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Seed Coat-Associated Invertases of Fava Bean Control Both Unloading and Storage Functions: Cloning of cDNAs and Cell Type-Specific Expression

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We have studied the molecular physiology of photosynthate unloading and partitioning during seed development of fava bean (*Vicia faba*). During the prestorage phase, high levels of hexoses in the cotyledons and the apoplastic endospermal space are correlated with activity of cell wall-bound invertase in the seed coat. Three cDNAs were cloned. Sequence comparison revealed genes putatively encoding one soluble and two cell wall-bound isoforms of invertase. Expression was studied in different organs and tissues of developing seeds by RNA gel analysis, in situ hybridization, enzyme assay, and enzyme activity staining. One extracellular invertase gene is expressed during the prestorage phase in the thin-walled parenchyma of the seed coat, a region known to be the site of photoassimilate unloading. We propose a model for an invertase-mediated unloading process during early seed development and the regulation of cotyledonary sucrose metabolism. After unloading from the seed coat, sucrose is hydrolyzed by cell wall-bound invertases. Thus, invertase contributes to establish sink strength in young seeds. The resultant hexoses are loaded into the cotyledons and control carbohydrate partitioning via an influence on the sucrose synthase/sucrose-phosphate synthase pathway. The developmentally regulated degradation of the thin-walled parenchyma expressing the invertase apparently initiates the storage phase. This is characterized by a switch to a low sucrose/hexoses ratio. Feeding hexoses to storage-phase cotyledons in vitro increases the sucrose-phosphate synthase/sucrose synthase ratio and changes carbohydrate partitioning in favor of sucrose. Concomitantly, the transcript level of the major storage product legumin B is downregulated.

INTRODUCTION

Sucrose plays an important role in the metabolism of plants. In many species, it is the main transport metabolite for carbohydrates. Sink organs that do not perform photosynthesis are dependent on its import. Sucrose hydrolysis is the first step toward either metabolism or storage product synthesis. Two enzymes are involved in the cleavage of sucrose: sucrose synthase and invertase. In the same sink organ, a polarity is often found in the distribution of these enzymes, signifying their different roles in regulating sugar metabolism (Ho et al., 1991). A change in activity of sucrose synthase and invertase is correlated with certain sink functions, such as import, cell division, and storage (Sung et al., 1994). Sucrose synthase catalyzing a reversible reaction (Geigenberger and Stitt, 1993) is associated with storage functions, such as starch synthesis (Heim et al., 1993). On the other hand, invertases (β-fructofuranosidases; EC 3.2.1.26) catalyze the irreversible cleavage of sucrose into glucose and fructose. In storage tissues, they have been correlated with developmental processes having their highest activity before storage processes start (Eschrich, 1980). Invertases can appear in different isoforms inside the same tissue: a nonglycosylated form, characterized by an alkaline pH optimum, is active in the cytosol (Stommel and Simon, 1990), whereas the highly glycosylated acid invertases with a pH optimum of ~4.5 either occur in soluble form inside the vacuole or are tightly bound to the cell wall (Sturm and Chrispeels, 1990).

Soluble acid invertases are often present in young seedlings and roots (Ricardo and ap Rees, 1970). They are involved in fruit ripening (Elliott et al., 1993) and are thought to regulate hexose levels in mature tissues and the mobilization of sucrose stored in vacuoles. The cell wall–bound isoforms are active in growing zones and extending tissues, such as root tips, internodes, and developing leaves. They are involved in phloem unloading and sucrose partitioning (Eschrich, 1989), osmoregulation (Meyer and Boyer, 1981), gravitropism (Wu et al., 1993), and wound response (Matsushita and Uritani, 1974). In sink organs, they can help to maintain sink strength, facilitating unloading by increasing the concentration gradient of sucrose between sink and source and lowering the water potential of the free space (Eschrich, 1989). They must play an important role in seed development, at least in maize, because the invertase-deficient *miniature1* seed mutation is associated with early embryo degeneration (Miller and Chourey, 1992).

A number of genes and cDNAs coding for vacuolar and cell wall–bound invertases have been cloned from different species

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(Unger et al., 1994). The sequences are characterized by N-terminal signals responsible for either vacuolar or apoplastic targeting. The two isoforms seem to be encoded by different small gene families. The mature proteins from both forms contain two conserved domains, but the bound form from carrot is more related to the bound form from potato than to the soluble form of carrot (Unger et al., 1994).

To understand the molecular physiology of seed development and storage product synthesis of fava bean (*Vicia faba*), we analyzed carbohydrate and storage protein metabolism in seeds. During development, the change from the cell division to the cell expansion phase at ~25 days after fertilization (DAF) is accompanied by a rapid synthesis of large amounts of both starch and storage proteins in the cotyledons (Borisjuk et al., 1995). During the storage phase of seed development, sucrose is transported symplastically through the seed coat (Patrick et al., 1995), where it is unloaded by an apoplastic step into the endospermal space (Wang et al., 1995). After being taken up by the embryo, sucrose is broken down by a sucrose synthase–specific pathway. Invertases play no important overall role in the cotyledons of fava bean (de Fekete, 1969) and pea (Edwards and ap Rees, 1986). However, an analysis of free sugars in different organs of the seed during the prestorage phase revealed high levels of hexoses in the endospermal space and in the embryo, probably due to cell wall–bound invertases in the maternal seed coat.

We cloned cDNAs encoding two bound invertases and one vacuolar invertase defined by sequence comparison and analyzed their expression by RNA gel blot and in situ hybridization. Analyses of sugars and the activities of invertases, sucrose synthase, and sucrose-phosphate synthase as well as transcript analysis of storage proteins were performed with the seed throughout development. The results suggest an important role of invertases for unloading, carbohydrate partitioning, and control of storage functions during seed development.

RESULTS

Ratios of Free Sugars Differ in Seed Organs during Development

During seed filling, photoassimilates are transported as sucrose via the phloem to the seed. The sieve elements end up in the seed coat, where sucrose is unloaded by an apoplastic step because there is no symplastic connection between the seed coat and the embryo. To analyze the photosynthate transfer in the developing seed, we measured the amounts of sucrose, glucose, and fructose in the seed coat, apoplastic endospermal space, and cotyledons of particular seeds on a fresh weight basis. Figure 1 shows that at 18 DAF, levels of sucrose were high in the seed coat and considerably lower in the apoplastic space and the cotyledons (72, 34, and 59 μmol/g fresh weight of tissue, respectively). On the other hand, levels of hexoses were low in the seed coat but dominated in the apoplastic space and the cotyledons (10, 76, and 82 μmol/g fresh weight of tissue or solute, respectively).

The results suggest a concentration gradient for sucrose between seed coat and cotyledons. The high levels of hexoses may result either from a rapid and selective uptake of sucrose by the cotyledons or more likely from a sucrose-degrading activity in the apoplastic space.

High Levels of Hexoses in the Cotyledons Correspond to Seed Coat-Associated Invertase

To analyze the sucrose-degrading activity of the seed coat and the cotyledons, we measured invertase in cell wall extracts at different stages of development. Figure 2A demonstrates that considerable invertase activity was found only in the cell wall fraction derived from the seed coat and not in the cotyledons. The activity increased from 12 DAF, reached the highest levels at ~15 to 22 DAF, and decreased to low levels between 30 and 40 DAF (Figure 2A). The optimal pH was 4.5, and the activity could not be released from the cell wall even after washing with 1 M NaCl overnight. These findings suggest the presence of cell wall–bound invertase in the seed coat. An analysis of the amount of free hexoses in the cotyledons and seed coat is given in Figure 2B and shows that levels were high in the prestorage phase cotyledons but low in the seed coat. Levels of sucrose, shown in Figure 2C, were low in the cotyledons of the prestorage phase and higher in the seed coat but increased dramatically in the cotyledons during the storage phase.

The results suggest that during early development, sucrose is cleaved after unloading from the seed coat into the apoplastic space by cell wall–bound invertase and that the resulting hexoses are taken up by the cotyledons. The early embryo itself seems not to be involved in sucrose cleavage because we have

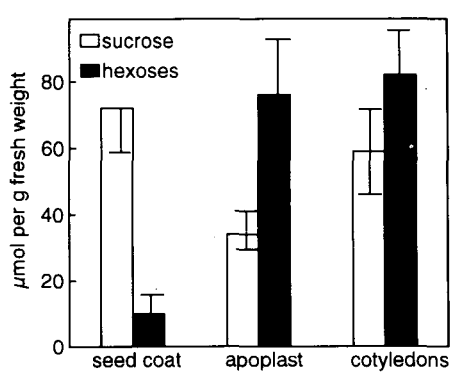

Figure 1. Free Sugars in Different Tissues of Prestorage Phase Seed.

Levels of sucrose and hexoses (glucose and fructose) were determined in the seed coat, the apoplastic endospermal space, and the cotyledons of particular seed of fava bean at 18 DAF. Values are means ± SD of three analyses.

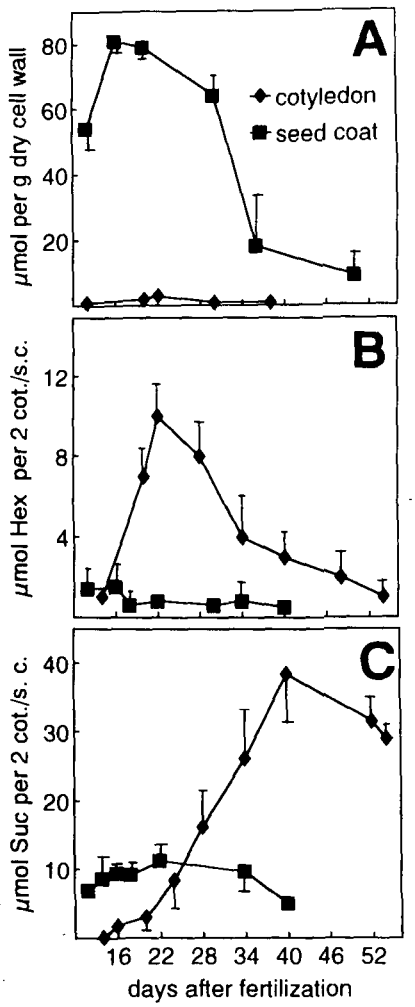

Figure 2. Cell Wall-Bound Invertase Activities and Free Sugars in Cotyledons and Seed Coats during Development.

(A) Cell wall-bound invertase, given in micromoles of glucose per gram of dry cell wall material per minute, was determined in the cotyledons and the respective seed coat during development. Values are means ± SD of at least three replicate experiments.

(B) and **(C)** Levels of hexose (Hex; sum of glucose and fructose) and sucrose (Suc) were determined in two cotyledons (cot.) as well as in the respective seed coats (s.c.). Values are means ± SD of at least three replicate experiments.

shown previously (Heim et al., 1993) that sucrose synthase is low before 20 DAF.

cDNA Cloning Reveals Three Different Invertase Genes Expressed in the Seed Coat

To isolate the cDNA of fava bean seed coat invertase, degenerate DNA primers were designed from conserved domains of known invertases (Sturm and Chrispeels, 1990), and polymerase chain reaction (PCR) was performed using as template double-stranded cDNA specific for seed coats at 10 to 20 DAF. Amplified DNA bands of ~560 bp were subcloned and sequenced. A comparison at the nucleotide level with the corresponding fragments of the cell wall-bound (Sturm and Chrispeels, 1990) and the soluble invertase (Unger et al., 1994) form of carrot revealed a higher identity of two sequences to the cell wall-bound form (69 and 67% versus 59%), whereas the third sequence was more related to the soluble form (57 and 61% versus 71% identity, respectively).

We therefore designated these sequences VfCWINV1 (for *V. faba* cell wall-bound invertase), VfCWINV2, and VfVCINV (for *V. faba* vacuolar invertase). The PCR fragments were used to screen a cDNA library specific for 10- to 20-DAF seed coats. We characterized three, four, and one positive clone, using VfCWINV1, VfCWINV2, and VfVCINV fragments, respectively, as the probes. Each isoform was found to be represented by one single open reading frame of 1725, 1743, and 1908 bp encoding 575, 581, and 636 amino acid residues, respectively. The deduced amino acid sequences of VfCWINV1 and VfCWINV2 were 50.6% identical, whereas the VfVCINV sequence was only 34 and 41% identical to VfCWINV1 and VfCWINV2, respectively. The calculated isoelectric points of the deduced proteins were 9.78, 8.08, and 5.48, respectively. The sequence of VfVCINV contained an N-terminal extension of 54 and a small C-terminal extension of seven amino acid residues, indicating a soluble isoform (Unger et al., 1994). All three putative amino acid sequences contain the invertase motif NDPNG as well as the putative catalytic domain WEC(P/V)D. The cell wall-bound isoforms, including VfCWINV1 and VfCWINV2, described to date contain a Pro residue at the fourth position, whereas all soluble forms, including VfVCINV, contain a Val residue at that position. The putative amino acid sequences were aligned to cell wall-bound isoforms of Arabidopsis (ATBSF1 and ATBSF2), *Chenopodium rubrum* (CRCIN1), carrot (DCINVA and DCINC1), tobacco (NTCWINV), and potato (STBFRCA) as well as to the soluble isoforms of carrot (DCSBFRU and DCABF), mung bean (VRINVA), tomato (LEINV1), potato (STBFS1), and maize (ZMIVR1) (see legend to Figure 3 for full protein names). A dendrogram of the alignment, shown in Figure 3, grouped all invertases into two clusters according to their origin from either the cell wall or the vacuolar compartment. Interestingly, the group including the only experimentally proven cell wall-bound isoform (DCINVA) is composed of two clearly separated subgroups (Unger et al., 1994). VfCWINV1 and VfCWINV2 grouped into each of the two subgroups of cell wall-bound forms, whereas VfVCINV classified as a vacuolar form.

Cell Wall-Bound Invertase VfCWINV1 Is Specifically Expressed in Seed Coats after Fertilization

To analyze the expression pattern of the invertase genes of fava bean in different organs of the seed during development and in other parts of the plant, the cDNAs were used as probes

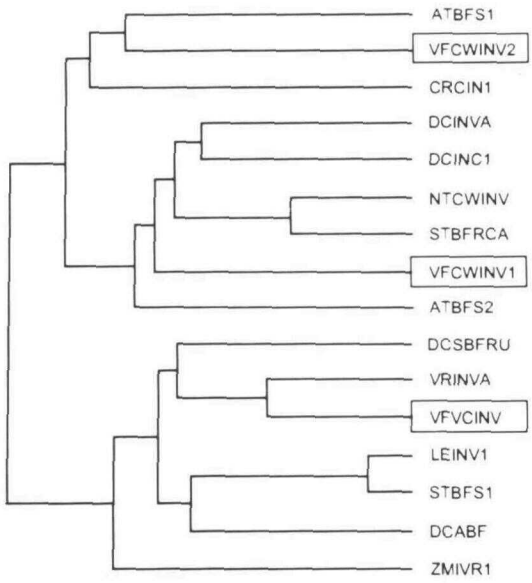

Figure 3. Comparison of Invertases from the Cell Wall and the Vacuolar Compartment.

A dendrogram of the alignment of cell wall–bound and soluble vacuolar invertases is shown. The putative amino acid sequences of VfCWINV1, VfCWINV2, and VfVCINV were aligned with the corresponding cell wall–bound isoforms as follows (GenBank/EMBL/DDBJ accession numbers are also provided): Arabidopsis (ATBSF1, accession number X74515; and ATBSF2, U11033), carrot (DCINVA, P26792; and DCINC1, X78424), tobacco (NTCWINV, X81834), and potato (STBFRCA, Z22645). The fava bean sequences were also aligned with the soluble isoforms of carrot (DCSBFRU, X67163; and DCABF, X75352), mung bean (VRINVA, P29001), tomato (LEINV1, P29000), potato (STBFS1, L29099), and maize (ZMIVR1, U16123). Alignments were performed using the CLUSTAL program of PCGENE version 6.8 software package (IntelliGenetics, Mountain View, CA). ATBSF 1 and ATBSF2, *Arabidopsis thaliana* β-fructosidases 1 and 2; CRCIN1, *Chenopodium rubrum* cell wall–bound invertase; DCABF, *Daucus carota* soluble acid β-fructosidase; DCINC1, *D. carota* cell wall–bound invertase 1; DCINVA, *D. carota* invertase; DCSBFRU, *D. carota* soluble fructosidase; LEINV1, *Lycopersicum esculentum* acid invertase; NTCWINV, *Nicotiana tabacum* cell wall–bound invertase; STBFRCA, *Solanum tuberosum* cell wall–bound fructosidase A; STBFS1, *S. tuberosum* acid fructosidase 1; VRINVA, *Vigna radiata* acid invertase; ZMIVR1, *Zea mays* soluble invertase.

in RNA gel blot experiments. Figure 4 shows that mRNA of VfCWINV1 was found only in developing anthers and seed coats. In the seed coat, levels increased from 8 DAF, reaching a peak at ~13 DAF, and decreased afterward. VfCWINV2 mRNA was present in gynoecia, pods, seed coats, and roots. No mRNA of any cell wall–bound isoform was detected in cotyledons during development and 6 days after germination. VfVCINV mRNA was present in anthers, gynoecia, pods, and seed coats at 13 to 17 DAF, in cotyledons at 15 DAF, and in stems and roots.

The data indicate that VfCWINV1 is expressed in anthers and seed coats after fertilization. The isoform VfCWINV2 reveals a broader specificity, also being expressed in vegetative parts of the plant. Equally, VfVCINV is active in both seeds and vegetative parts.

Different organs of flowers and developing seeds were assayed for soluble acid invertase in crude extracts and for the bound isoform in cell wall extracts. The results are given in Table 1 in nanomoles of glucose per gram fresh weight of tissue. Activity of both soluble and cell wall invertases was present in developing anthers and gynoecia as well as in pods. However, in seed coats at 8 and 15 DAF, activity was detected nearly exclusively in the cell wall fraction. No cell wall–bound invertase was present in cotyledons at either 18 or 25 DAF, but a low amount of the soluble form was measured at 18 DAF originating from contaminating endosperm (see below).

VfCWINV1 Is Expressed in a Seed Coat Cell Layer Involved in Assimilate Unloading

Seed coat anatomy of fava bean and the pathway of photosynthate transfer have been described in detail by Offler et al. (1989). The seed coat consists of three layers: the single cell–layered palisade and hypodermis and the underlying parenchymatous tissue in which the vascular system is embedded. The parenchymatous tissue is composed of chlorenchyma, ground parenchyma, and thin-walled parenchyma. Ground parenchyma cells are characterized by deposits of probably phenolic composition. The vascular system separates the ground parenchyma from the thin-walled parenchyma. The latter consists of a number of cell rows covering the entire inner surface of the seed coat. During seed coat maturation, the number of cell rows is reduced probably due to crushing by the enlarging cotyledons. Photosynthates enter the seed coat through the funiculus into one chalazal and two lateral veins. Symplastic transfer toward the embryo is either radially through the vascular to the thin-walled parenchyma or laterally through ground and thin-walled parenchyma. The thin-walled parenchyma is thought to be the site of unloading into the apoplastic space. Differentiation into transfer cells supports its important

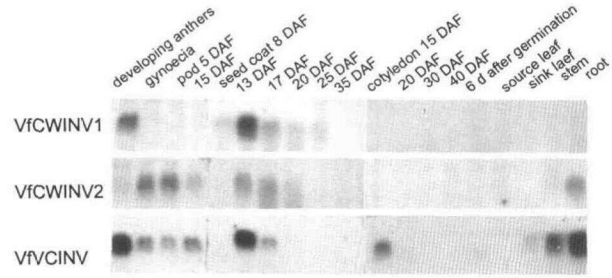

Figure 4. Transcript Analysis of Fava Bean Invertases.

The steady state levels of mRNA from invertases were analyzed in different tissues of the seed and other vegetative parts of the plant. Total RNA was analyzed on gel blots using the PCR products of fava bean invertases as probes.

Table 1. Enzyme Activity of Cell Wall–Bound and Soluble Acid Invertases in Various Seed Tissues of Fava Bean

Tissue	Invertase Activity[a] (nmol Glucose per g Fresh Weight per Min)	
	Cell Wall–Bound Invertase	Soluble Acid Invertase
Developing anthers	2.7 ± 0.9	3.6 ± 1.2
Gynoecia	0.77 ± 0.2	1.56 ± 0.05
Pods 5 DAF	0.47 ± 0.31	0.73 ± 0.13
Pods 8 DAF	0.38 ± 0.18	0.99 ± 0.27
Pods 10 DAF	0.38 ± 0.1	1.55 ± 0.15
Pods 20 DAF	0.09 ± 0.01	0.45 ± 0.13
Seed coats 8 DAF	1.7 ± 0.35	>0.01
Seed coats 15 DAF	1.17 ± 0.3	>0.01
Embryos 18 DAF	>0.01	0.13 ± 0.05
Embryos 25 DAF	>0.01	>0.01

[a] Values are means ± SD of at least three replicate experiments.

role for membrane exchange of photosynthates (Offler and Patrick, 1993).

To analyze the role of invertases in photosynthate transport and unloading with respect to different cell layers of the seed coat, we used the cloned PCR products as probes for in situ hybridization with sections of seeds from different developmental stages. In situ hybridization results are shown in Figures 5A, 5C, 5E, 5G, 5I, 5K, and 5M; a comparison of tissue structure after toluidine blue staining is shown in Figures 5B, 5D, 5F, 5H, 5J, 5L, and 5N. In Figures 5A, 5C, 5E, and 5G, the VfCWINV1 fragment was used as probe. Labeling was detected as early as 1 to 2 DAF in the cells of the integument (Figure 5A). At 10 DAF, the labeling covered the cells of the thin-walled parenchyma and the chalazal vein (Figure 5C). At 15 DAF, labeling was distributed over several of the innermost cell rows of the thin-walled parenchyma (Figure 5E), whereas at 30 DAF, it became reduced to a narrow band (Figure 5G). Figure 5O shows that between 20 and 30 DAF, the inner cell rows of the thin-walled parenchyma were degraded where the enlarging embryo contacted the seed coat. Therefore, the reduction of labeling with ongoing development may be due to lysis of cells expressing VfCWINV1.

In Figure 5I, VfCWINV2 was used as a probe. At 20 DAF, silver grains were localized exclusively in the chlorenchyma and the ground parenchyma of the seed coat but not in the thin-walled parenchyma (compare with Figure 5J). In situ analysis of the vacuolar invertase form VfVCINV is shown in Figures 5K and 5M. At 15 DAF, labeling was localized in the thin-walled parenchyma and the endosperm (Figure 5K as compared with Figure 5L), whereas at 25 DAF, mainly the chlorenchyma and to a lesser extent the ground and thin-walled parenchyma were labeled (Figure 5M as compared with Figure 5N). The embryo itself was never found to be labeled with any of the invertase probes. Histochemical activity staining for cell wall–bound invertase was performed on hand-cut sections of seeds at 25 DAF. Figure 5P shows an intense blue staining restricted to the innermost layer of the seed coat.

The data provide evidence for a temporal and spatial specificity of expression of the two cell wall–bound invertases in developing seed coats. During the prestorage phase, VfCWINV1 is specifically expressed in the vein and in the thin-walled parenchyma representing the unloading area of the seed coat (Offler et al., 1989; Offler and Patrick, 1993; Wang et al., 1995). This suggests an important role of VfCWINV1 in early seed development.

Seed Coat Invertases Control the Partitioning of Photoassimilates in the Cotyledons

The increase of sucrose in the cotyledons (Figure 2C) concomitant with the decrease of hexoses (Figure 2B) suggests a resynthesis of sucrose. We therefore analyzed sucrose-phosphate synthase and sucrose synthase in crude extracts. Figure 6A demonstrates that sucrose-phosphate synthase increased from 15 DAF to slightly higher levels than sucrose synthase until 22 DAF. The sucrose synthase level was low at ~14 DAF and increased to high levels only after 25 DAF. As reported by Reimholz et al. (1994), sucrose-phosphate synthase can be activated by hexose-phosphate in storage tissues, whereas sucrose synthase from fava bean cotyledons is inhibited strongly by free hexoses (H. Weber, P. Buchner, L. Borisjuk, and U. Wobus, unpublished data). Therefore, when sucrose is cleaved before transfer into the embryo by invertases, the resulting high hexoses/sucrose ratio in the cotyledons could favor sucrose synthesis via the sucrose-phosphate synthase pathway rather than cleavage via the sucrose synthase pathway, thereby leading to carbohydrate partitioning into sucrose.

Sucrose synthase mRNA levels increased when sucrose became the dominating sugar in the cotyledons after 25 DAF. The enzyme is important for the mobilization of sucrose for storage product synthesis (Heim et al., 1993). As shown in Figures 6B and 6C, both storage starch and the transcript levels of the storage proteins legumin B and vicilin accumulated after 30 DAF in the embryo. This occurred at the time when levels of hexoses decreased and those of sucrose increased (Figures 2B and 2C, respectively), indicating that the seed coat invertase could be indirecty involved in the regulation of storage product synthesis by controlling the hexoses/sucrose ratio.

Feeding Hexoses to Storage Phase Cotyledons Directs Carbohydrate Partitioning into Sucrose and Downregulates Legumin B mRNA

To study the proposed role of the invertase-derived hexoses on storage metabolism, we used an in vitro culture system. Cotyledons that had entered the phase of storage product synthesis at ~30 DAF were cultured in the presence of elevated levels of hexoses. One cotyledon of a particular seed was

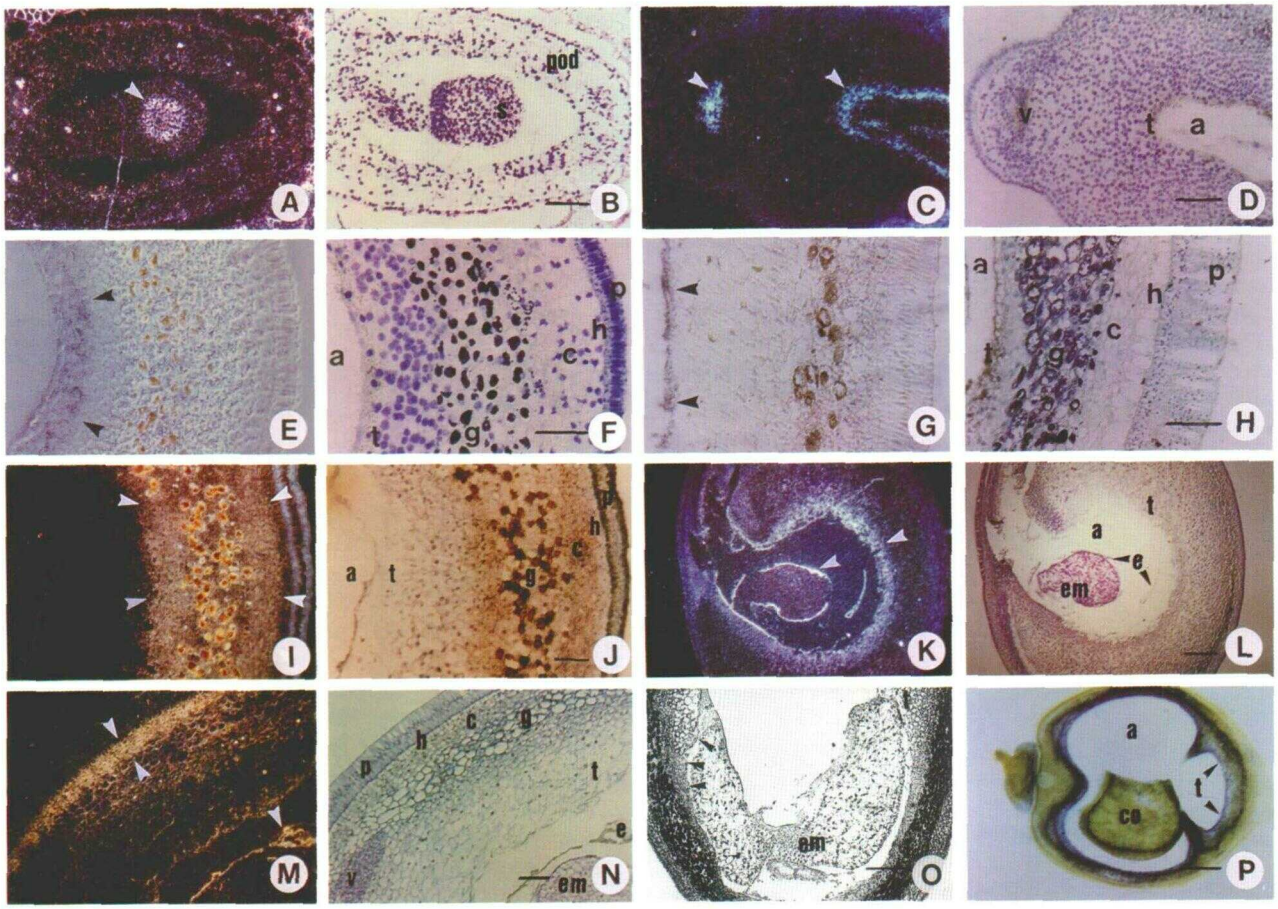

Figure 5. Localization of Invertase mRNA by in Situ Hybridization and Activity Staining.

(A), **(C)**, **(E)**, **(G)**, **(I)**, **(K)**, and **(M)** show tissue sections after in situ hybridization; **(B)**, **(D)**, **(F)**, **(H)**, **(J)**, **(L)**, and **(N)** show the same sections after staining with toluidine blue.

(A) and **(B)** Cross-section through a young pod and seed around fertilization. Dark-field microscopy after in situ hybridization with VfCWINV1 probe DNA is shown in **(A)**. The toluidine blue–stained section is shown in **(B)**.

(C) and **(D)** Cross-section through a seed coat at ~10 DAF. Dark-field microscopy after in situ hybridization with VfCWINV1 probe DNA is shown in **(C)**, with the toluidine blue–stained section shown in **(D)**.

(E) and **(F)** Cross-section through a seed coat at 15 DAF. Dark-field microscopy after in situ hybridization with VfCWINV1 probe DNA is shown in **(E)**, with the toluidine blue–stained section shown in **(F)**.

(G) and **(H)** Cross-section through a seed coat at 30 DAF. Bright-field microscopy of an in situ hybridization with VfCWINV1 probe DNA is shown in **(G)**, with the toluidine blue–stained section shown in **(H)**.

(I) and **(J)** Cross-section through a seed coat at 25 DAF. Dark-field microscopy after in situ hybridization with VfCWINV2 probe DNA is shown in **(I)**, with the toluidine blue–stained section shown in **(J)**.

(K) and **(L)** Cross-section through a seed at 15 DAF. Dark-field microscopy after in situ hybridization with VfVCINV probe DNA is shown in **(K)**, with the toluidine blue–stained section shown in **(L)**.

(M) and **(N)** Cross-section through a seed coat at 25 DAF. Dark-field microscopy after in situ hybridization with VfVCINV probe DNA is shown in **(M)**, with the toluidine blue–stained section shown in **(N)**.

(O) Tangential section through a seed at 25 DAF. The degradation zone of the cells of the thin-walled parenchyma is indicated by arrowheads.

(P) Hand-cut longitudinal section through a seed at 25 DAF stained for cell wall–bound invertase.

Silver grains are indicated with arrowheads. Bars = 0.05 mm in **(B)**, **(D)**, **(F)**, and **(J)**; 0.1 mm in **(H)** and **(N)**; 0.5 mm in **(L)** and **(O)**; and 1 mm in **(P)**. a, apoplastic space; c, chlorenchyma; co, cotyledon; e, endosperm; em, embryo; g, ground parenchyma; h, hypodermis; p, palisade; s, seed; t, thin-walled parenchyma; v, chalazal vein.

incubated with glucose and fructose, at 75 mM each, and 50 mM of sucrose was added to the basal medium. The second cotyledon was cultured in a medium supplemented with 150 mM sucrose and served as a control. After 4 days, cotyledons were assayed for sucrose-phosphate synthase and sucrose synthase and hexoses, sucrose, and starch. Transcript levels of legumin B and vicilin, the main storage proteins, were analyzed on RNA gel blots. Table 2 shows that culturing in hexose medium decreased the sucrose synthase/sucrose-phosphate synthase ratio from 26.2 to 5.9. This decrease was accompanied by an accumulation of hexoses from 11.9 to 33.8 μmol/g fresh weight. Sucrose increased from 46 to 104.5 μmol/g fresh weight, starch from 2.2 mg to 2.8 mg per cotyledon, and the sucrose/starch ratio from 1.4 to 2.23. Levels of legumin B mRNA were strongly reduced in comparison with the control, whereas vicilin levels remained unchanged (as shown in Figure 7).

The data indicate that feeding hexoses leads to carbohydrate partitioning in favor of sucrose due to an activation of sucrose-phosphate synthase and an inactivation of sucrose synthase. Furthermore, expression of certain storage protein genes is influenced, as indicated by a downregulation of legumin B mRNA.

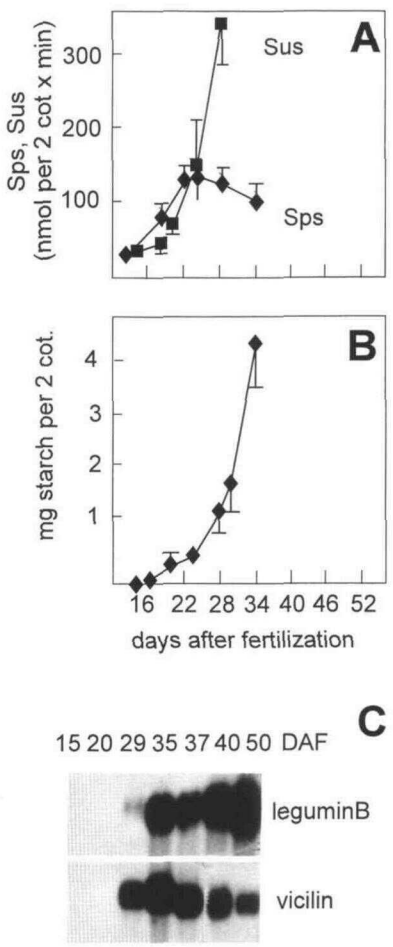

Figure 6. Changes in Sucrose Synthase and Sucrose-Phosphate Synthase Enzymes, Starch Content, and Storage Protein mRNA Levels of Cotyledons during Development.

(A) Enzyme activity of sucrose synthase (Sus) and sucrose-phosphate synthase (Sps) in cotyledons (cot). Values are means ±SD of three experiments.

(B) Accumulation of starch in cotyledons (cot). Values are means ±SD of at least three replicate experiments.

(C) RNA gel blot analysis of legumin B4 (top lane) and vicilin (bottom lane). Total RNA (3 μg per lane) from developing cotyledons was analyzed.

DISCUSSION

During seed development of fava bean, the end of the prestorage phase is characterized by a decreasing mitotic rate and by cell differentiation. Whereas the embryo of the prestorage phase develops only a small sink capacity, rapid fresh weight increase occurs during the storage phase (Borisjuk et al., 1995). The developmental switch from the differentiation to the storage phase is therefore accompanied by a physiological switch. In this study, we provide evidence that cell wall–bound invertase in the seed coat is important for this process.

Seed Coat Apoplastic Invertase Controls the Ratio of Hexose to Sucrose Delivered to the Embryo

During the prestorage phase, the distribution of free sugars in the seed coat, the apoplastic space, and the embryo indicates that after unloading from the seed coat, most of the sucrose must be hydrolyzed. We found considerable activity of cell wall–bound invertase in the seed coat and correspondingly high levels of hexoses in the embryo. The embryo itself

Table 2. Influence of Feeding Hexoses to Storage Phase Cotyledons in Vitro

Parameters Measured	Culture Conditions[a] Low Hexoses/ Sucrose Ratio (0:150)	High Hexoses/ Sucrose Ratio (150:50)
Sus/Sps[b]	26.2 ± 9	5.9 ± 2.2
Hexoses (μmol/g)	11.9 ± 5.3	33.8 ± 12.4
Sucrose (μmol/g)	46 ± 20	104.5 ± 31
Starch (mg/g)	2.2 ± 1.4	2.8 ± 1.3
Sucrose/starch	1.4 ± 0.9	2.23 ± 1.4

[a] Values are means ± SD of at least three replicate experiments.
[b] Sucrose synthase/sucrose-phosphate synthase ratio.

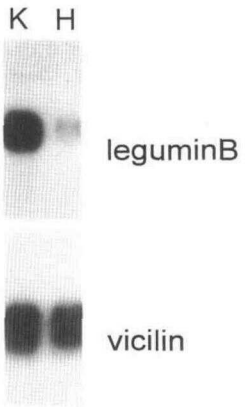

Figure 7. Influence of Feeding Hexoses to Developing Cotyledons.

One cotyledon from a 30-DAF seed was cultured in basal medium (K), and the other was cultured in basal medium supplemented with glucose and fructose at 75 mM each (H). After 3 days of culture, total RNA from the cotyledons was analyzed on gel blots using cDNA fragments of legumin B4 and vicilin as probes.

has only a low potential for sucrolysis during the prestorage phase because (1) the activity of both isoforms of acid invertases as well as neutral invertase is very low (Table 1; H. Weber, unpublished results), (2) the activity of sucrose synthase is also very low before 20 DAF (Figure 6A), and (3) the enzyme may also be inhibited by hexoses directly and by an unfavorably high hexose/sucrose ratio present in the cotyledons (H. Weber, P. Buchner, L. Borisjuk, and U. Wobus, unpublished data; Ross and Davies, 1992). We conclude that the high ratio of hexose to sucrose during the prestorage phase is caused by apoplastic invertase associated with the seed coat and not by the embryo.

Expression of the Seed Coat-Associated Invertases Is Spatially and Developmentally Regulated

A PCR-based approach was used to clone cDNAs of the seed coat–specific invertases. We amplified three products with 60 to 70% homology with known invertases from other species, indicating the invertase nature of the cDNA fragments. These fragments were used to screen a seed coat–specific library for the corresponding full-length cDNAs. A comparison of the characterized sequences and the clearly different expression patterns evident from RNA gel blot and in situ hybridization experiments indicated three different genes. Several lines of evidence suggest that VfCWINV1 and VfCWINV2 represent two cell wall–bound isoforms, whereas VfVCINV is a soluble acid invertase. First, a dendrogram including seven other known cell wall–bound and six soluble invertases clearly displayed two groups of sequences (Figure 3). One group comprises all cell wall–bound forms, including VfCWINV1 and VfCWINV2; the other one comprises all soluble forms, including VfVCINV. Second, sequence analysis of VfCWINV1 and VfCWINV2 revealed the catalytic domain WECPD of the cell wall–bound type, whereas VfVCINV possesses the WECVD motif of the soluble form. Third, VfVCINV has C- and N-terminal extensions typical of the soluble-type invertases (Unger et al., 1994). Fourth, the calculated isoelectric points of VfCWINV1 and VfCWINV2 are 9.87 and 8.08, respectively. This basic character is important for the binding to the extracellular matrix and typical for cell wall–bound invertases (Unger et al., 1994). VfVCINV, on the other hand, has a calculated acidic pI of 4.48.

Expression analysis of the three invertase genes by RNA gel blot and in situ hybridization provided important hints for their function. Whereas all three invertases are expressed in the seed coat, they differ strongly with respect to cell type (Figure 5). Most interestingly, VfCWINV1 is expressed exclusively in the chalazal vein and the inner rows of cells of the thin-walled parenchyma of the seed coat. These cells represent the end of the sieve element system (Offler et al., 1989; Wang et al., 1995). The inner five to seven rows of cells of the thin-walled parenchyma are supposed to be the site for photosynthate exchange to the apoplastic space (Offler et al., 1989). According to our analyses, exactly this cell type expresses VfCWINV1. VfCWINV2 is expressed in roots and seed coats and predominantly during pod development. Within the seed coat, expression is temporally similar to that of VfCWINV1 but is spatially different. mRNA was detected in the chlorenchyma and the ground parenchyma but not in the thin-walled parenchyma (Figure 5I). VfCWINV2 may therefore be more important for the metabolism and development of the seed coat itself.

The vacuolar form—VfVCINV—is expressed in most of the sink organs such as roots, stems, and sink leaves and in flowers, pods, seed coats, and the endosperm attached to the embryo (Figure 5K). This broad specificity indicates a general role for sink tissues. None of the invertases is expressed in embryonic cells, although a signal on an RNA gel blot was observed with cotyledons at 15 DAF for the vacuolar form (Figure 4). However, in situ hybridization showed labeling located in the embryo-attached endosperm and not in the embryo itself (Figure 5K). The positive signal therefore most likely resulted from contaminating endospermal RNA.

Seed Coat Apoplastic Invertase Contributes To Establish Sink Strength in the Young Seed

The early embryo of fava bean does not represent a strong sink because sucrolysis (see above) and the synthesis of storage products are low (Figure 6). The specific expression of VfCWINV1 in the unloading area could help to establish sink strength according to a model proposed by Eschrich (1989): due to a high sucrose concentration in the sieve cells, the unloading pressure is high, but sink activity would develop only after sucrose is released into the apoplastic space and then is taken up by the cells of the storage parenchyma. Sucrose would be reloaded into the sieve elements if not hydrolyzed

by apoplastic invertases. The resultant hexoses cannot be reloaded but are taken up by and accumulate in the embryo (Eschrich, 1989). Thus, in fava bean, seed sink strength may develop (1) by increasing the concentration gradient of sucrose between cells of the thin-walled parenchyma and the apoplastic space by invertase-mediated sucrolysis and (2) by lowering the water potential in the apoplast due to accumulation of hexoses. Patrick (1993) reported that, at least in seeds from the storage phase, the sucrose efflux from the coats was elevated at low osmotic potential. Also in maize and sorghum, sucrose hydrolysis by extracellular invertases seems to be part of the import mechanism (for review, see Wolswinkel, 1992).

During early development, a resynthesis of sucrose from imported hexoses may occur in the embryo via the sucrose-phosphate synthase pathway. The evidence for this is severalfold: (1) high levels of sucrose-phosphate synthase (Figure 6A) correspond to an increase of sucrose (Figure 2C); (2) levels of sucrose-phosphate synthase and sucrose synthase are within the same range in the embryo before 23 DAF (Figure 6A), but sucrose-phosphate synthase is activated by glucose 6-phosphate (Reimholz et al., 1994), the level of which is high at a high ratio of hexoses to sucrose (H. Weber, unpublished results); whereas (3) sucrolysis by sucrose synthase can be inhibited at least in vitro by free hexoses (H. Weber, P. Buchner, L. Borisjuk, and U. Wobus, unpublished data). Therefore, the high hexoses/sucrose ratio present in the embryo of the prestorage phase due to invertase action would partition carbohydrates into sucrose due to a differential effect on both sucrose-phosphate synthase and sucrose synthase. Resynthesis of sucrose in storage parenchyma cells from hexoses produced by apoplastic invertase was also described for sugar cane (Hatch et al., 1963).

We want to emphasize that our model of invertase-mediated control of seed development is based only on correlative evidence. The levels of free sugars given in Figures 2B and 2C may not reflect the effective concentrations present in the cytoplasm because during development the dry weight/fresh weight ratio increases as well as cell volumes. Furthermore, the exact subcellular concentration of free sugars is unknown.

Storage Product Synthesis Is Dependent on the Sucrose Synthase Pathway and a Low Hexoses/Sucrose Ratio

When the enlarging embryo attaches the seed coat, the inner rows of cells of the thin-walled parenchyma become degraded (Figure 5O). This process is accompanied by a decline of VfCWINV1 mRNA (compare Figures 5E and 5G). The reduction of the expression of VfCWINV1 after 15 DAF may therefore be the result of the lysis of cell layers expressing the gene. This phenomenon occurs during the switch from the prestorage to the storage phase and is accompanied by a dramatic increase of fresh weight and a decrease of the hexoses/sucrose ratio in the embryo. Bonnemain et al. (1991) reported the differentiation of transfer cells in the epidermal layer of fava bean cotyledons during a period immediately preceding the phase of fresh weight gain. They hypothesized that the change in the predominant sugars bathing the cotyledons from hexoses to sucrose may be responsible for the initiation of transfer cell development. Thus, a morphological and physiological change seems to result in the embryo being no longer dependent on an invertase-mediated unloading process but acquiring the competence to import large amounts of sucrose directly for the synthesis of storage products.

We proposed earlier (Heim et al., 1993) that storage product synthesis is dependent on the sucrose synthase pathway and a low ratio of hexoses to sucrose. In wheat endosperm, the rate of storage starch accumulation was reported to be a function of the concentration of sucrose (Jenner et al., 1991). When we fed hexoses to storage phase cotyledons, the sucrose synthase/sucrose-phosphate synthase ratio decreased and the sucrose/starch ratio increased (Table 2). We concluded that partitioning of carbohydrates is altered by a differential effect on both sucrose-phosphate synthase and sucrose synthase. Most interestingly, storage protein synthesis is also influenced because culturing in high hexoses specifically downregulates the mRNA level of a main storage protein, legumin B, whereas that of vicilin remains uninfluenced (Figure 7). Thus, seed coat–associated invertase may exert indirectly via hexoses a downregulating control of storage product synthesis. A similar effect on legumin mRNA was reported for the *rugosus* pea seed, which has been characterized as having an increased sucrose/starch ratio (Turner et al., 1990).

METHODS

Plant Material

Fava bean (*Vicia faba* var minor cv Fribo; Genbank, Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany) plants were grown in growth chambers under a 16-hr-light/8-hr-dark regime at 20°C. Pods were harvested in the middle of the light phase, chilled on ice immediately, dissected, and frozen in liquid nitrogen.

RNA Isolation and RNA Gel Blot Hybridization

RNA was isolated and hybridized as described in detail by Heim et al. (1993). Briefly, plant tissue was homogenized in 2.5 volumes of 1 M Tris-HCl, pH 7, 10 mM EDTA, 1% SDS, 0.7 mL of phenol-chloroform. After centrifugation, the aqueous phase was extracted with phenol-chloroform, and nucleic acids were precipitated with ethanol. After centrifugation, the nucleic acid pellet was dissolved in water, and the RNA was selectively precipitated in 2 M LiCl. The RNA from seed coats was prepared according to John (1992) as modified by Heim et al. (1993). RNA was separated in agarose gels containing 15% formaldehyde and blotted on nylon filters. RNA gel blot hybridization was performed according to Church and Gilbert (1984). The following cDNA fragments were used as probes after labeling with ^{32}P-dCTP according

to Feinberg and Vogelstein (1983): a 616-bp EcoRI-HindIII fragment from pVfCWINV1 containing a 566-bp polymerase chain reaction (PCR) product of VfCWINV1, a 615-bp EcoRI-HindIII fragment from pVfCWINV2 containing a 565-bp PCR product of VfCWINV2, a 587-bp EcoRI-BamHI fragment from pVfVCINV containing the 564-bp PCR product of VfVCINV, a 500-bp PstI fragment from pVfc28 containing a fava bean vicilin sequence (see Borisjuk et al., 1995), and a 520-bp PstI fragment from pVfc70 containing a fava bean legumin B4 sequence (Wobus et al., 1986).

Invertase Cloning and Sequencing

For PCR amplification of partial cDNAs of invertases, the following primers were used: primer A, 5′-AAAC/TTGGATG/TAACGATCCTAA-TGGA/TCC-3′; primer B, 5′-AAATCA/TGGACATTCCCA-3′; primer C, 5′-AAAA/TCTA/GGGACAT/CTCCCAAT-3′. The primer combination A plus B was used to amplify cell wall–bound isoforms, and the combination A plus C was used to amplify the vacuolar isoform. PCR was performed under standard conditions with 1.5 mM $MgCl_2$, 0.4 μM each primer, and 10 ng of double-stranded cDNA from seed coat (at 10 to 20 days after fertilization [DAF]) as a template. The temperature regime was 95°C for 0.5 min, 39°C for 1 min, 72°C for 1 min, and 94°C for 0.5 min 40 times, and 72°C for 5 min. Amplified DNA bands of ~650 bp were gel purified and subcloned as blunt-end fragments into the SmaI site of pUC18. A cDNA library was constructed using a Superscript Lambda System (Gibco BRL) according to the manufacturer's directions and screened under high-stringency conditions using the PCR-derived partial cDNAs VfCWINV1, VfCWINV2, and VfVCINV as probes. Positive inserts were cloned into Lambda ZIP LOX (Gibco BRL). Sequencing and data analysis were performed as described by Heim et al. (1993). The cDNA sequences of VfCWINV1, VfCWINV2, and VfVCINV have EMBL/GenBank/DDBJ accession numbers Z35162, Z35163, and Z35164, respectively.

Extraction and Determination of Sugars and Starch

Procedures were performed as described by Heim et al. (1993). Briefly, soluble carbohydrates were extracted in 80% ethanol at 80°C, and sucrose, glucose, and fructose were determined enzymatically. The remaining insoluble material was used for starch determination after solubilizing in 1 M KOH and hydrolyzing with amyloglucosidase. For the analysis of sugars in the apoplastic endospermal space, a known volume (2 μL) was removed by needle and syringe from seed immediately after harvesting and placed in 100 μL of 80% of ethanol for extraction.

Histochemical Staining of Cell Wall-Bound Invertase

Histochemical staining of cell wall–bound invertase was performed according to Dahlqvist and Brun (1962). Whole seed at 25 DAF were sectioned by hand followed by an intensive washing in distilled water. The sections were then incubated in a mixture of 0.38 M of sodium phosphate, pH 6, 0.24 mg/mL nitro blue tetrazolium, 0.14 mg/mL phenazine methosulfate, 25 units of glucose oxidase, and 5 g/mL sucrose. Control sections were heat treated before the incubation (10 min at 65°C). Blue precipitates occurred after several hours. The sections were washed in water and photographed using a Zeiss photomicroscope (Carl Zeiss, Oberkochen, Germany).

Enzyme Assays

For extraction of enzymes, the tissue was ground by mortar and pestle in a fivefold volume of extraction buffer at 0°C. Enzyme assays were checked for their dependence on each reaction component and on the linearity of their action with respect to time and amount of extract.

Acid Invertases

Invertases were extracted in 20 mM of sodium acetate, pH 5.2. After centrifugation, the supernatant was used to determine soluble acid invertase. After washing twice in 20 mM sodium acetate, pH 5.2, and four times in distilled water, the remaining pellet was used fresh or in freeze-dried form to determine the cell wall–bound invertase. The assay buffer consisted of 50 mM citric acid–Na_2HPO_4, pH 4.5, 100 mM sucrose, and the reaction was started with 150 μL of extract in a total volume of 300 μL for the soluble enzyme. The bound invertase was determined by suspending 3 mg of cell wall material in 150 μL of 20 mM sodium acetate, pH 5.2. The reaction was started by adding 150 μL of assay buffer. After incubation for 20 min at 30°C, the reaction was stopped in a boiling water bath for 2 min, and an aliquot was used to determine the reducing sugars with a Boehringer Mannheim kit.

Sucrose-Phosphate Synthase

Sucrose-phosphate synthase was measured in a spectrophotometric assay monitoring the synthesis of UDP (Stitt et al., 1988). The extraction buffer consisted of 50 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.9, 5 mM $MgCl_2$, 2% soluble PVP, 4 mM DTT, and 0.02% BSA. After centrifugation, the reaction was started by adding 50 μL of crude extract to 150 μL of assay buffer I (50 mM Hepes, pH 7.1, 5 mM $MgCl_2$, 4 mM fructose 6-phosphate, 20 mM glucose 6-phosphate, 3 mM UDP-glucose, 0.02% BSA). After 20 min at 30°C, the reaction was stopped in a boiling water bath. Control reactions were stopped immediately after starting. Extract (100 μL) was added to 1 mL of assay buffer II (100 mM Tris, pH 8.1, 0.5 mM phosphoenolpyruvate, 0.15 mM NADH, 1 mM $MgCl_2$, 50 mM KCl, 1.25 μg per 10 mL NAD-linked lactate dehydrogenase, 62.5 μL per 10 mL pyruvate kinase). After 30 min at 37°C, absorption at 340 nm was determined. Extracts were assayed for UDP-degrading activity with finding rates ~70%.

Sucrose Synthase

Sucrose synthase was measured in a continuous spectrophotometric assay, according to Xu et al. (1989), in the cleavage reaction linked to the conversion of UDP-glucose to glucose 1-phosphate by the endogenous UDP-glucose pyrophosphorylase. Tissue was ground in 100 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.2, 5 mM $MgCl_2$, 0.02% BSA. After centrifugation, 5 to 30 μL of the supernatant was added to 500 μL of assay buffer (75 mM Hepes-KOH, pH 7.9, 7 mM $MgCl_2$, 50 mM sucrose, 1 mM UDP, 1 mM NAD, 2 units per mL phosphoglucomutase, 1 unit per mL glucose-6-phosphate dehydrogenase). After 5 min, the reaction was started with 2 μL of 0.5 M sodium pyrophosphate. NADH synthesis was linear for at least 10 min.

In Situ Hybridization

Dissected seeds were immersed in 4% (w/v) paraformaldehyde, and 50 mM potassium phosphate buffer, pH 7, for 4 hr at room temperature following another 4 hr of incubation under slight vacuum. The

tissue was rinsed in 50 mM phosphate buffer, pH 7, dehydrated in a graded ethanol series, and embedded in Paraplast Plus (Sherwood Medical, St. Louis, MO). Sections (7 to 10 μm thick) were cut with a steel knife, transferred to glass slides coated with 0.1% (w/v) poly-L-lysine in 10 mM of Tris, pH 8, and dried overnight at 45°C. The slides were cleared with xylene, blocked with 1% BSA, treated with 10 μg/mL of proteinase K, and acetylated to block nonspecific charge interactions.

In situ hybridization was performed according to Harris and Wilkinson (1990) overnight at 45°C using the ^{33}P-labeled DNA probes described above. For seed tissues, DNA probes were used rather than antisense RNA probes because they require shorter exposure times and lower background levels. The slides were washed for 1 hr at 45°C in 50% formamide, 0.1 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), and 0.3 M NaCl, coated with photoemulsion (type LM-1: Amersham Corp.), dried, and exposed for 3 to 20 days at 4°C. Slides were developed using D-19 developer (Eastman Kodak, Stuttgart, Germany) and counterstained briefly with toluidine blue (0.05%, aqueous). For photomicrographs, a Zeiss photomicroscope equipped with bright- and dark-field condensers and phase contrast optics was used. Slides treated with RNase before hybridization revealed no detectable labeling of any cell type by any probe. To confirm the specificity of our in situ hybridization, we used a ^{33}P-labeled cDNA encoding an available human methyltransferase with no known homology with genes of higher plants (Rydberg et al., 1990). We never observed any labeling of our plant tissues with this probe.

In Vitro Culture of Developing Cotyledons

Seeds were removed from freshly harvested pods, and two cotyledons from a single seed were placed in separate Petri dishes containing 9 mL of basal medium with 62.5 mM L-glutamine according to Millerd et al. (1975). The medium was supplemented with either 150 mM sucrose and 150 mM sorbitol (control) or 50 mM sucrose and glucose and fructose at 75 mM each. The dishes were sealed and placed in growth chambers under dim light at 21°C. After 3 days, the cotyledons were removed, washed several times in sterile water, blotted dry on filter paper, weighed, and frozen in liquid nitrogen until used for sugar or starch determination, enzyme assays, or RNA extraction.

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