10 daf, with lower levels observed at 3 daf, and were expressed at low levels in the other tissues examined (Fig. 1B, C). Young immature rosette leaves contained the highest amount of *AtSUS4* transcript in comparison with its level in the other tissues examined (Fig. 1D). At 3 and 10 daf, developing siliques showed slightly higher expression of *AtSUS5* compared with other tissues, which is in agreement with the result of Bieniawska *et al*. (2007). However, these findings were not confirmed by comprehensive microarray analysis using Genevestigator (Laule *et al*., 2006) (data not shown). *AtSUS5* transcript was also found to be expressed in cauline leaves and young rosette leaves (Fig. 1E). The *AtSUS6* transcript was found to be expressed at relatively low levels in all the tissues examined (Fig. 1F).

SUS activity in Arabidopsis *tissues*

SUS activity was subsequently measured in the same tissue samples used for the quantitative PCR analysis (Fig. 2). The highest level of SUS activity was present in developing siliques compared with the vegetative tissues including mature leaves harvested after flowering, stem, flower, cauline leaves, and seedlings; however, SUS activity was also high in young immature rosette leaves.

Immunolocalization of SUS protein in developing seeds and siliques

To determine the cellular localization and temporal expression pattern of SUS protein in developing *Arabidopsis* seed, immunolocalization was performed on longitudinal sections of siliques at 3, 10, and 13 daf. The three time points selected represent early, mid, and late stages of seed development in *Arabidopsis* (see Discussion). An antibody raised against a rice SUS2 peptide was used to detect SUS proteins in the *Arabidopsis* samples. Table 2 shows a comparison between the predicted peptide sequences for the six *Arabidopsis* SUS proteins and that of rice SUS2. *Arabidopsis* AtSUS1, AtSUS2, AtSUS3, and AtSUS4 showed high homology with the rice SUS2 protein, with 79, 73, 76, and 82% identity, respectively, while AtSUS5 and AtSUS6 had lower levels of identity, 61% and 66%, respectively. The antibody detected a band at the expected size of 92–95 kDa for SUS on a protein blot of the same range of *Arabidopsis* tissues as used for SUS activity measurements (Fig. 3A). However, it is not possible to determine which and how many of the SUS isoform(s) were recognized by the antibody, and, as a consequence, the distribution and hence potential roles of SUS may be underestimated. Figures 3B–G illustrate the results of immunolocalization in longitudinal sections of siliques. At 3 daf, SUS labelling was present in the funiculus, seed coat, endosperm, and in the vascular bundle of the silique wall (Fig. 3B). SUS protein was present in the same tissues at 10 daf (Fig. 3D). However, at 13 daf, SUS protein was detected in the embryo, the aleurone layer, and cotyledons (Fig. 3F), but was absent from the funiculus and the seed coat. Labelling was absent in the sections probed with the pre-immune serum (Fig. 3C, E, G).

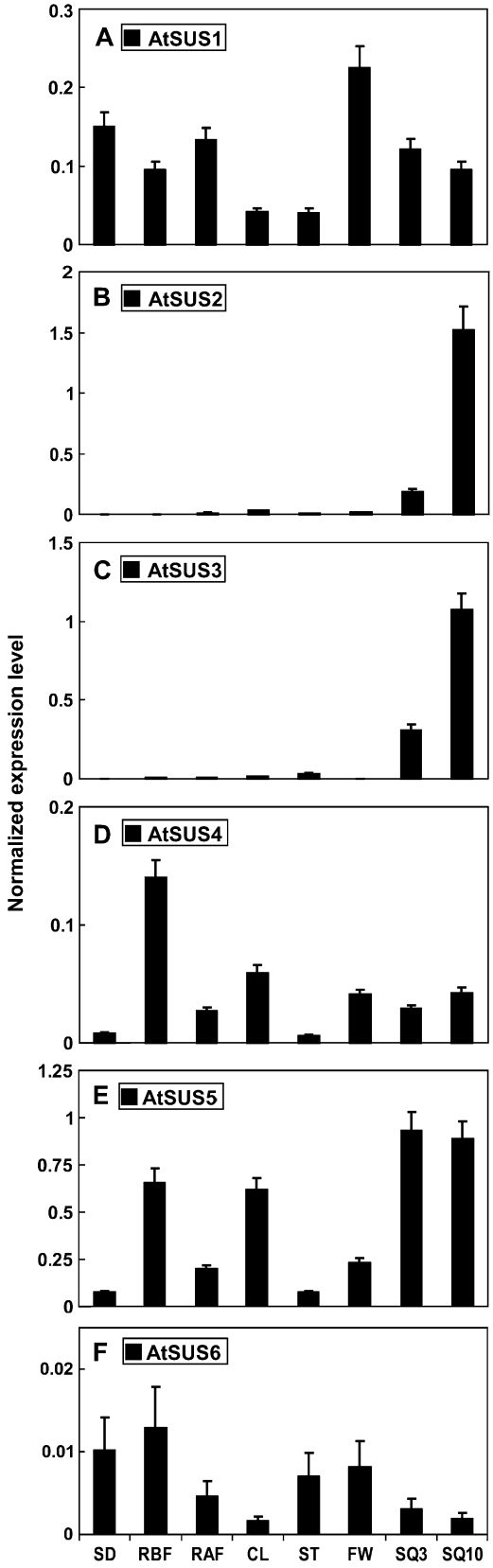

Fig. 1. Relative expression levels of the six *AtSUS* transcripts in a range of *Arabidopsis* tissues. (A– F) The normalized transcript levels of the *AtSUS* isoforms 1–6 against cyclophilin, respectively. Relative expression of *AtSUS2* and *AtSUS3* was higher in the developing siliques compared with other tissues. SD, seedling; RBF, young rosette leaves before flowering; RAF, mature rosette leaves after flowering; CL, cauline leaves; ST, stem; FW, flower at the day of flowering; SQ3, siliques 3 daf; SQ10, siliques 10 daf. Error bars indicate the SE (n=3).

Starch localization during Arabidopsis *seed development*

Starch grain localization was carried out on sections of developing seed to examine a possible role for SUS in starch biosynthesis (Fig. 4). Starch grains were present in the seed coat and funiculus of the seed at 3 daf (Fig. 4A). At 10 daf, starch grains localized in the seed coat (Fig. 4B). Interestingly, at 13 daf, starch grains were absent in these tissues (Fig. 4C). Repeatable results were obtained in serial sections on three slides from each of three biological replicate samples. Carbohydrate content was determined in whole silique tissue at similar time points used for starch staining (Fig. 5). In all three developmental stages, the amount of hexose was higher than that of sucrose; however, the ratio of hexose to sucrose declined toward maturation. Glucose and fructose levels in siliques at 3 daf were almost 10 times higher than those at 10 and 13 daf. The sucrose content of the samples remained relatively unchanged during silique development. Whole silique tissues were used for the sugar measurement, and in considering these data it should be pointed out that the silique tissue at 3 daf is somewhat different from those at 10 and 13 daf, as by the later time points seed development is at advanced stages.

Immunolocalization of SUS protein in the companion cells

The vascular bundle of the silique walls (arrow in Fig. 3B), in which SUS protein was localized, is composed of phloem, xylem, and vascular parenchyma cells. Further experiments were therefore conducted to determine the cellular location of the SUS protein within the vascular bundle. Figure 6A shows labelling of SUS protein in the CCs of the phloem but not in the sieve elements or xylem. Labelling was absent in sections probed with the pre-immune serum (Fig. 6B). Since CCs contain nuclei, which are absent from sieve elements, DAPI staining was used to verify the identity of the cells in the phloem (data not shown).

SUS protein is present in the nectaries of Arabidopsis *flowers*

To determine whether SUS protein is expressed in *Arabidopsis* carpels prior to the development of siliques, longitudinal sections of whole flowers, harvested at the day of flowering, were probed with the anti-SUS antibody (Fig. 7). SUS labelling was found to be absent from carpels at this stage. However, strong labelling of SUS

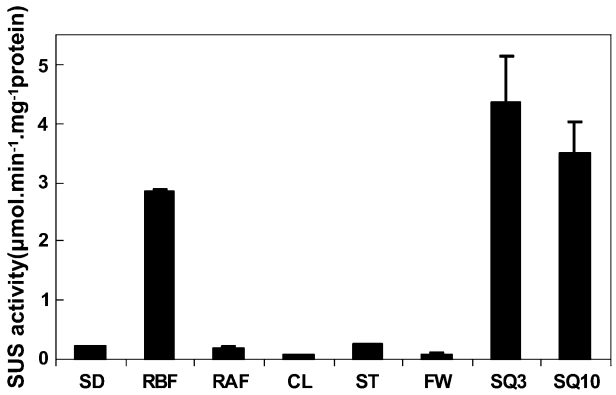

Fig. 2. SUS activity in a range of tissues of *Arabidopsis thaliana*. SUS activity was higher in the sink tissues including young immature rosette leaves and developing siliques compared with other tissues. SD, seedling; RBF, young rosette leaves before flowering; RAF, mature rosette leaves after flowering; CL, cauline leaves; ST, stem; FW, flower at the day of flowering; SQ3, siliques 3 daf; SQ10, siliques 10 daf. Error bars indicate the SE (n=3).

Table 2. *Comparison of the predicted peptide sequences of the six* Arabidopsis *SUS isoforms with that of rice SUS2*

The antibody used in this work was raised against a polypeptide corresponding to amino acids 205–542 of rice SUS2.

Isoform	Protein ID	Molecular weight (kDa)	Identity to rice SUS2 (CAA78747)	Amino acid sequence used for alignment
AtSUS1	NP197583	92.98	79%	210–547
AtSUS2	NP199730	92.05	73%	205–544
AtSUS3	AAK93678	91.98	76%	210–547
AtSUS4	AAK59464	92.98	82%	210–547
AtSUS5	NP198534	95.77	61%	206–540
AtSUS6	NP177480	106.86	66%	228–551

epitopes was present in the lateral nectaries located at the base of the flower (Fig. 7A). Labelling was absent from sections probed with the pre-immune serum (Fig. 7B). SUS labelling was also found in the phloem of sepals and petals, where it localized to the CCs (data not shown).

Discussion

The importance of SUS for sucrose metabolism in starch-storing reproductive structures of legumes such as *Vicia* (Heim *et al*., 1993) and *Pisum* sp. (Barratt *et al*., 2001), and in cellulose synthesis in cotton fibre (Ruan *et al*., 2003) and seeds (Ruan *et al*., 1997), has been clearly established by enzyme activity, transcript measurements, and protein localization, and through mutant (Chourey and Nelson, 1976) and transgenic plant analysis (Zrenner *et al*., 1995; D'Aoust *et al*., 1999). Recently, the surprising absence of a phenotype in reproductive tissues of multiple knockout lines of *AtSUS1*, *AtSUS2*, *AtSUS3*, and *AtSUS4* isoforms in *Arabidopsis* (Bieniawska *et al*., 2007) has cast doubt on the view that this enzyme is indispensable in starch and cellulose biosynthesis, in contrast to the above evidence. While reverse genetics would be expected to provide definitive evidence of the importance of SUS in *Arabidopsis*, lack of a phenotype could also be due to plasticity in plant metabolism and utilization of alternative pathways of sucrose breakdown (such as invertase, the transcript of which is very high in *Arabidopsis* sink tissues (Tymowska-Lalanne and Kreis, 1998). Why such plasticity in sucrose metabolism is not apparent in the crop species mentioned above is unknown.

Studies on SUS in *Arabidopsis* have focused mainly on measurements of transcript abundance (due to a focus on the complexity of the gene family in *Arabidopsis* and the availability of the genome sequence) (Baud *et al*., 2004) and, less commonly, by immunological quantification of individual SUS isoforms (Bieniawska *et al*., 2007). Both these techniques are extremely challenging to quantify when comparing different tissues over development. This is in part due to the difficulty of determining a suitable normalization control transcript. In addition to this technical problem, transcript levels often do not reflect absolute protein level or enzyme activity due to post-transcriptional/translational regulation. Estimating flux through SUS and determination of its physiological role in *Arabidopsis* tissues would be substantially furthered by bulk enzyme activity measurement and protein localization in the appropriate tissues. In the present study, these tools were used to investigate the potential role(s) of SUS in developing siliques and seeds of *Arabidopsis*.

To calibrate the protein measurements against transcript levels in the same tissue samples, expression levels of all six AtSUS isoforms and SUS activity in a range of tissues were measured, revealing that SUS was predominantly present in sink tissues. Importantly, there was little direct correlation between transcript level of the six genes and SUS activity. This lack of correlation with SUS activity was evident in all tissues examined, except for siliques, where high expression levels of *AtSUS2*, *AtSUS3*, and *AtSUS5* could account for the higher SUS activity, and in young rosette leaves. As discussed above, this inconsistency between gene expression and enzyme activity indicates that SUS protein measurements are crucial in extrapolating transcriptional data to physiological function. High SUS activity was found in vegetative and reproductive sink tissues, including young rosette leaves and developing siliques where the high levels of *AtSUS2* and *AtSUS3* transcripts were found. Interestingly, SUS activity slightly declined towards silique maturity, while the expression of both isoforms increased. Baud and Graham (2006) also observed a decline in enzyme activity staining towards the maturity stages of *Arabidopsis* embryo development. These results are in agreement with those reported by Hill *et al*. (2003) in oilseed rape. High SUS activity has been reported in reproductive sink

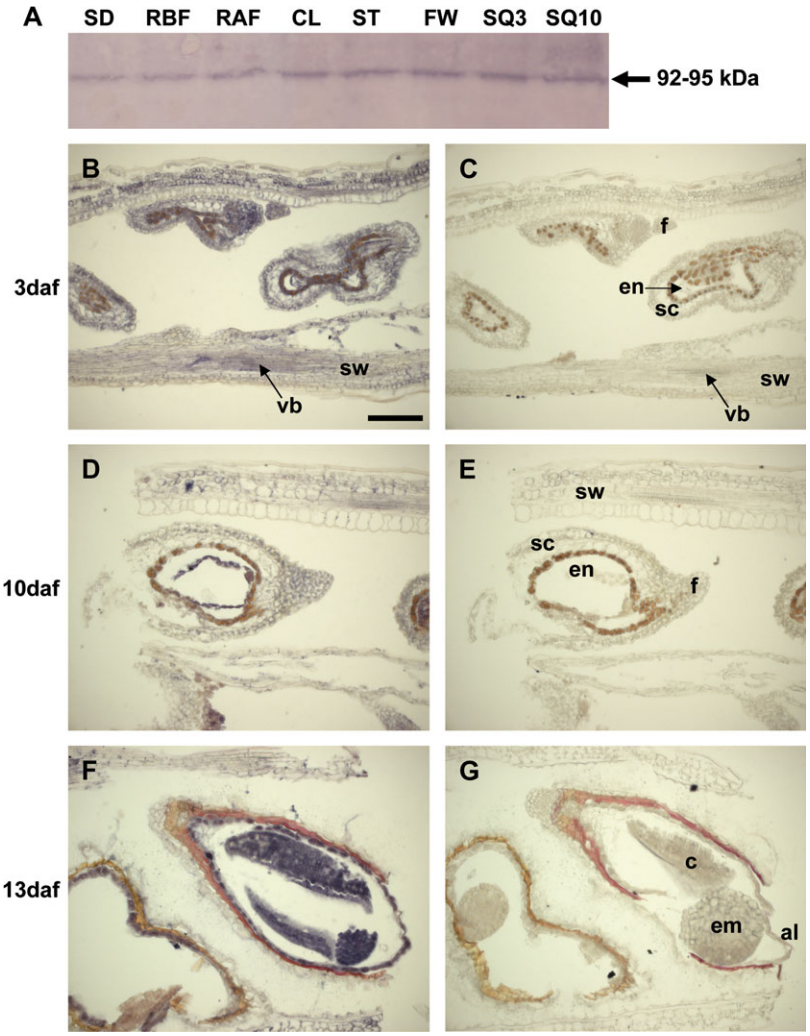

Fig. 3. Immunolocalization of SUS protein in *Arabidopsis* tissues. (A) A western blot containing protein samples from a range of *Arabidopsis* tissues was probed with an antibody raised against a rice SUS2 peptide. A single band at ~ 92–95 kDa was detected in all samples. SD, whole seedlings at the four true leaves stage; RBF, rosette leaves before flowering; RAF, rosette leaves after flowering; CL, cauline leaves; ST, stem; FW, flowers at the day of flowering; SQ3, siliques at 3 daf; SQ10, siliques at 10 daf. Longitudinal sections of siliques at 3 (B, C), 10 (D, E), and 13 (F, G) daf were probed with either the same anti-rice SUS2 antibody (B, D, F) or a pre-immune serum (C, E, G). Antibody labelling of SUS protein, indicated by purple colour development, was present in the funiculus, endosperm, seed coat, and silique wall at 3 and 10 daf, but it was localized in the embryo and aleurone layer at 13 daf. sw, silique wall; en, endosperm; al, aleurone layer; f, funiculus; c, cotyledon; sc, seed coat; em, embryo; vb, vascular bundle. Bar = 100 μm.

tissues of several other plant species including wheat grain (Dale and Housley, 1986), rice (Chan *et al*., 1990), tomato fruit (Sun *et al*., 1992), cotton fibre and seed (Ruan *et al*., 1997), and canola seed and silique (King *et al*., 1997). High SUS activity in these tissues indicates a potential role for SUS as a major enzyme of sucrolysis in these tissues in determining sink strength. During early stages of seed development, high SUS activity establishes a strong sink for sucrose by cleavage of imported sucrose to fructose and UDP-glucose. The preference of SUS over invertase in sucrose cleavage in these tissues might be partly due to a decreased inhibitory effect of fructose on SUS in comparison with invertase (Isla *et al*., 1991). In addition, UDP-glucose produced by the SUS reaction could be channelled to other important pathways such as starch (Munoz *et al*., 2005) and cellulose biosynthesis (Amor *et al*., 1995) during cellularization of endosperm, where the demand for cell wall components including UDP-glucose is increasing (Ruan *et al*., 2008). The resultant fructose from SUS activity may also be used in provision of energy for cell division and expansion of the filial tissues. A significant increase in fructokinase activity and not glucokinase activity has been reported during early to mid stages of seed development (Hill *et al*., 2003), indicating a possible increase in fructose utilization at these stages.

The *Arabidopsis* SUS gene family consists of six isoforms as previously reported through genome analysis

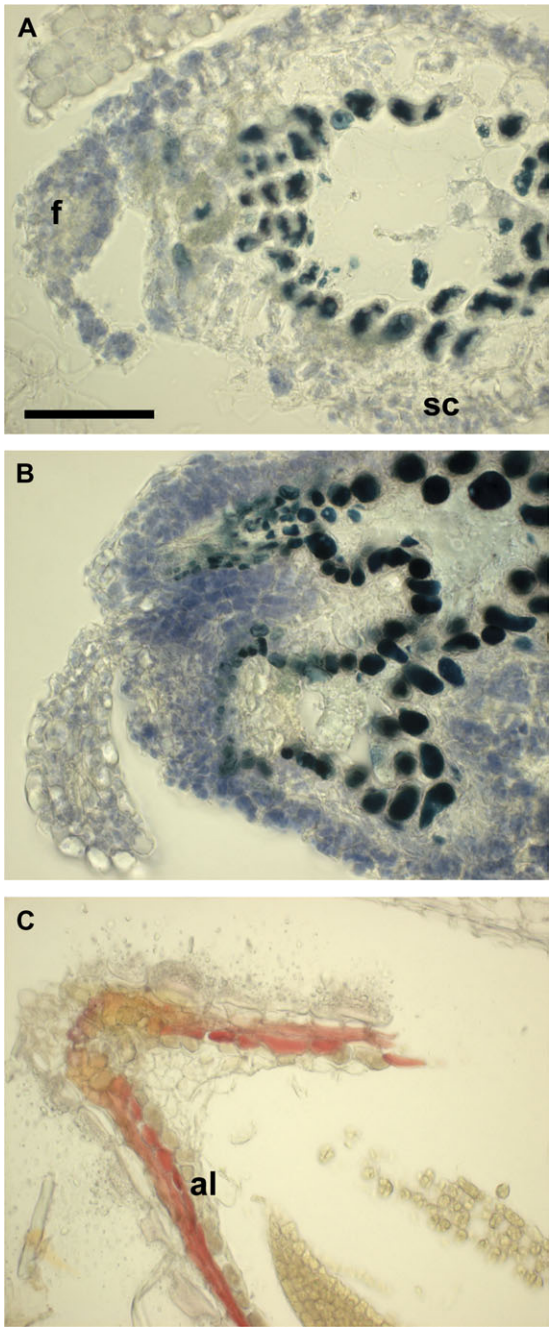

Fig. 4. Localization of starch grains in longitudinal sections of developing *Arabidopsis* siliques at 3 (A), 10 (B), and 13 (C) daf. Starch grains, indicated by the blue coloration, were present in the funiculus and seed coat during early stages of seed development but were absent from 13 daf sections. f, funiculus; sc, seed coat; al, aleurone layer. Bar = 50 μm.

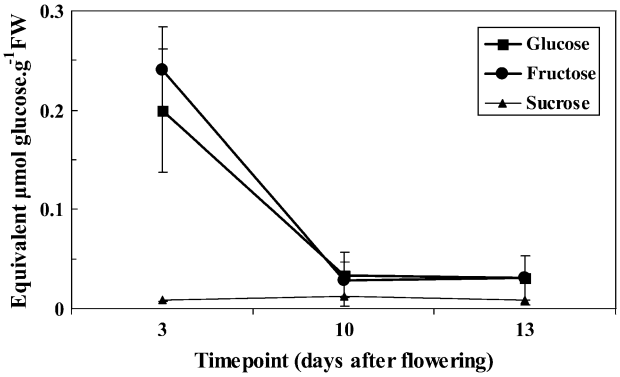

Fig. 5. Carbohydrate analysis in siliques. The glucose, fructose, and sucrose content of developing siliques were determined at 3, 10, and 13 daf. Error bars indicate the SE (n=3).

(Barratt *et al*., 2001). Baud *et al*. (2004) found distinct but partially redundant expression profiles for the six *AtSUS* genes. Bieniawska *et al*. (2007) used T-DNA insertion mutants to elucidate the role of each of these isoforms, and measured the transcript levels using quantitative and semi-quantitative RT-PCR. The results of transcript level measurements obtained in these two studies were slightly different. In the current study, the transcript level of all six *AtSUS* isoforms was measured in several tissues using the same primer sets used by Beiniawska *et al*. (2007). Except for AtSUS2 and AtSUS3, the isoforms did not show strong tissue-specific expression patterns, which is in agreement with Bieniawska *et al*. (2007). The presence of the *AtSUS1* transcript at similar levels in most of the tissues indicates a possible housekeeping role for this isoform. The transcript levels of *AtSUS2* and *AtSUS3* increased in developing siliques during seed development. Semi-quantitative RT-PCR in a previous study showed a similar pattern for these two isoforms (Bieniawska *et al*., 2007). Data analysis obtained from multiple microarrays, accessed via http://www.genevestigator.ethz.ch/mm/ (Laule *et al*., 2006), also revealed an increase in the transcript level of *AtSUS2* and *AtSUS3* during mid and late stages of silique and seed development, respectively. These findings indicate a role for *AtSUS2* and *AtSUS3* during seed development. The *AtSUS4* transcript was most highly expressed in young rosette leaves in comparison with its expression level in the other tissues examined. Studies by Baud *et al*. (2004) and Bieniawska *et al*. (2007) have reported relatively high *AtSUS4* transcript expression in root samples compared with other tissues. These results suggested a role for *AtSUS4* in the vegetative rather than reproductive sink tissues. The expression of *AtSUS5* was found to be high during silique development; however, young immature rosette and cauline leaves also contained high levels of *AtSUS5* transcript. As the kinetics of these enzymes have not been established, it is not known whether higher activity in siliques at 3 daf compared with that in siliques at 10 daf is correlated to the presence of more *AtSUS5* transcript at this stage. *AtSUS6* expression was found to be relatively low in all the tissues examined. Based on these expression patterns, it is not possible to conclude a specific role for each of these latter two isoforms.

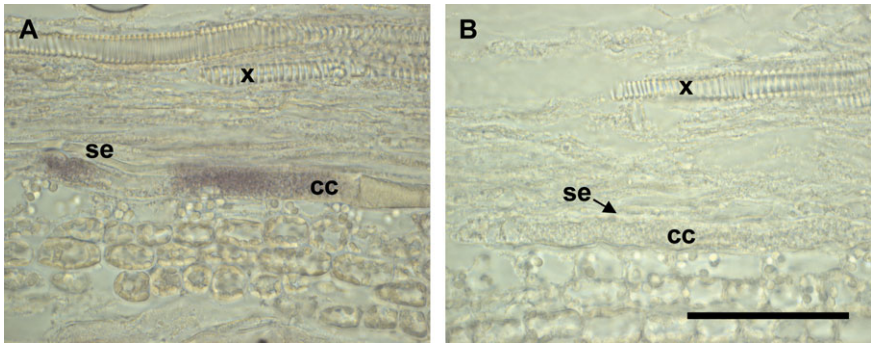

Fig. 6. Immunolocalization of SUS in companion cells. Longitudinal sections of *Arabidopsis* silique wall at 10 daf were probed with either the anti-rice SUS antibody (A) or the pre-immune serum (B). x, xylem; cc, companion cell; se, sieve element. Bar = 100 μm.

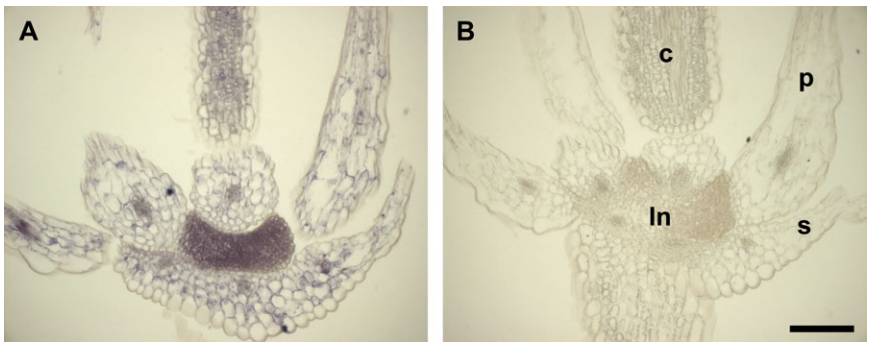

Fig. 7. Immunolocalization of SUS protein in lateral nectaries of *Arabidopsis* flowers. Longitudinal sections of an *Arabidopsis* flower, harvested on the day of flowering, were probed with either the anti-rice SUS antibody (A) or the pre-immune serum (B). p, petal; s, sepal, ln, lateral nectary; c, carpel. Bar = 100 μm.

To extend the present study beyond enzyme activity and transcript determinations in bulk tissue samples, immunolocalization of SUS in *Arabidopsis* tissue sections was performed, using an antibody raised against rice SUS2, to localize SUS protein. The aim was not to separate individual SUS isoforms in these experiments but to compare total SUS protein location with tissue enzyme measurements. Comparison of the predicted peptide sequences of the six AtSUS isoforms with that of rice SUS2 revealed high levels of identity for AtSUS1, AtSUS2, AtSUS3, and AtSUS4, and lower levels of identity for AtSUS5 and AtSUS6. Analysis at four different stages of silique development revealed both spatial and temporal differences in SUS protein localization. The results indicated the involvement of SUS in a range of metabolic processes in reproductive tissues.

In carpels, labelling of SUS protein was found to be absent on the day of flowering. However, SUS protein was found to be present in the lateral nectaries of flowers. The role of SUS in this tissue is not known. It has been shown that the carbohydrate content and composition of nectaries vary greatly between different *Arabidopsis* ecotypes (Davis *et al*., 1998). In Columbia, the sucrose to hexose ratio is low, whereas in Landsberg and Wassilewskija the ratio is significantly higher. High SUS activity may reduce the accumulation of sucrose in the nectaries of Columbia, resulting in a higher hexose concentration. Ren *et al*. (2007) have also reported elevated expression of SUS transcript and protein in developing nectaries of ornamental tobacco. Their results suggested a role for SUS in starch biosynthesis during early stages of nectary development. This starch later remobilized to provide precursors for other pathways in this tissue through hexose production (Ren *et al*., 2007).

In the silique wall, SUS protein was shown to be present in the CCs of phloem at 10 daf, when the silique is mature and fully expanded. This result is consistent with previous localization studies in citrus fruit, maize leaves (Nolte and Koch, 1993), and radish hypocotyl (Rouhier and Usuda, 2001), where it has been suggested that SUS protein is involved in providing energy for phloem loading/unloading processes (Van Bell *et al*., 2000). It has previously been shown that photosynthesis occurs in the mature silique walls of *Arabidopsis* (Robinson and Hill, 1999) and canola (King *et al*., 1997). Assimilates derived from this source tissue may then be transported to the developing seed sink tissues via the silique wall phloem. SUS protein may therefore be involved in providing energy for uploading of assimilates

into the phloem. SUS protein was also observed in CCs of the phloem in flower sepals and petals, which are sink tissues. In these tissues, SUS may have a similar role in the provision of energy for active unloading of assimilate, evidence for the possibility of which was provided by Zhang *et al*. (2004).

In the developing seed, at 3 daf the embryo is at the torpedo developmental stage and the endosperm is undergoing rapid cell division. By 10 daf, the embryo is at the stick-like developmental stage and the cellularized endosperm is rapidly used by the expanding embryo. Accumulation of storage protein and lipids commences at this stage. By 13 daf, cell division declines, the embryo is at the end of the bent cotyledon stage, the endosperm disappears and is replaced by a single cell aleurone layer, and the seed reaches its maximum size (Raz *et al*., 2001). Therefore, in the current work these three stages were selected as early, mid, and late stages of seed development, respectively. SUS protein was found to be present in the endosperm, seed coat, funiculus, and silique walls during early to mid stages of silique development. Later, at 13 daf, SUS protein was found in the embryo and aleurone layer. These temporal changes in cellular localization of SUS protein during *Arabidopsis* seed development are comparable with those reported in cotton seed (Nolte *et al*., 1995), and are consistent with the intracellular localization of SUS in developing kernels of maize (Duncan *et al*., 2006). SUS protein was also localized in the endosperm, but not in young embryos, during early stages of cotton seed development (Ruan *et al*., 2008). Previously, *in situ* staining of SUS activity during maize kernel development (Wittich and Vreugdenhil, 1998) indicated the presence of active SUS protein in a similar pattern to the subsequent protein immunolocalization study. Results from sugar measurements in the current work indicated that a decrease in the hexose to sucrose ratio is a result of a decline in hexose concentration rather than an increase in sucrose accumulation. Starch grains were co-localized with SUS proteins during early to mid stages of seed development. These findings are in agreement with the results of starch measurement in *Arabidopsis* seeds reported by Focks and Benning (1998). It is known that during the early stages of seed development in oilseed plants, starch accumulates temporarily in the seed coat, which is later remobilized for use in lipid and storage protein biosynthesis (Norton and Harris, 1975; Da Silva *et al*., 1997; King *et al*., 1997; Vigeolas *et al*., 2003; Lin *et al*., 2006).

Based on the results presented here and those from previous studies, a model is proposed for sucrose partitioning in developing *Arabidopsis* seed, which might be applicable to other oilseed plants (Fig. 8). During the early to mid stages, sucrose delivered to the seed coat and endosperm, via the funiculus, is utilized by SUS for starch deposition in the seed coat and for cell wall biosynthesis

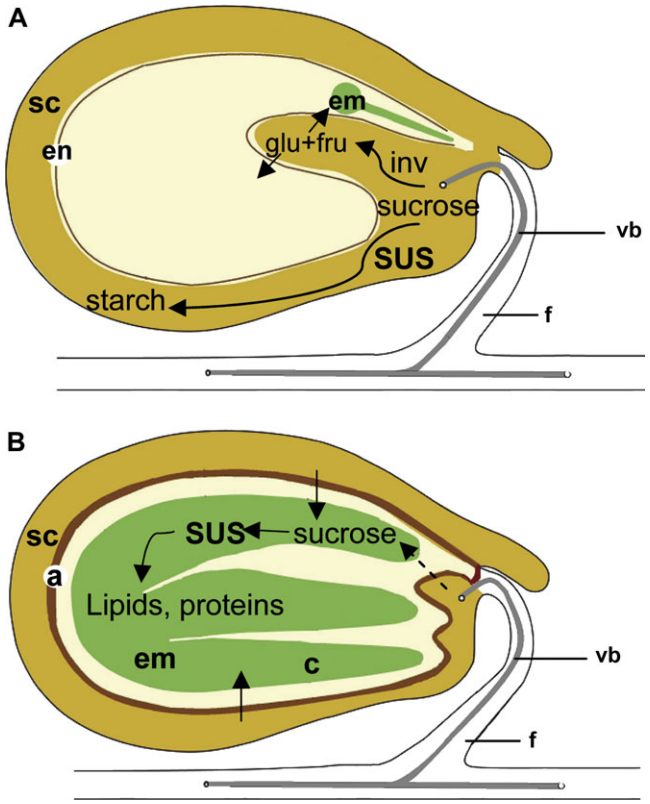

Fig. 8. A model illustrates the diverse role of SUS in sucrose metabolism during *Arabidopsis* seed development. (A) During the early stages of seed development sucrose enters the seed coat via the vascular bundle of the funiculus. Sucrose is cleaved by either sucrose synthase (SUS) or invertase (Inv). The UDP-glucose produced by SUS activity is used in biosynthesis of starch. The starch is temporarily stored in the seed coat, whereas the resultant hexoses, produced by Inv activity, are used by developing embryo and endosperm. (B) During later stages of seed development, sucrose is delivered directly to the embryo from the maternal tissues. SUS activity in the embryo uses sucrose to provide precursors for the biosynthesis of storage protein and lipids. In addition, the temporary starch reserves are remobilized from the seed coat to the embryo via the aleurone layer. SUS activity in the aleurone layer may be involved in this remobilization process. al, aleurone layer; c, cotyledon; em, embryo; en, endosperm; fru, fructose; f, funiculus; glu, glucose; Inv, invertase; SUS, sucrose synthase; sc, seed coat; vb, vascular bundle.

in the endosperm (Fig. 8A). There is evidence to indicate that a sucrose transporter is expressed specifically in the micropylar region of the endosperm up to 8 daf (Baud *et al*., 2005). However, some of the sucrose may be cleaved by invertase activity in the seed coat, and the resultant hexoses are delivered to the developing embryo and endosperm. Previous studies indicated that acid invertase activity was higher in the seed coat during early stages of seed development compared with that of later stages (Sturm and Tang, 1999; Hill *et al*., 2003). Temporary storage of assimilates during early stages in the form of starch will maintain constant and strong sink strength by preventing accumulation of sucrose and hexoses in the seed coat and embryo. It may also prevent

negative feedback regulation of photosynthesis in the source tissues due to accumulation of unused sugars in the sink tissue. Later during seed development, sucrose may be delivered to the embryo by maternal tissues (Fig. 8B). The sucrose may be cleaved by SUS in embryo for lipid and protein biosynthesis. At this stage, the starch reserves in the seed coat may be remobilized and utilized by the embryo and aleurone layer. An increase in the transcript expression of amylase genes has been reported during mid to late stages of seed development (Kim *et al*., 2005). In addition, a centripetal transport of assimilates from outer integument towards filial tissues has been demonstrated in developing *Arabidopsis* seed from expression of β-glucuronidase protein under different promoters (Stadler *et al*., 2005).

In summary, from expression analysis at the transcript, protein, and enzyme activity level, SUS is likely to be involved in several separate cellular processes in flower and developing seeds of *Arabidopsis*. Co-localization of SUS protein and starch grains indicates a possible role in carbon partitioning during early to mid stages of seed development, as a process to maintain sink strength. In later stages, SUS protein might have a role within the embryo, cotyledon, and aleurone layer in sucrose utilization. The current work also provided further evidence for SUS involvement in the provision of energy for phloem loading and unloading, as indicated by localization of the protein in CCs. SUS protein was also localized to the floral nectary, which is previously unreported, indicating a potential role in carbon partitioning within this tissue.

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References

Amor Y, Haigler C, Johnson S, Wainscott M, Delmer D. 1995. A membrane-associated form of sucrose synthase and its potential role in synthesis of cellulose and callose in plants. *Proceedings of the National Academy of Sciences, USA* **92,** 9353–9357.

Barratt DHP, Barber L, Kruger NJ, Smith AM, Wang TL, Martin C. 2001. Multiple, distinct isoforms of sucrose synthase in pea. *Plant Physiology* **127,** 655–664.

Baud S, Graham IA. 2006. A spatiotemporal analysis of enzymatic activities associated with carbon metabolism in wild-type and mutant embryos of Arabidopsis using *in situ* histochemistry. *The Plant Journal* **46,** 155–169.

Baud S, Vaultier M-N, Rochat C. 2004. Structure and expression profile of the sucrose synthase multigene family in Arabidopsis. *Journal of Experimental Botany* **55,** 397–409.

Baud S, Wuilleme S, Lemoine R, Kronenberger J, Caboche M, Lepiniec L, Rochat C. 2005. The AtSUC5 sucrose transporter specifically expressed in the endosperm is involved in early seed development in Arabidopsis. *The Plant Journal* **43,** 824–836.

Bieniawska Z, Barratt P, Garlick AP, Thole V, Kruger NJ, Martin C, Zrenner R, Smith AM. 2007. Analysis of the sucrose synthase gene family in Arabidopsis. *The Plant Journal* **49,** 810–828.

Carlson SJ, Chourey PS, Helentjaris T, Datta R. 2002. Gene expression studies on developing kernels of maize sucrose synthase (SuSy) mutants show evidence for a third SuSy gene. *Plant Molecular Biology* **49,** 15–29.

Chan H-Y, Ling T-Y, Juang R-H, Ting I-N, Sung H-Y, Su J-C. 1990. Sucrose synthase in rice plants: growth-associated changes in tissue specific distributions. *Plant Physiology* **94,** 1456–1461.

Chourey P, Nelson OE. 1976. The enzymatic deficiency conditioned by the shrunken-1 mutations in maize. *Biochemical Genetics* **14,** 1041–1055.

Chourey P, Taliercio EW, Carlson SJ, Ruan Y-L. 1998. Genetic evidence that the two isozymes of sucrose synthase present in developing maize endosperm are critical, one for cell wall integrity and the other for starch biosynthesis. *Molecular and General Genetics* **259,** 88–96.

Counce PA, Gravois KA. 2006. Sucrose synthase activity as a potential indicator of high rice grain yield. *Crop Science* **46,** 1501–1507.

Craig J, Barratt P, Tatge H, *et al*. 1999. Mutations at the rug4 locus alter the carbon and nitrogen metabolism of pea plants through an effect on sucrose synthase. *The Plant Journal* **17,** 353–362.

D'Aoust M-A, Yelle S, Nguyen-Quoc B. 1999. Antisense inhibition of tomato fruit sucrose synthase decreases fruit setting and the sucrose unloading capacity of young fruit. *Plant Cell* **11,** 2407–2418.

Da Silva PMFR, Eastmond PJ, Hill LM, Smith AM, Rawsthorne S. 1997. Starch metabolism in developing embryos of oilseed rape. *Planta* **203,** 480–487.

Dale EM, Housley TL. 1986. Sucrose synthase activity in developing wheat endosperms differing in maximum weight. *Plant Physiology* **82,** 7–10.

Davis AR, Pylatuik JD, Paradis JC, Low NH. 1998. Nectar-carbohydrate production and composition vary in relation to nectary anatomy and location within individual flowers of several species of Brassicaceae. *Planta* **205,** 305–318.

Doehlert DC, Kuo TM, Felker FC. 1988. Enzymes of sucrose and hexose metabolism in developing kernels of two inbreds of maize. *Plant Physiology* **86,** 1013–1019.

Duncan KA, Hardin SC, Huber SC. 2006. The three maize sucrose synthase isoforms differ in distribution, localization, and phosphorylation. *Plant Cell Physiology* **47,** 959–971.

Focks N, Benning C. 1998. wrinkled1: a novel, low-seed-oil mutant of Arabidopsis with a deficiency in the seed-specific regulation of carbohydrate metabolism. *Plant Physiology* **118,** 91–101.

Fu H, Kim SY, Park WD. 1995. A potato Sus3 sucrose synthase gene contains a context-dependent 3′ element and a leader intron with both positive and negative tissue-specific effects. *The Plant Cell* **7,** 1395–1403.

Haigler CH, Ivanova-Datcheva M, Hogan PS, Salnikov VV, Hwang S, Martin K, Delmer DP. 2001. Carbon partitioning to cellulose synthesis. *Plant Molecular Biology* **47,** 29–51.

Harada T, Satoh S, Yoshioka T, Ishizawa K. 2005. Expression of sucrose synthase genes involved in enhanced elongation of pondweed (*Potamogeton distinctus*) turions under anoxia. *Annals of Botany* **96,** 683–692.

Heim U, Weber H, Baumlein H, Wobus U. 1993. A sucrose-synthase gene of *Vicia faba* L—expression pattern in developing seeds in relation to starch synthesis and metabolic-regulation. *Planta* **191,** 394–401.

Hill LM, Morley-Smith ER, Rawsthorne S. 2003. Metabolism of sugars in the endosperm of developing seeds of oilseed rape. *Plant Physiology* **131,** 228–236.

Hirose T, Scofield GN, Terao T. 2008. An expression analysis profile for the entire sucrose synthase gene family in rice. *Plant Science* **174,** 534–543.

Horst I, Welham T, Kelly S, Kaneko T, Sato S, Tabata S, Parniske M, Wang TL. 2007. TILLING mutants of *Lotus japonicus* reveal that nitrogen assimilation and fixation can occur in the absence of nodule-enhanced sucrose synthase. *Plant Physiology* **144,** 806–820.

Huang J, Chen J, Yu W, Shyur L, Wang A, Sung H, Lee P, Su JC. 1996. Complete structures of three rice sucrose synthase isogenes and differential regulation of their expressions. *Bioscience, Biotechnology, and Biochemistry* **60,** 233–239.

Isla MI, Vattuone MA, Sampietro AR. 1991. Modulation of potato invertase activity by fructose. *Phytochemistry* **30,** 423–426.

Kim Y-C, Nakajima M, Nakayama A, Yamaguchi I. 2005. Contribution of gibberellins to the formation of Arabidopsis seed coat through starch degradation. *Plant and Cell Physiology* **46,** 1317–1325.

King SP, Lunn JE, Furbank RT. 1997. Carbohydrate content and enzyme metabolism in developing canola siliques. *Plant Physiology* **114,** 153–160.

Koch K. 2004. Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. *Current Opinion in Plant Biology* **7,** 235–246.

Laule O, Hoffmann MH, Hruz T, Gruissem I, Zimmermann P. 2006. Web-based analysis of the mouse transcriptome using Genevestigator. *BMC Bioinformatics* **7,** 311–319.

Lerchl J, Geigenberger P, Stitt M, Sonnewald U. 1995. Impaired photoassimilate partitioning caused by phloem-specific removal of pyrophosphate can be complemented by a phloem-specific cytosolic yeast-derived invertase in transgenic plants. *The Plant Cell* **7,** 259–270.

Lin Y, Ulanov AV, Lozovaya V, Widholm J, Zhang G, Guo J, Goodman HM. 2006. Genetic and transgenic perturbations of carbon reserve production in *Arabidopsis* seeds reveal metabolic interactions of biochemical pathways. *Planta* **225,** 153–164.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\tilde{\Delta}\Delta C}{}_{T}$ method. *Methods* **25,** 402–408.

Lunn JE, Ap Rees T. 1990. Apparent equilibrium constant and mass–action ratio for sucrose-phosphate synthase in seeds of *Pisum sativum*. *Biochemical Journal* **267,** 739–743.

Millar AA, Jacobsen JV, Ross JJ, Helliwell CA, Poole AT, Scofield G, Reid JB, Gubler F. 2006. Seed dormancy and ABA metabolism in Arabidopsis and barley: the role of ABA 8′-hydroxylase. *The Plant Journal* **45,** 942–954.

Munoz FJ, Baroja-Fernandez E, Moran-Zorzano MT, Viale AM, Etxeberria E, Alonso-Casajus N, Pozueta-Romero J. 2005. Sucrose synthase controls both intracellular ADP glucose levels and transitory starch biosynthesis in source leaves. *Plant and Cell Physiology* **46,** 1366–1376.

Nolte KD, Hendrix DL, Radin JW, Koch KE. 1995. Sucrose synthase localization during initiation of seed development and trichome differentiation in cotton ovules. *Plant Physiology* **109,** 1285–1293.

Nolte KD, Koch KE. 1993. Companion-cell specific localization of sucrose synthase in zones of phloem loading and unloading. *Plant Physiology* **101,** 899–905.

Norton G, Harris JF. 1975. Compositional changes in developing rape seed (*Brassica napus* L.). *Planta* **123,** 163–174.

Raz V, Bergervoet J, Koornneef M. 2001. Sequential steps for developmental arrest in Arabidopsis seeds. *Development* **128,** 243–252.

Ren G, Healy RA, Horner HT, James MG, Thornburg RW. 2007. Expression of starch metabolic genes in the developing nectaries of ornamental tobacco plants. *Plant Science* **173,** 621–637.

Robinson CK, Hill SA. 1999. Altered resource allocation during seed development in *Arabidopsis* caused by the *abi3* mutation. *Plant, Cell and Environment* **22,** 117–123.

Rouhier H, Usuda H. 2001. Spatial and temporal distribution of sucrose synthase in the radish hypocotyl in relation to thickening growth. *Plant Cell Physiology* **42,** 583–593.

Ruan Y-L, Chourey PS. 1998. A fiberless seed mutation in cotton is associated with lack of fiber cell initiation in ovule epidermis and alterations in sucrose synthase expression and carbon partitioning in developing seeds. *Plant Physiology* **118,** 399–406.

Ruan Y-L, Chourey PS, Delmer DP, Perez-Grau L. 1997. The differential expression of sucrose synthase in relation to diverse patterns of carbon partitioning in developing cotton seed. *Plant Physiology* **115,** 375–385.

Ruan Y-L, Llewellyn DJ, Furbank RT. 2003. Suppression of sucrose synthase gene expression represses cotton fiber cell initiation, elongation, and seed development. *The Plant Cell* **15,** 952–964.

Ruan Y-L, Llewellyn DJ, Liu Q, Xu S-M, Wu L-M, Wang L, Furbank RT. 2008. Expression of sucrose synthase in developing endosperm is essential for early seed development. *Functional Plant Biology* in press.

Ruuska SA, Girke T, Benning C, Ohlrogge JB. 2002. Contrapuntal networks of gene expression during Arabidopsis seed filling. *The Plant Cell* **14,** 1191–1206.

Scofield GN, Aoki N, Hirose T, Takano M, Jenkins CLD, Furbank RT. 2007. The role of the sucrose transporter, OsSUT1, in germination and early seedling growth and development of rice plants. *Journal of Experimental Botany* **58,** 483–495.

Stadler R, Lauterbach C, Sauer N. 2005. Cell-to-cell movement of green fluorescent protein reveals post-phloem transport in the outer integument and identifies symplastic domains in Arabidopsis seeds and embryos. *Plant Physiology* **139,** 701–712.

Sturm A, Tang G-Q. 1999. The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. *Trends in Plant Science* **4,** 401–407.

Sun J, Loboda T, Sung S-JS, Black CC Jr. 1992. Sucrose synthase in wild tomato, *Lycopersicon chmielewskii*, and tomato fruit sink strength. *Plant Physiology* **98,** 1163–1169.

Tymowska-Lalanne Z, Kreis M. 1998. Expression of the *Arabidopsis thaliana* invertase gene family. *Planta* **207,** 259–265.

Van Bel AJE, Knoblauch M. 2000. Sieve element and companion cell: the story of the comatose patient and the hyperactive nurse. *Functional Plant Biology* **27,** 477–487.

Vigeolas H, van Dongen JT, Waldeck P, Huhn D, Geigenberger P. 2003. Lipid storage metabolism is limited by the prevailing low oxygen concentrations within developing seeds of oilseed rape. *Plant Physiology* **133,** 2048–2060.

Wang A-Y, Yu W-P, Juang R-H, Huang J-W, Sung H-Y, Su J-C. 1992. Presence of three rice sucrose synthase genes as revealed

by cloning and sequencing of cDNA. *Plant Molecular Biology* **18,** 1191–1194.

Wittich P, Vreugdenhil D. 1998. Localization of sucrose synthase activity in developing maize kernels by *in situ* enzyme histochemistry. *Journal of Experimental Botany* **49,** 1163–1171.

Xu D-P, Sung S-JS, Black CC. 1989. Sucrose metabolism in lima bean seeds. *Plant Physiology* **89,** 1106–1116.

Zhang L-Y, Peng Y-B, Pelleschi-Travier S, Fan Y, Lu Y-F, Lu Y-M, Gao X-P, Shen Y-Y, Delrot S, Zhang D-P. 2004. Evidence for apoplasmic phloem unloading in developing apple fruit. *Plant Physiology* **135,** 574–586.

Zrenner R, Salanoubat M, Willmitzer L, Sonnewald U. 1995. Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (Solanum tuberosum L.). *The Plant Journal* **7,** 97–107.