# A Role for Sugar Transporters during Seed Development: Molecular Characterization of a Hexose and a Sucrose Carrier in Fava Bean Seeds

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To analyze sugar transport processes during seed development of fava bean, we cloned cDNAs encoding one sucrose and one hexose transporter, designated *VfSUT1* and *VfSTP1*, respectively. Sugar uptake activity was confirmed after heterologous expression in yeast. Gene expression was studied in relation to seed development. Transcripts were detected in both vegetative and seed tissues. In the embryo, *VfSUT1* and *VfSTP1* mRNAs were detected only in epidermal cells, but in a different temporal and spatial pattern. *VfSTP1* mRNA accumulates during the midcotyledon stage in epidermal cells covering the mitotically active parenchyma, whereas the *VfSUT1* transcript was specific to outer epidermal cells showing transfer cell morphology and covering the storage parenchyma. Transfer cells developed at the contact area of the cotyledonary epidermis and the seed coat, starting first at the early cotyledon stage and subsequently spreading to the abaxial region at the late cotyledon stage. Feeding high concentrations of sugars suppressed both *VfSUT1* expression and transfer cell differentiation in vitro, suggesting a control by carbohydrate availability.

## INTRODUCTION

Postphloem assimilate transport could be exclusively sink controlled by a mechanism in which a high capacity to metabolize assimilates in the embryo lowers sugar concentrations and creates a gradient that drives import. However, growing evidence favors the view that sink-located transfer and transport processes control assimilate partitioning (Fisher, 1995; Patrick and Offler, 1995; Truernit et al., 1996). In legume seeds, sugars are unloaded from the coat cells into the seed apoplast and must be taken up by the apoplastically isolated embryo. Active transport systems are present in different parts of the seed (Gahrtz et al., 1996; McDonald et al., 1996) and could represent control points. Active sugar transport involves $H^+$ sugar transporters (reviewed in Bush, 1993; Buckhout and Tubbe, 1996). Two types of carriers have been characterized: disaccharide transporters (Riesmeier et al., 1992) and monosaccharide transporters (Sauer et al., 1990b). Both protein families have a typical 12 membrane–spanning domain structure (Marger and Saier, 1993). Sucrose transporter cDNAs were isolated from sugar beet, spinach, potato, Arabidopsis, *Plantago major*, and *Ricinus communis*. There seems to be only one gene present in spinach and potato, but *P. major* and Arabidopsis have two. PmSUC1 and AtSUC1 are both insensitive to changes in pH

and were therefore designated as neutral transporters. All other sucrose transporters are highly pH sensitive, showing increased transport rates when the pH is decreased. The acid transporters are localized in the sieve element companion cells and probably carry sucrose from the apoplast (Stadler et al., 1995a; Stadler and Sauer, 1996).

Hexose transporter cDNAs are encoded by gene families of up to 12 members in *R. communis* (Weig et al., 1994), *Chenopodium rubrum* (Roitsch and Tanner, 1994), and Arabidopsis (Sauer et al., 1994). β-Glucuronidase fusions of promoters from Arabidopsis hexose transporter genes show tissue-specific expression (Sauer et al., 1994), and one of these transporters (STP4) has been shown to be sink specific and regulated by environmental stress (Truernit et al., 1996).

Thus far, little is known of the molecular biology of sugar transport during seed development. However, transport physiology and the cellular pathway of sucrose were analyzed in some detail in fava bean seeds at the filling stage (reviewed in Patrick and Offler, 1995). The sieve elements of the phloem end up in the seed coat and are symplastically connected with coat cells. Phloem unloading and postphloem transport through the seed coat are symplastic (Patrick et al., 1995). Inhibitor studies and selective removal of the inner cell layers of the coat, the so-called thin-walled parenchyma, have been used to identify these cells as the site of unloading into the apoplast (Wang et al., 1995). However, the possible mechanism for unloading remains controversial.

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For pea, a passive process of seed coat unloading mediated by a porin-like transporter has been suggested (De Jong and Wolswinkel, 1995; De Jong et al., 1996). For fava bean seed coats, on the other hand, carrier-mediated transport has been proposed (Wang et al., 1995).

Sugar uptake in the embryo is catalyzed by a saturable and a nonsaturable system (reviewed in Patrick and Offler, 1995). In situ uptake kinetics, inhibitor studies, and pH sensitivity point to an $H^+$ sucrose symporter as being responsible for the saturable component. In fava bean cotyledons, carrier-specific inhibitors as well as the removal of the outer cell layers of the cotyledons reduced sucrose uptake considerably (McDonald et al., 1995), indicating that sucrose is taken up predominantly by the outer epidermis. From in vitro studies, it was concluded that uptake is primarily controlled by an $H^+$ sucrose symporter located in the outer epidermal transfer cell complex, whereas the passive uptake system may be less important (McDonald et al.,1996). The required proton motive force in the epidermal cells is provided by a colocalized $H^+$-ATPase (Bouché-Pillon et al., 1994). On the other hand, sugar uptake by the storage parenchyma cells seems to be passive (McDonald et al., 1996).

The physiological studies addressed only the later phase of seed development. The early embryo, however, is a weak sink because both sucrolysis and storage activity are low (Weber et al., 1995, 1996a). During the prestorage phase, sink strength is provided by seed coat–associated cell wall–bound invertase, which controls the carbohydrate state of the apoplast environment, bathing the embryo and causing a high hexose/sucrose ratio in the cotyledons. When invertase activity declines, the storage phase is initiated and is accompanied by an increase in fresh weight and a decrease in the hexose/sucrose ratio in the embryo (Weber et al., 1995). Immediately before the phase of fresh weight gain, the epidermal cell layer of fava bean cotyledons differentiates into transfer cells (Bonnemain et al., 1991).

To shed some light on the role of sugar carriers in seed development, we cloned cDNAs encoding a hexose and a sucrose transporter and analyzed gene expression. Sucrose transporter gene expression in the embryo is specific to the cotyledonary epidermis and is coupled to transfer cell differentiation, a process that is probably under carbohydrate control. Hexose transporter gene expression is seen in parts of the cotyledonary epidermis covering mitotically active parenchyma cells.

## RESULTS

### cDNA Cloning Reveals One Sucrose and One Hexose Transporter Gene Expressed in Cotyledons

Fava bean seed development has been divided into seven stages: the globular stage (I), the early and late heart stages (II and III), and four stages covering early to late cotyledon

development (IV to VII; Borisjuk et al., 1995). To isolate seed-specific sugar transporter cDNAs, degenerate primers were designed from conserved domains of both sucrose and hexose transporters, and polymerase chain reaction (PCR) was performed, using as a template a fava bean cDNA library specific for stage V to VI cotyledons. A comparison of the amplified sequences at the nucleotide level revealed homologies of a 780-bp fragment to sucrose transporters and of a 280-bp fragment to hexose transporters.

The PCR fragments were used to isolate the full-length cDNAs from the cotyledon-specific cDNA library. Clones encoding the sucrose transporter and the hexose transporter were isolated, characterized, and designated as *VfSUT1* and *VfSTP1*, respectively. *VfSUT1* and *VfSTP1* are represented by single transcripts of 2004 and 1672 bases encoding proteins of 523 (55 kD) and 516 (57 kD) amino acids, respectively. Both *VfSUT1* and *VfSTP1* possess the typical 12 membrane-spanning domain structure (Marger and Saier, 1993).

The putative amino acid sequence of VfSUT1 was aligned with the deduced protein sequences of nine known sucrose transporters (EMBL data bank). Figure 1A shows that VfSUT1 clustered together with the acid transporters (Stadler et al., 1995a). Accordingly, VfSTP1 was aligned with deduced protein sequences of seven known hexose transporters (Figure 1B; EMBL data bank).

### Heterologous Expression of Transporter cDNAs in Yeast

The cDNAs encoding VfSTP1 and VfSUT1 were expressed in fission or in baker's yeast, respectively, both in the sense and in the antisense orientation. Figure 2A shows that yeast strain VFY-SS, which expresses *VfSUT1* in a sense orientation, was able to transport sucrose at a high rate across the yeast plasma membrane, whereas only a very slow uptake rate was seen in the VFY-SA antisense strain. Sucrose transport by VFY-SS is energy dependent. Uptake rates in the absence of the metabolizable energy source D-glucose are ~70% lower (data not shown). The transport rates of VfSUT1 increase dramatically with decreasing pH (relative rates at pH values of 6.0, 5.5, 5.0, and 4.5 are 1, 1.8, 3.4, and 3.8, respectively), indicating that VfSUT1 belongs to the group of acidic sucrose carriers. Because the $K_m$ value of VfSUT1 for sucrose decreases with the pH ($K_m$ of 3.3 mM at a pH of 6.0; $K_m$ of 1.4 mM at a pH of 4.5), these increasing rates are due at least in part to an increasing affinity of VfSUT1 for sucrose.

Figure 2B shows that VFY-HS cells expressing *VfSTP1* in a sense orientation can catalyze the uptake of D-glucose across their plasma membrane, whereas only a negligible rate is seen in glucose transport–deficient VFY-HA antisense cells. Like other plant monosaccharide $H^+$ symporters, VfSTP1 has an extremely low $K_m$ for D-glucose ($K_m$ of 30 μM), and like other plant hexose transporters, it can transport a number of substrates. (The relative transport rates at 100 μM substrate concentration for D-glucose, D-mannose, D-galactose, 3-O-
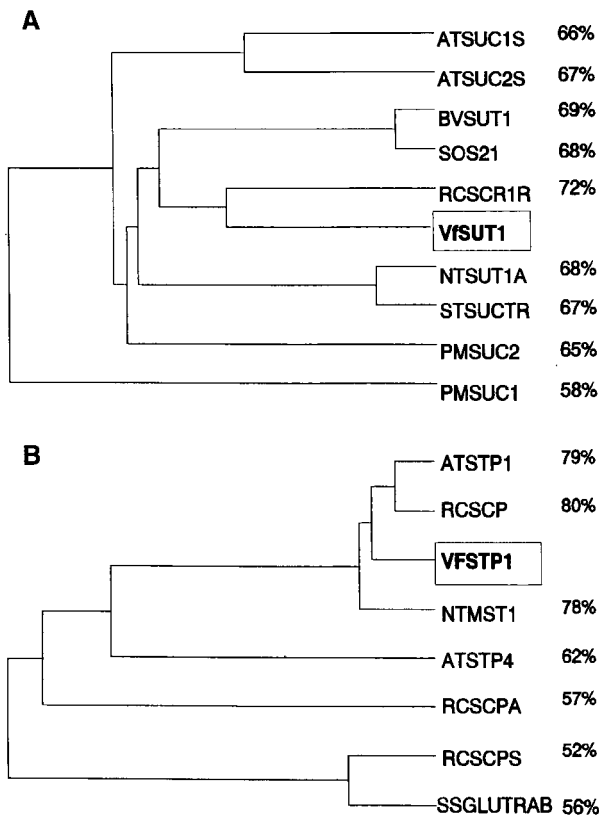
# A



| | |
|---|---|
| ATSUC1S | 66% |
| ATSUC2S | 67% |
| BVSUT1 | 69% |
| SOS21 | 68% |
| RCSCR1R | 72% |
| VfSUT1 | |
| NTSUT1A | 68% |
| STSUCTR | 67% |
| PMSUC2 | 65% |
| PMSUC1 | 58% |

# B

| | |
|---|---|
| ATSTP1 | 79% |
| RCSCP | 80% |
| VFSTP1 | |
| NTMST1 | 78% |
| ATSTP4 | 62% |
| RCSCPA | 57% |
| RCSCPS | 52% |
| SSGLUTRAB | 56% |

**Figure 1.** Comparison of Sucrose and Hexose Transporters.

**(A)** A dendrogram of the alignment of sucrose transporters. The amino acid sequence of VfSUT1 was aligned with the corresponding sucrose transporters as follows (with their GenBank, EMBL, and DDBJ accession numbers): Arabidopsis (ATSUC1S, No. X75365; ATSUC2S, No. X75382), sugar beet (BVSUT1, No. X83850), spinach (SOS21, No. X67125), *R. communis* (RCSCR1R, No. Z31561), tobacco (NTSUT1A, No. X82276), potato (STSUCTR, No. X69165), and *P. major* (PMSUC1, No. X84389; PMSUC2, No. X75764).
**(B)** A dendogram of the alignment of hexose transporters. The putative amino acid sequence of VfSTP1 was aligned with the corresponding hexose transporters as follows: Arabidopsis (ATSTP1, No. X55350; ATSTP4, No. X66857), *R. communis* (RCSCP, No. L08196; RCSCPA, No. L08188; RCSCPS, No. L08197), tobacco (NTMST1, No. X66856), and sugar cane (SSGLUTRAB, No. L21753).
Indicated are the percent identities of the amino acid residues of the sucrose and hexose transporters.

methylglucose, L-glucose, and D-fructose are 100, 74, 48, 13, 7, and 6, respectively.)

## Expression Analysis of Sugar Transporter Genes

The full-length cDNAs were used as probes in RNA gel blot experiments. Figure 3A shows the quantified signals of VfSUT1 mRNA present in cotyledons, seed coats, pods,

sink leaves, source leaves, and roots. In cotyledons, levels increased from 13 days after flowering (DAF; stage IV from Borisjuk et al., 1995) until the beginning of the storage phase (~20 DAF, stage V to VI, midcotyledon stage) and decreased afterward until maturation (38 DAF, stage VII, late cotyledon stage). Maximal levels in the seed coat are delayed by ~4 days. Figure 3B shows the signals of VfSTP1 mRNA in cotyledons, seed coats, pods, sink leaves, source leaves, and roots. RNA levels were high in stage V cotyledons (~16 DAF) and decreased rapidly thereafter. In the seed coat, VfSTP1 mRNA was detected at ~9 DAF until 35 DAF. Expression in vegetative tissues revealed slight sink specificity, with higher levels in roots and in sink leaves compared with source leaves. The expression level of
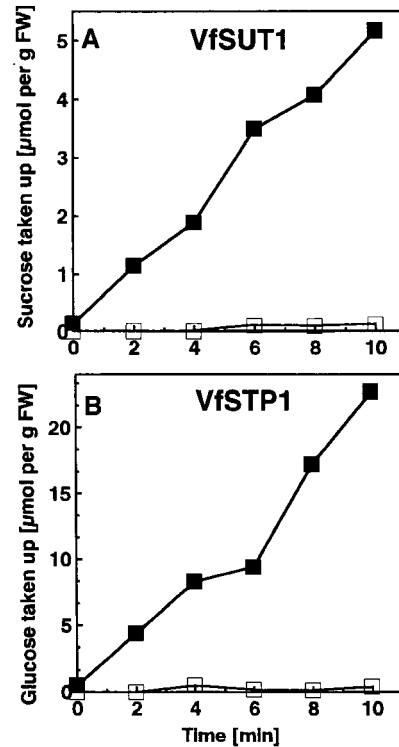


**Figure 2.** Sugar Uptake of Fava Bean Transporters Expressed in Yeast.

Sense and antisense constructs of VfSUT1 and VfSTP1 cDNAs were expressed in baker's or fission yeast, respectively, and transport rates for sucrose and glucose were determined in the resulting yeast strains.
**(A)** Uptake of sucrose (initial concentration of 1 mM) by VFY-SS (filled squares; expressing VfSUT1 in a sense orientation) and VFY-SA (open squares; expressing the antisense construct) cells.
**(B)** Uptake of D-glucose (initial concentration of 100 μM) by VFY-HS (filled squares; expressing VfSTP1 in a sense orientation) and VFY-HA (open squares; expressing the antisense construct) cells.
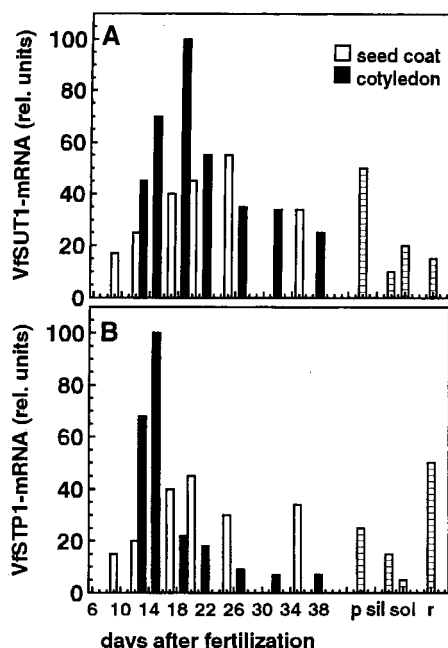FW, fresh weight.

**Figure 3.** Transcript Analysis of *VfSUT1* and *VfSTP1*.

**(A)** Accumulation of *VfSUT1* mRNA in cotyledons, seed coats, pods (p), sink leaves (sil), source leaves (sol), and roots (r). Hybridization signals from RNA gel blot analysis were quantified using a Phosphor-Imager (Fuji Photo Film Co., Tokyo, Japan).

**(B)** Accumulation of *VfSTP1* mRNA detected on the same blot. Experiments were performed three times. Representative data from a single experiment are shown.

rel. units, relative units.

*VfSUT1* was found to be higher than that of *VfSTP1*. However, the VfSTP1 transcript was sufficiently abundant to be analyzed on gel blots containing 5 μg of total RNA per lane.

Both genes are expressed in the embryo, maternal seed tissues, and vegetative parts of the plant. To determine whether the hybridizing RNA was heterogenous, blots were rehybridized using 510- and 268-bp fragments from the 3' ends of *VfSUT1* and *VfSTP1*, respectively, as probes. These fragments are assumed to be gene-specific probes because their homology to the corresponding region of the most homologous sequences of *RCSCR1R* and *RCSCP* is 30 and 60%, respectively. These gene-specific 3' end probes gave results similar to those observed when full-length probes were used (data not shown), indicating that *VfSUT1* and *VfSTP1* are expressed in both embryonic and vegetative tissues.

### Expression of the Sucrose Transporter in the Embryo Is Epidermis Specific

Cell type–specific expression of VfSUT1 was analyzed in developing embryos by in situ hybridization, using full-length

cDNAs as probes. Figure 4A shows that during the heart stage, a narrow layer of cells surrounding the entire embryo is labeled. After the initiation of cotyledon development, this uniform pattern changed. As seen in Figure 4B, the outer epidermis of the originating cotyledons is much more heavily labeled compared with the region between the cotyledons. In addition, the endosperm is labeled. A cross-section through a stage V embryo is shown in Figure 4C. Figure 4D shows the same tissue after in situ hybridization and reveals strong labeling restricted to the outer epidermis of the cotyledons. No signal was detected in the epidermal cells covering the hypocotyl, the axis, and the inner region between and the most abaxial part of the cotyledons. An enlarged sector, provided in Figure 4E, shows more clearly the signal present in the inner cell layers of the seed coat and the endosperm and the outer epidermal cell layer of the cotyledon (right side), whereas the epidermis at the most abaxial end is not labeled. Figure 4F shows a section through a stage VII embryo. The corresponding hybridization, given in Figure 4G, reveals a faint but clear *VfSUT1*-specific signal in the most abaxial part of the cotyledonary epidermis.

During stage V, storage activity begins in the adaxial parenchyma cells of the cotyledons, thereby forming a gradient across the cotyledon. Figures 4H and 4I show stage V to VI embryos. Figure 4I shows the results of an in situ hybridization analysis using a vicilin cDNA as a probe to determine storage activity. A comparison of Figures 4D and 4I shows that the *VfSUT1*-expressing epidermis covers storage-active parenchyma. During stage VII, when the storage activity in the parenchyma finally spreads to the outermost abaxial ends (Borisjuk et al., 1995), *VfSUT1*-specific labeling can be detected in abaxial epidermal cells (Figure 4G).

In summary, the data indicate that *VfSUT1* expression in the embryo is epidermis specific. However, during the heart stage, the epidermal cell layer is evenly labeled, whereas during the cotyledon stage, a strong signal is present only in the outer epidermis and is correlated with storage activity in the underlying parenchyma.

### Hexose and Sucrose Transporters Are Differentially Expressed within Developing Seeds

Figure 5A represents a longitudinal section through the chalazal part of a stage III seed coat. The results of in situ hybridization analyses using *VfSTP1* and *VfSUT1* as probes are shown in Figures 5B and 5C, respectively. Whereas only the thin-walled parenchyma was labeled by the *VfSTP1*-specific probe (Figure 5B), a *VfSUT1*-specific signal was found in the cells of the parenchyma and the thin-walled parenchyma (Figure 5C). A section through the chalazal part of a stage V to VI seed coat is seen in Figure 5D, and the corresponding hybridizations using *VfSTP1*- and *VfSUT1*-specific probes are provided in Figures 5E and 5F, respectively. Both probes labeled the endosperm and cells of the thin-walled parenchyma in the seed coat. However, the number of cell

rows in the seed coat carrying the *VfSUT1*-specific signal is higher (Figure 5F). Figure 5G shows a control after pretreatment with RNase and in situ hybridization, using *VfSUT1* as a probe. No signal was observed. The bright fluorescence seen in the ground parenchyma layer is derived from phenolic compounds present in these cells.

Figure 5H represents an embryo at stage III. After hybridization, as shown in Figure 5I, no *VfSTP1*-specific signal could be detected, whereas the thin-walled parenchyma of the seed coat and the endosperm was labeled, indicating that *VfSTP1* is not expressed in the epidermis of the heart-stage embryo. This is in contrast to *VfSUT1*, which is expressed in the cotyledonary epidermis at stage III (Figure 4B). The abaxial part of a stage V to VI embryo is presented in Figure 5J. The hybridization pattern in Figure 5K reveals clear *VfSTP1*-specific labeling of the cotyledonary epidermis.

It is of interest that epidermal cells at the abaxial end and of the inner region of the cotyledons are predominantly labeled. This result is in contrast to the *VfSUT1* probe, which exclusively labels the outer epidermal cells in stage V to VI cotyledons (Figure 4D). No *VfSTP1*-specific label could be detected in late stage VI and stage VII embryos (data not shown).

Taken together, both transporters are expressed in the cotyledonary epidermis. However, the location and timing are different. Whereas the sucrose transporter is generally epidermis specific during the heart stage, later on it is restricted to the outer epidermis of the cotyledons covering the storage-active parenchyma. *VfSTP1* expression, on the other hand, can be detected only at midcotyledon stage and is confined to the abaxial epidermis covering the mitotically active parenchyma.

## Epidermal Transfer Cell Differentiation Is Correlated with Increased Expression of the Sucrose Transporter

We analyzed the temporal and spatial pattern of cell wall modification as a marker for transfer cell differentiation (Bonnemain et al., 1991). Figure 6A demonstrates that during the early cotyledon stage, the epidermal cells covering the shoulder of the cotyledon show asymmetrical thickening of the cell wall. Figure 6B shows the particular asymmetrical shape of the transfer cells. This shape is distinct from that of the underlying parenchyma cells. The modification spreads out and down to the abaxial epidermis, thereby accompanying the growth of the cotyledons. When the time course of cell wall modification is compared with the distribution of the *VfSUT1*-specific label (Figures 4D and 4G), a similar pattern is evident, indicating that expression of *VfSUT1* increases in epidermal cells when acquiring transfer cell morphology. The enlarged sector from the outer epidermal region of a stage VI cotyledon (shown in Figure 6C) clearly demonstrates cell wall thickening, whereas the epidermal cells covering the internal side between the cotyledons, as demonstrated in Figure 6D, and the axis (data not shown) are not modified.

Only the outer layer where transfer cell morphology develops is labeled by the *VfSUT1* probe (Figure 4D), indicating transfer cell–specific expression of the sucrose transporter. On the other hand, expression of *VfSTP1* is not specifically confined to the modified epidermis (Figures 5J and 5K).

## Epidermal Transfer Cells Develop at the Contact Zone of the Embryo and the Seed Coat

The fava bean embryo develops inside the endospermal cavity. During late heart stage, the growing embryo contacts the seed coat near the shoulders of the cotyledons. Later on, as seen in Figure 6E, the contact zone enlarges, spreading along the outer surface of the cotyledons down to the abaxial region. Although continuously decreasing in size, the endospermal vacuole is still present in the abaxial part of the seed coat up to stage VII. Only at the latest developmental stage is the entire embryo tightly attached to the seed coat, with the endospermal vacuole having disappeared (Borisjuk et al., 1995). Transfer cells start to develop in the region in which the cotyledon first contacts the seed coat, and the progressive contact between the epidermis and coat is always accompanied by wall modification of the respective epidermal cells.

The data indicate that transfer cells form in the epidermis at the seed coat contact zone. This process is coupled with a large increase in *VfSUT1* expression (Figure 4D). These observations suggest that transfer cell differentiation and *VfSUT1* expression in epidermal cells may be induced by signals coming from the maternal seed coat or elicited by tissue contact.

## High Sugar Levels Suppress Sucrose Transporter Expression and Transfer Cell Differentiation

To analyze the influence of the carbohydrate state on sugar transporter gene expression, we applied different sugar conditions to cotyledons in vitro. Cotyledons were separated and cultured in the presence of low (10 mM) or high (150 mM) sucrose concentrations. In controls, the identical osmotic conditions were achieved by adding sorbitol to the medium. Figure 7A presents quantitative data from RNA gel blot analysis and reveals that *VfSUT1* transcript levels decreased in cotyledons after we applied high concentrations of sucrose, whereas low sugar concentrations resulted in transcript levels similar to those in freshly harvested cotyledons. When glucose was used, a similar effect was achieved (data not shown), indicating that high levels of soluble sugars repress *VfSUT1* expression in cotyledons. Figure 7B shows that only *VfSUT1* mRNA levels were reduced, whereas *VfSTP1* mRNA levels decreased only slightly. Figure 7C demonstrates that vicilin mRNA levels did not change significantly after either high or low concentrations of sucrose were applied.
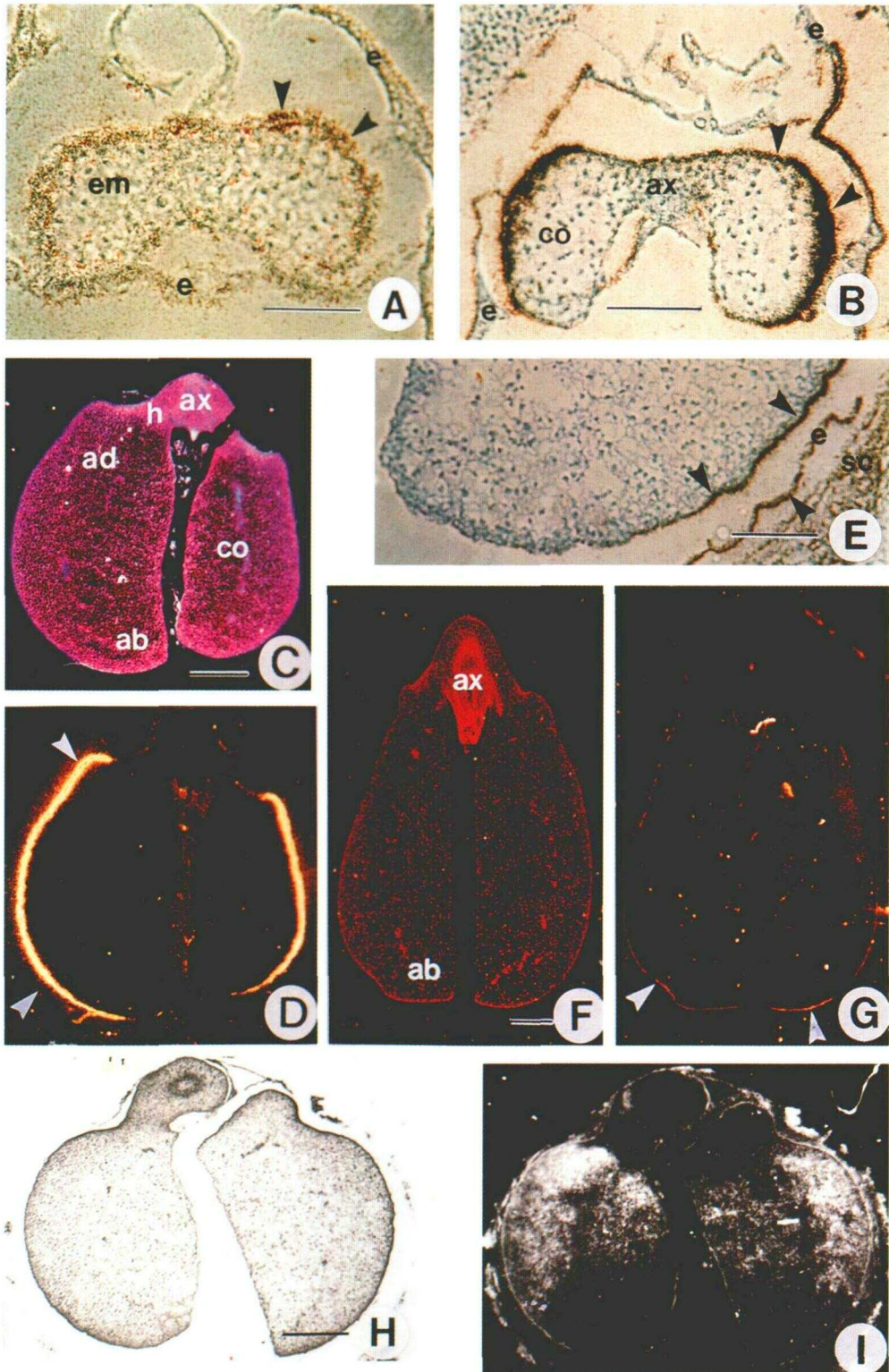
**Figure 4.** Localization of *VfSUT1* mRNA in Developing Embryos.

Sections after in situ hybridization are shown in **(A)**, **(B)**, **(D)**, **(E)**, **(G)**, and **(I)**. **(C)**, **(F)**, and **(H)** show toluidine blue–stained sections of the tissue used for **(D)**, **(G)**, and **(I)**.

The repression of *VfSUT1* mRNA levels was more dramatic in younger cotyledons. A possible reason may be that stage IV to V cotyledons are still developing their epidermal transfer cell complex expressing the *VfSUT1* gene, whereas in stage VI to VII cotyledons, transcript levels of *VfSUT1* are already decreasing in vivo (Figure 3A).

From these results we inferred an influence of high sugar concentrations on transfer cell formation and analyzed histologically the epidermal transfer cell complex after in vitro culture. Figure 8A shows that after applying high sugar concentrations, cell wall thickening in the epidermal transfer cell complex was much less pronounced than in the other cotyledon of the same embryo, shown in Figure 8B, which was fed low sucrose. This suggests that high sucrose concentrations in contrast to low ones affect the formation of transfer cell morphology.

## DISCUSSION

PCR-mediated cloning and subsequent screening of a fava bean seed-specific library revealed only one cDNA species from each class of transporters, suggesting that only one sucrose and one hexose transporter gene are expressed in developing cotyledons. VfSUT1 belongs to the group of acidic transporters and is more distantly related to the neutral transporters, which may play a role in seed development of *P. major* (Gahrtz et al., 1996). RNA gel blot analysis using as probes either the full-length cDNAs or less conserved 3′ fragments detected no differences in the RNA signal pattern, indicating that transporter genes are expressed in both seeds and leaves. In addition, DNA gel blot analysis performed under high-stringency conditions revealed only a simple pattern of one or two bands per lane (data not shown). Therefore, we conclude that under our hybridization conditions, the full-length probes are specific for only a single transcript. Accordingly, in other species in which gene fami-lies of sugar transporters have been identified, high-stringency hybridization using full-length cDNA probes has also been shown to be specific for only one member (Weig et al., 1994).

## Expression of *VfSUT1* and *VfSTP1* in Different Seed Tissues Is Developmentally Regulated

In the developing embryo, both transporters are expressed only in the epidermal cell layer and can therefore be considered as epidermal markers. During cotyledon development, *VfSUT1* expression is restricted to the outer epidermis. mRNA signals appear first in the shoulder region of the cotyledons (stage IV) and spread progressively to the outer abaxial end (stage VII), thereby accompanying cotyledon differentiation. Expression is correlated with storage activity in the underlying parenchyma, indicating an important role for storage product accumulation.

On the other hand, expression of *VfSTP1* in the epidermis could be detected only during the midcotyledon stage and is, in contrast to *VfSUT1*, confined to epidermal cells covering developmentally younger, mitotically active regions at the abaxial tip of the cotyledons (see Borisjuk et al., 1995; Figure 7A). Opposite to this region in the thin-walled parenchyma of the seed coat, a cell wall–bound invertase is expressed. At midcotyledon stage, invertase is predominantly active in the chalazal part, whereas adaxially the activity is lower (Weber et al., 1995; H. Weber and L. Borisjuk, unpublished results). The mitotically active parenchyma at the abaxial tips of the cotyledon is characterized by higher levels of hexoses, whereas adaxially in the storage-active region, mainly sucrose is present (L. Borisjuk, unpublished results). The high ratio of hexoses to sucrose is important in promoting mitotic activity (Weber et al., 1996b). Possibly the invertase-derived hexoses are taken up via VfSTP1 to supply the mitotically active parenchyma. Coordinated expression of hexose transporter and invertase has also been reported for

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**Figure 4.** (continued).

**(A)** Cross-section, seen by phase contrast microscopy, through a heart-stage embryo (stage III) after in situ hybridization. Labeling of the epidermis is indicated by arrowheads. Bar = 100 μm; magnification ×140.

**(B)** Cross-section through a stage III to IV embryo after in situ hybridization, as seen by toluidine blue staining and phase contrast microscopy. Strong labeling of the epidermis is indicated by arrowheads. Bar = 100 μm; magnification ×70.

**(C)** and **(D)** Dark-field microscopy of cross-sections through the cotyledon and hypocotyl of a stage V embryo. **(C)** shows toluidine blue staining, with the section after in situ hybridization shown in **(D)**. Labeling of the outer epidermis is indicated by arrowheads in **(D)**. Bar in **(C)** = 1 mm; magnification ×9.

**(E)** Enlargement of an abaxial section of a stage V cotyledon after in situ hybridization and toluidine blue staining. Labeling of the outer epidermis is indicated by arrowheads. Bar = 100 μm; magnification ×70.

**(F)** and **(G)** Dark-field microscopy of cross-sections through a late-cotyledon-stage embryo at stage VII. **(F)** shows toluidine blue staining, with the section following in situ hybridization shown in **(G)**. Labeling of the epidermis covering the most abaxial part of the cotyledon is indicated by arrowheads. Bar in **(F)** = 1 mm; magnification ×7.6.

**(H)** and **(I)** Cross-sections through midcotyledon-stage embryos (stage V to VI). **(H)** shows toluidine blue staining of tissue corresponding to that used in **(I)** for in situ hybridization, using the vicilin cDNA as a probe. The bar in **(H)** = 1 mm; magnification ×9.

ab, abaxial region; ad, adaxial region; ax, axis; co, cotyledon; e, endosperm; em, embryo; h, hypocotyl; sc, seed coat.
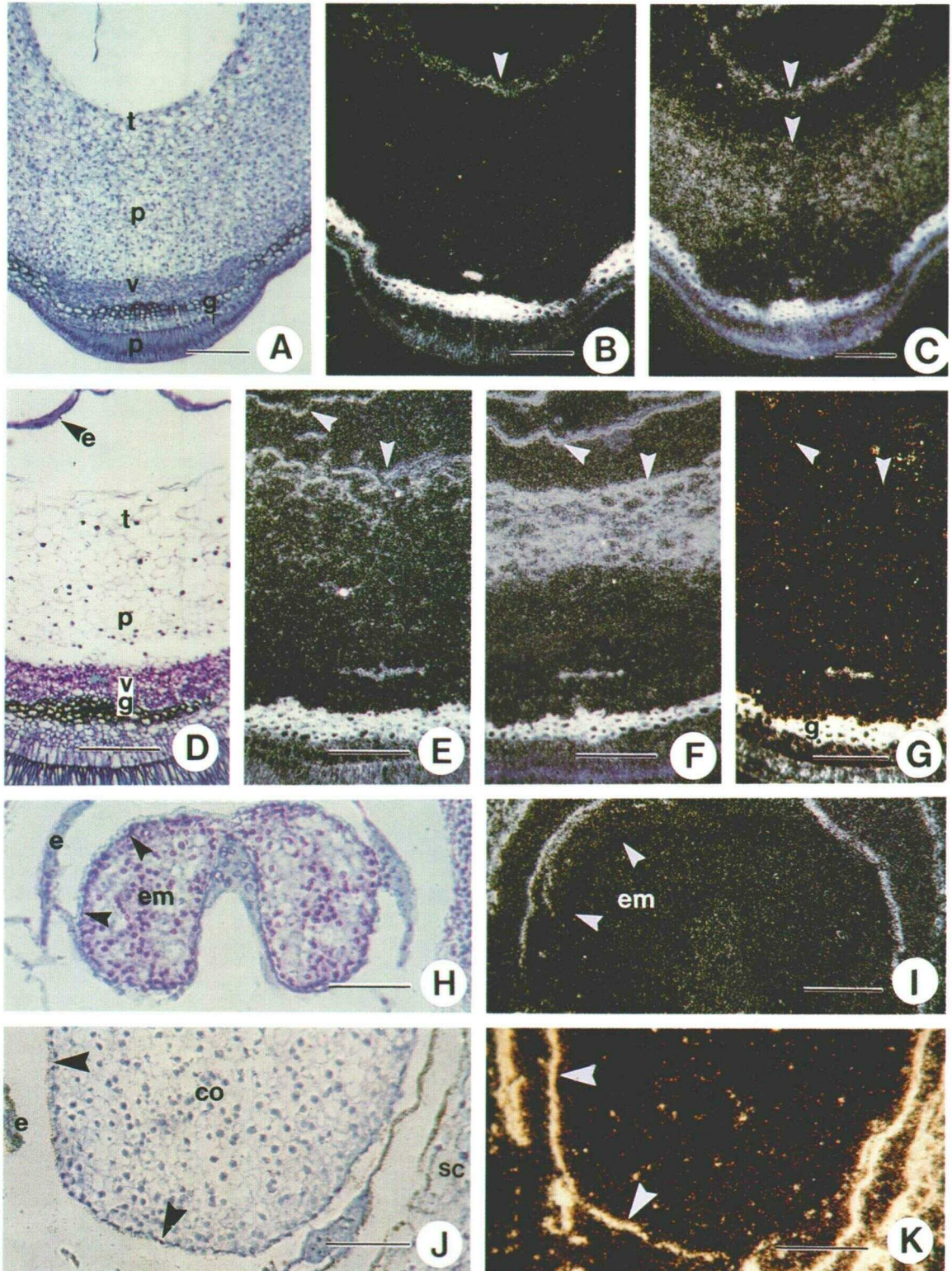
**Figure 5.** Localization of *VfSTP1* and *VfSUT1* mRNAs in Developing Seeds.

Sections after in situ hybridization are shown in **(B)**, **(C)**, **(E)**, **(F)**, **(G)**, **(I)**, and **(K)**. Sections stained with toluidine blue are shown in **(A)**, **(D)**, **(H)**, and **(J)**.

Arabidopsis, with both invertases and hexose transporters being induced during sink formation in response to wounding and infection (Sturm and Chrispeels, 1990; Truernit et al., 1996). This observation could indicate that such cooperation may generally provide sink tissues with hexoses.

Both *VfSUT1* and *VfSTP1* were found to be expressed predominantly in the thin-walled parenchyma of the seed coat during stages III to VI. There is no clear evidence for a role of the transporters in seed coat unloading. Patrick and Offler (1995) have suggested a predominantly energy-dependent process catalyzed by a putative sucrose/H+ antiporter localized in the thin-walled parenchyma. However, this hypothesis was not directly proven, and the same authors also calculated that 50% of assimilate unloading in fava bean seed coats may occur passively (Wang et al., 1995). De Jong et al. (1996) have suggested an entirely passive mechanism for pea seed coats. In this study, we provide molecular evidence that in fava bean, a sucrose/H+ symporter is expressed in the thin-walled parenchyma. This finding suggests that both active and passive processes contribute to assimilate unloading and that the respective contribution of one or the other process may change during development. The exact role of *VfSUT1* expressed in the fava bean seed coat is therefore unclear at the moment but could be approached by sense and antisense expression under the control of a seed coat–specific promoter.

Both transporters are expressed in the endosperm. However, because very little information is available on how this tissue functions during legume seed development, a detailed discussion of the functional significance is not possible. The endosperm develops until the early cotyledon stage and reaches a multinucleate, coenocytic stage. The embryo, which develops inside the endospermal cavity, is covered by the cytoplasmic strands of the endosperm (Borisjuk et al., 1995; Panitz et al., 1995). Sugars transferred to the embryo must therefore pass the endosperm—a process that may require a transport system.

## *VfSUT1* Is a Marker of Embryonic Epidermal Transfer Cells

In fava bean, the epidermal cell layer of the embryo is established during the globular stage. Cells divide anticlinally and form a separate layer distinct from the parenchyma (Borisjuk et al., 1995). During fava bean cotyledon formation (stage IV), epidermal cells in the outer region facing the seed coat differentiate into transfer cells. This modification enlarges the transport-active surface area and occurs at the beginning of the phase of rapid fresh weight gain (Offler et al., 1989; Bonnemain et al., 1991). Transfer cell development is correlated with strong hyperpolarization of the outer membrane (Bonnemain et al., 1991) due to the presence and activity of H+-ATPases (Bouché-Pillon et al., 1994). We report here that transfer cell formation in fava bean cotyledons is correlated with strong *VfSUT1* expression (Figures 4 and 6).

During cotyledon development, an effective sucrose uptake system is established in the outer cotyledonary epidermis to provide the necessary substrate for storage product synthesis in the cotyledons. The uptake system is characterized by (1) amplification of the surface area of the plasmalemma membrane, (2) hyperpolarization of the membrane, and (3) strong expression of a sucrose transporter gene. Thus, *VfSUT1* expression is a marker for cotyledonary transfer cells and accompanies their differentiation.

## Transfer Cell Formation and *VfSUT1* Expression Are Induced by Carbohydrate Availability during Epidermal Contact of the Growing Embryo with the Seed Coat

The sucrose uptake system develops during the switch to the storage phase and is probably a prerequisite to render cotyledon development independent from the invertase-mediated unloading process that provides the sink strength

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**Figure 5.** (continued).

**(A)** to **(C)** Cross-sections through a stage III seed coat. A toluidine blue–stained section is shown in **(A)**, and dark-field microscopy after in situ hybridization using *VfSTP1* and *VfSUT1* as probes is shown in **(B)** and **(C)**, respectively. In **(B)**, labeling of the inner cell layer of the seed coat is indicated by an arrowhead. Labeling of the seed coat cell layer in **(C)** is marked by arrowheads.

**(D)** to **(G)** Cross-sections through a stage V to VI seed coat. A toluidine blue–stained section is shown in **(D)**, and dark-field microscopy after in situ hybridization using *VfSTP1* and *VfSUT1* as probes is depicted in **(E)** and **(F)**, respectively. Dark-field microscopy after RNase treatment and in situ hybridization using *VfSUT1* as a probe is shown in **(G)**. The arrowhead in **(D)** marks the endosperm. Labeling of the endosperm and the inner layer of the seed coat in **(E)** is indicated by arrowheads, and labeling of the endosperm and seed coat in **(F)** is indicated by arrowheads. In **(G)**, the positions of the endosperm and inner layer are shown by arrowheads, with no labeling being visible.

**(H)** and **(I)** Cross-sections through a late-heart-stage cotyledon. Toluidine blue staining is shown in **(H)**, and dark-field microscopy after in situ hybridization using *VfSTP1* as a probe is depicted in **(I)**. The arrowheads in **(H)** and **(I)** indicate the outer epidermis of the cotyledon. No labeling of this tissue is visible in **(I)**.

**(J)** and **(K)** Cross-sections through the abaxial part of a stage V to VI cotyledon. **(J)** shows toluidine blue–stained tissue, and **(K)** shows dark-field microscopy after in situ hybridization using *VfSTP1* as a probe. In **(J)**, arrowheads indicate the inner epidermis (left) and the abaxial epidermis (right) of the cotyledon. Labeling of the inner and abaxial epidermis is also shown by arrowheads in **(K)**.

co, cotyledon; e, endosperm; em, embryo; g, ground parenchyma; p, parenchyma; sc, seed coat; t, thin-walled parenchyma; v, vein. Bars = 200 µm; magnifications in **(A)** to **(C)** are ×52; **(D)** to **(G)**, ×55; **(H)** to **(K)**, ×64.
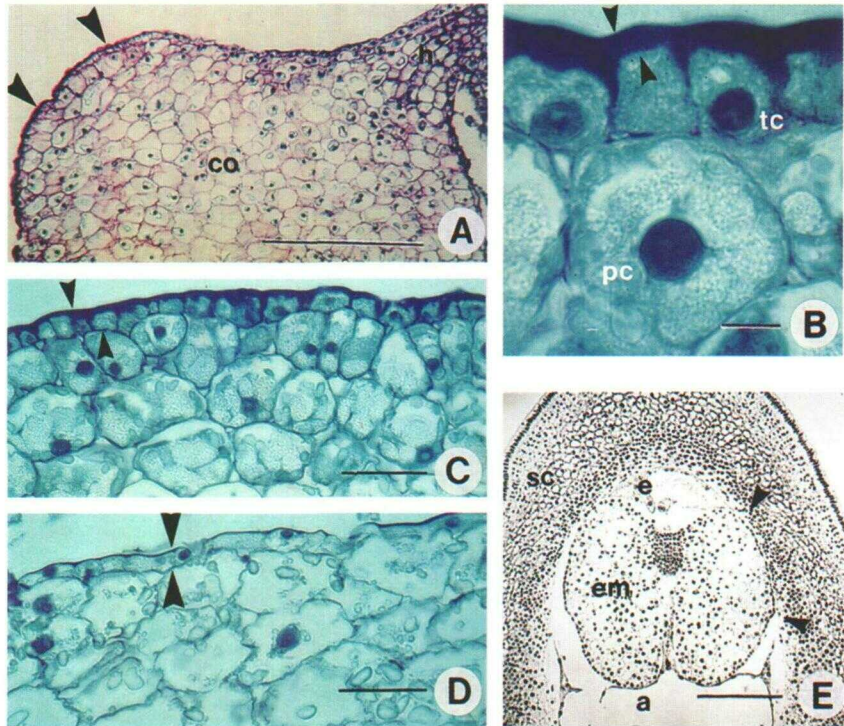
**Figure 6.** Transfer Cell Differentiation during Embryo Development.

**(A)** Cross-section through the adaxial part of a stage IV embryo. Arrowheads indicate cell wall thickening. Bar = 100 μm; magnification ×270.
**(B)** Enlargement of the outer epidermal region of a stage V embryo. Arrowheads indicate wall thickening of the epidermal cell layer. Bar = 10 μm; magnification ×1000.
**(C)** Enlargement of the outer epidermal region, marked by arrowheads, of a stage V embryo. Bar = 50 μm; magnification ×250.
**(D)** Enlargement of the inner epidermal region, marked by arrowheads, of a stage V embryo. Bar = 50 μm; magnification ×250.
**(E)** Cross-section through a stage IV seed after toluidine blue staining. Arrowheads indicate the contact zone between the epidermis and the seed coat. Bar = 500 μm; magnification ×30.
a, apoplast; co, cotyledon; e, endosperm; em, embryo; h, hypocotyl; pc, parenchyma cell; sc, seed coat; tc, transfer cell.

during the prestorage phase (Weber et al., 1995, 1996b). Our data show that transfer cell formation (Figure 6) and expression of *VfSUT1* (Figure 4) parallel the progressive contact between the epidermis and the seed coat during cotyledon growth (Figure 6). Possibly, the epidermal contact with the seed coat provides a signal for transfer cell differentiation. An interesting analogy can be drawn from studies of pathogen–plant interactions. The parasites Orobanche and Cuscuta retrieve sugars from their hosts, probably by establishing a sucrose uptake system involving transfer cells. In both parasites, transfer cells form only after contact with the host vascular tissue (Ayres et al., 1996).

Applying high sugar concentrations to explanted cotyledons disturbed transfer cell morphology and suppressed *VfSUT1* expression, whereas low concentrations of sugars maintained normal development and *VfSUT1* transcript levels. Possibly, a sucrose transporter–transfer cell complex can develop only when the exogenous sugar concentrations

are low, indicating control by sugar availability. Such a response is a widespread phenomenon (reviewed in Koch, 1996). Carbohydrate depletion can up- or downregulate different glucose transporters in yeast (Özcan and Johnston, 1995) and Chlorella (Stadler et al., 1995b). A role for sugars in regulating transfer cell development in fava bean has already been suggested by Liet and Offler (1996). These authors found that differentiation was selectively suppressed in cotyledons cultured with high sucrose concentrations. In the endospermal cavity of legume seeds, sugar concentrations of 5 to 200 mM were found (De Jong et al., 1996). Our own measurements with fava bean consistently showed a high concentration of sugar (∼100 mM) in the endospermal cavity (H. Weber and L. Borisjuk, unpublished results). This may explain why epidermal cells adjacent to and in contact with the endospermal cavity never show transfer cell morphology. In contrast, high sugar concentrations may be reduced in the region where contact is tight between the seed coat and the

cotyledonary epidermis, thus establishing the sucrose trans-porter–transfer cell complex. In pea, during the seed filling phase, a sucrose concentration of ~15 mM was suggested in the apoplast. This concentration is kept low by an active H+/sucrose symporter in the cotyledonary cells and is needed to drive passive seed coat unloading (De Jong et al., 1996).

It has recently been shown that sugar transporters can be involved in sugar sensing in yeast (Özcan et al., 1996). Al-though evidence of such a role for VfSTP1 and VfSUC1 is missing, we have shown that VfSTP1 and VfSUT1 are highly homologous to other plant transporters responsible for sugar uptake. Therefore, a possible involvement in sugar sensing cannot be excluded either for VfSTP1 and VfSUT1 or for any plant sugar transporter described to date.
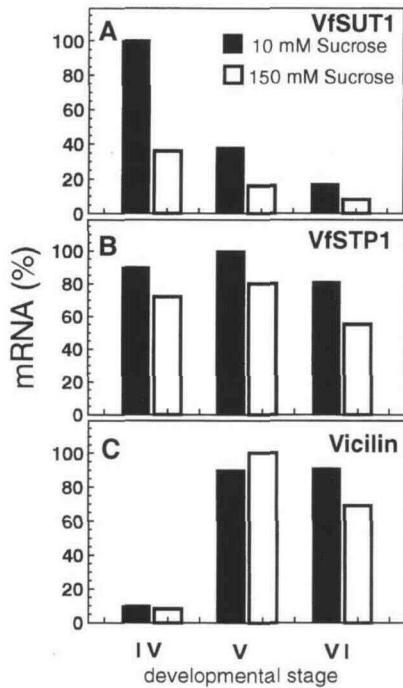


**Figure 8.** Transfer Cell Morphology after Feeding with Low or High Sugar Concentrations.

Cotyledons of a stage V embryo were separated and cultured in vitro for 3 days in 150 and 10 mM sucrose, respectively. Sections were stained with toluidine blue.

**(A)** Cross-section through the outer epidermal region of a stage V cotyledon after applying 150 mM sucrose in vitro.
**(B)** Section through the other cotyledon cultured in 10 mM sucrose. The epidermal transfer cell complex is shown by arrowheads.
ep, epidermis; sp, storage parenchyma. Bars = 50 μm; magnifica-tion ×188.



**Figure 7.** Transcript Analysis of VfSUT1, VfSTP1, and Vicilin after Feeding with Low or High Sugar Concentrations.

**(A)** Accumulation of VfSUT1 mRNA in cotyledons of stage IV, V, and VI embryos after applying 10 mM sucrose to one and 150 mM su-crose to the other cotyledon of the same seed in vitro. Hybridization signals from RNA gel blot analysis were quantified using a Phosphor-Imager.
**(B)** Accumulation of VfSTP1 mRNA detected on the same blot.
**(C)** Accumulation of vicilin mRNA detected after a third round of hy-bridization.
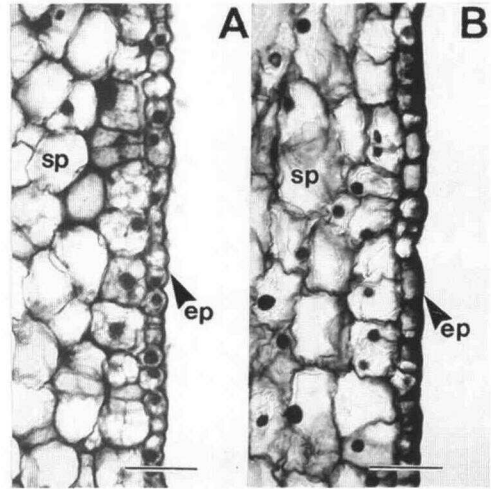Experiments were repeated twice. Representative data from a single experiment are shown.

## METHODS

### Plant Material

Fava bean (Vicia faba var minor cv Fribo; Genbank, IPK) was grown in growth chambers under a light-and-dark regime of 16 hr of light and 8 hr of dark at 20°C.

### RNA Isolation and Hybridization Techniques

RNA was isolated and hybridized as described in Heim et al. (1993). The following cDNA fragments were used as probes after labeling with $^{32}$P-dCTP, according to Feinberg and Vogelstein (1983): a 1672-bp fragment encoding the full-length cDNA of the hexose transporter (VfSTP1), a 2004-bp cDNA fragment encoding the sucrose trans-porter (VfSUT1), a 510-bp cDNA fragment encoding the 3' end of VfSUT1 (nucleotides 1481 to 1991), a 268-bp cDNA fragment encod-ing the 3' end of VfSTP1 (nucleotides 1385 to 1653), and a 500-bp PstI fragment from pVfc28 containing a fava bean vicilin sequence (Bassüner et al., 1987). In situ hybridizations were performed under high-stringency conditions, using full-length cDNA probes as de-scribed in Borisjuk et al. (1995). Hybridization signals were quantified as described in Weber et al. (1996b) and are given in relative units.

## Polymerase Chain Reaction–Mediated Cloning of Fava Bean Sugar Transporters

The following primers were deduced from conserved regions of transporter cDNAs (EMBL data library). Sucrose transporter primer I, 5'-GCIGCIGG/TICAA/GTTC/TGGITGGGC-3'; sucrose transporter primer II, 5'-CCCATCCAA/GCTIGTA/GCT-3'; hexose transporter primer I, 5'-GGA/TTTC/TGCA/TTGGTCA/TTGGGGA/TCC-3'; and hexose transporter primer II, 5'-A/TGGA/G/TATA/TCCC/TTTA/G/TGTC/TTCA/TGG-3'. Subsequent hot start polymerase chain reaction (PCR) was performed using 1.5 mM $MgCl_2$, 0.4 $\mu$M of each primer, and an aliquot of a cDNA library ($\lambda$ ZAP Express; Stratagene, La Jolla, CA) specific for stage V to VI cotyledons (T. Wohlfahrt, IPK). The temperature regime was 98°C for 5 min (50°C for 0.5 min, 72°C for 0.5 min, and 94°C for 0.5 min) for 30 cycles and 72°C for 10 min. Amplified DNA bands of ~780 and ~280 bp were subcloned after using the primer combinations of sucrose I and II and hexose I and II, respectively. Homologies of the PCR fragments with the sucrose and hexose transporter cDNAs were confirmed by sequencing. Fragments were subsequently used to screen the cotyledon-specific cDNA library under high-stringency conditions. Three and two positive plaques representing the VfSUT1 and VfSTP1, respectively, were isolated, and inserts were subcloned into plasmids. Sequencing and data analysis were performed as described by Heim et al. (1993). The cDNA sequences of the sucrose transporter (VfSUT1) and the hexose transporter (VfSTP1) have EMBL, GenBank, and DDBJ accession numbers Z93774 and Z93775, respectively.

## Heterologous Expression in Yeast

The VfSTP1 cDNA was cloned in sense and antisense orientations into the Schizosaccharomyces pombe/Escherichia coli shuttle vector SAP-E (Truernit et al., 1996). The resulting constructs were used to transform S. pombe YGS-5 (Milbradt and Höfer, 1994) by the method of Ito et al. (1983), with the strains VFY-HS (sense orientation) and VFY-HA (antisense orientation) being produced.

The VfSUT1 cDNA was cloned in sense and antisense orientations into the Saccharomyces cerevisiae/E. coli shuttle vector NEV-E (Sauer and Stolz, 1994). The resulting constructs were used to transform S. cerevisiae SEY2102 (Emr et al., 1983) by the method of Gietz et al. (1992), with the strains VFY-SS (sense orientation) and VFY-SA (antisense orientation) being produced.

Transport tests with transgenic yeast strains were performed in 50 mM sodium phosphate buffer (Sauer et al., 1990a). All transport tests with VFY-HS or VFY-HA cells were performed in the presence of 120 mM ethanol to guarantee optimal energization of the plasma membrane. For the same reason, all transport tests with VFY-SS and VFY-SA cells were performed in the presence of 10 mM D-glucose. Relative affinities of VfSTP1 for various potential hexoses were determined at initial substrate concentrations of 100 $\mu$M. $K_m$ values for sucrose of VfSUT1 were determined at pH 4.5 and 6.0.

## Fixation, Sectioning, and Histochemical Staining

Seeds or parts of seeds were fixed in 2.5% glutaraldehyde and 50 mM sodium cacodylate, pH 7, or 4% (w/v) paraformaldehyde and 50 mM potassium phosphate buffer, pH 7.0, under a slight vacuum for 4 hr at room temperature, rinsed in cacodylate buffer, dehydrated, and embedded in Paraplast Plus (Sherwood Medical, St. Louis, MO). Sections were cut at 1 to 10 $\mu$m on a microtome, transferred to poly-

L-lysine–treated slides (Sigma), and dried overnight at 45°C. Staining was performed with toluidine blue, according to Gerlach (1977) and Carson (1990).

## In Vitro Culture of Cotyledons

Cotyledons were cultured as described by Weber et al. (1995) in a medium, according to Millerd et al. (1975), containing 70 mM L-glutamine, 0.1 mg/L benzyladenine, and 0.02 mg/L naphthaleneacetic acid. Sugars were supplemented as indicated. After 3 days under dim light at 21°C, the cotyledons were fixed, embedded, and sectioned.

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