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A 62-kD Sucrose Binding Protein Is Expressed and Localized in Tissues Actively Engaged in Sucrose Transport

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Sucrose transport from the apoplasm, across the plasma membrane, and into the symplast is critical for growth and development in most plant species. Phloem loading, the process of transporting sucrose against a concentration gradient into the phloem, is an essential first step in long-distance transport of sucrose and carbon partitioning. We report here that a soybean 62-kD sucrose binding protein is associated with the plasma membrane of several cell types engaged in sucrose transport, including the mesophyll cells of young sink leaves, the companion cells of mature phloem, and the cells of the developing cotyledons. Furthermore, the temporal expression of the gene and the accumulation pattern of the protein closely parallel the rate of sucrose uptake in the cotyledon. Molecular cloning and sequence analysis of a full-length cDNA for this 62-kD sucrose binding protein indicated that the protein is not an invertase, contains a 29-amino acid leader peptide that is absent from the mature protein, and is not an integral membrane protein. We conclude that the 62-kD sucrose binding protein is involved in sucrose transport, but is not performing this function independently.

INTRODUCTION

The regulation of sucrose transport throughout the plant has a tremendous impact on plant growth and productivity (Gifford and Evans, 1981). Through photosynthesis, a plant fixes atmospheric CO_2 into triose phosphates, which are then utilized to produce sucrose and other carbohydrates. Once these carbohydrates are synthesized, they are partitioned throughout the plant where they can be used as an energy source, serve as carbon skeletons for synthesis of other organic compounds, or be stored either short or long term for future growth needs. In most higher plant species, sucrose is the major form of transported carbohydrate (Giaquinta, 1983).

The ability of certain cells to actively transport sucrose across the plasma membrane is fundamental to the functioning of vascular plants. For instance, long-distance transport in the plant is dependent upon moving the sucrose into the phloem against a concentration gradient and unloading the sucrose into various sink tissues. A considerable body of evidence now exists that favors, in most plant species, the presence of an apoplastic transport step (i.e., transport across the plasma membrane) just prior to entry of sucrose into the minor vein phloem (phloem loading; Giaquinta, 1983). Such an apoplastic transport step would involve facilitated efflux of sucrose from mesophyll cells into the apoplastic space and active transport of sucrose into the phloem against a sucrose concentration gradient (Riens et al., 1991).

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Several lines of evidence support the hypothesis for an active, energy-requiring loading of sucrose into the phloem from the apoplastic space in many species. Incipient plasmolysis studies coupled with freeze-substitution electron microscopy demonstrated that the sieve element–companion cell complexes have an osmotic pressure of ~30 bars compared to only ~10 bars for the adjacent mesophyll cells (Geiger et al., 1973). Hence, the concentration of sucrose in the minor veins is approximately three times higher than in the surrounding mesophyll cells. Other studies using autoradiography of either fixed $^{14}CO_2$ or exogenously supplied [^{14}C] sucrose in several species show that sucrose accumulates in the phloem compared to surrounding mesophyll cells. Geiger et al. (1974) estimated that the sieve element–companion cell complex contains 80% of the sucrose present in the leaf. Geiger and coworkers demonstrated that high levels of $^{14}CO_2$-derived assimilates (sucrose) were present in the leaf apoplast (Geiger et al., 1974; Geiger, 1975), and these levels increased when the rate of photosynthesis was increased by raising the light intensity. Furthermore, addition of sucrose to leaves (Giaquinta, 1977a, 1977b; Wright and Fisher, 1981) and isolated phloem (Daie, 1987, 1989) results in a rapid depolarization of the plasma membrane, suggesting that a sucrose-H^+ cotransporter exists at the plasma membrane (Komor et al., 1977; Hutchings, 1978; Williams et al., 1990).

Giaquinta (1976, 1983) used the nonpermeant sulfhydryl group modifier, p-chloromercuribenzene sulfonic acid (PCMBS), to inhibit sucrose translocation during photosynthesis. PCMBS

markedly inhibited sucrose transport when supplied to the apoplast, indicating that sucrose is moved from the apoplast into the symplast of the phloem and that a protein is involved. Studies using isolated membrane vesicles demonstrated active transport of sucrose that was dependent upon ΔpH and $\Delta\psi$ (Buckhout, 1989; Bush, 1989, 1990; Slone and Buckhout, 1991). Finally, the pool of apoplastic sucrose can be decreased by expressing a foreign invertase in the apoplastic compartment of leaves. When this is done, sucrose is cleaved into hexoses in the leaves, disappears in the sieve tube exudate, and results in a distinctive "slow-growth" phenotype because of the plant's inability to load sucrose into the phloem (van Schaewen et al., 1990; Dickinson et al., 1991; Sonnewald et al., 1991). These results provide a basis for concluding that there is an active sucrose carrier in some higher plants. The existence of this apoplastic loading pathway, however, should not minimize recent work demonstrating that certain plant species, such as the cucurbits, translocate sucrose via the symplastic compartment (Madore et al., 1986; Madore and Lucas, 1987; Turgeon and Hepler, 1989; Turgeon and Beebe, 1991; van Bel et al., 1992).

The identification of the proteins responsible for transporting sucrose across the plasma membrane of plant cells is critical to understanding the regulation of sucrose transport. At this time, only three candidates exist for proteins involved in sucrose transport. The first is a 36- to 50-kD protein group identified by Delrot and his colleagues. Antiserum raised against this protein group inhibits sucrose transport somewhat, and reconstitution of the group results in partial sucrose transport (Gallet et al., 1988, 1992; Lemoine et al., 1988; Li et al., 1991). The second is a protein with a predicted M_r of 55 kD that was identified by complementation of an engineered yeast vector which was invertase deficient and contained the gene for the potato sucrose synthase. The gene for this protein was sequenced and appears to have 12 membrane-spanning domains (Riesmeir et al., 1993).

The third protein is the focus of this manuscript. Initially, this 62-kD protein was identified because of its strong affinity for a sucrose analog, 6′-deoxy-6′-(4-azido-2-hydroxy)-benzamidosucrose, which competitively inhibited the influx of radiolabeled sucrose into protoplasts from developing soybean cotyledons (Card and Hitz, 1986; Ripp et al., 1988). Photoaffinity labeling of this protein was protected by the inclusion of 100 mM sucrose, thus establishing substrate protectability. Other sugars, including glucose, raffinose, and phenyl α-D-3-deoxy-3-fluoroglucopyranoside, did not afford even partial protection from labeling (Ripp et al., 1988). We call this protein the 62-kD sucrose binding protein (62-kD SBP), referring to its M_r and its proven ability to bind sucrose. The 62-kD SBP has many features that suggest its involvement in sucrose transport, including its affinity for sucrose, the correlation between protein level and active sucrose transport rate in the soybean cotyledon, immunocytochemical evidence demonstrating its association with cells actively engaged in sucrose uptake (Ripp et al., 1988; Warmbrodt et al., 1989, 1991), and, importantly, the observation that affinity-purified antiserum against the 62-kD SBP inhibits sucrose uptake into *Vicia faba* transfer cells (Fieuw et al., 1992). In this study, we report the molecular cloning of the gene for the 62-kD SBP, the nucleotide and deduced amino acid sequence of the gene and its protein, and elaborate on the expression and immunocytochemical association of the 62-kD SBP in and with various cell types of the soybean plant.

RESULTS

Isolation and Characterization of a cDNA Encoding the 62-kD SBP

A λZAP cDNA library representing the mRNA population of soybean cotyledons actively engaged in sucrose transport was screened with an affinity-purified antibody against the 62-kD SBP. Several clones were obtained that cross-reacted with this antibody. After initial characterization, one clone, designated *sbp*, was used for subsequent characterization. The 1.7-kb insert was excised from λZAP, propagated in the plasmid pBluescript II SK+, and subjected to DNA sequence analysis. The resulting nucleotide sequence is shown in Figure 1. The 1572-bp open reading frame encodes a protein of 524 amino acid residues with an M_r of 65,500 (Figure 1).

Several approaches were used to confirm that the *sbp* cDNA encodes for the 62-kD SBP protein. Purified SBP was subjected to amino-terminal sequencing and a 20-amino acid sequence was obtained that is shown in italics in Figure 2A. These 20 amino acids are identical to a region near the amino terminus of the deduced amino acid sequence from the *sbp* cDNA (Figure 2A, in italics). This alignment indicates that the *sbp* cDNA encodes the SBP protein and that there is a 29-amino acid leader peptide that apparently is absent from the mature protein. If this leader peptide is subtracted from the 524 deduced amino acid sequence, the resulting polypeptide has a molecular weight of ~62,000, which corresponds to the M_r of the 62-kD SBP. To confirm the relationship between this cDNA and the 62-kD SBP protein, the *sbp* cDNA was transformed into *Escherichia coli* and expressed as a fusion protein. The resulting protein appeared to be degraded. However, when the *E. coli* proteins were separated by SDS-PAGE (Laemmli, 1970), transferred to nitrocellulose, and reacted with antiserum, we detected proteinaceous material in the transformed *E. coli* that was not present in the untransformed bacteria. However, when the nucleotides encoding the 29-amino acid leader peptide were removed and the remaining cDNA was expressed in *E. coli*, this degradation problem was reduced somewhat and a significant amount of M_r 62,000 protein was synthesized. From these experiments, we concluded that the *sbp* cDNA encodes the 62-kD SBP.

The 29 amino-terminal residues of the 62-kD SBP deduced amino acid sequence contain many features normally associated with signal sequences (Watson, 1984; von Heijne,

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TGGAGCTCCACCGCGGTGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCG   -28

TTTTTTTTGTTCCTCACTGACCAAGCCATGGGCATGAGAACCAAACTTTCTTTAGCTATC    32
                          M  G  M  R  T  K  L  S  L  A  I

TTTTTCTTTTTTCTTTTAGCCTTATTTTCAAACCTAGCCTTTGGCAAATGTAAAGAAACC    92
F  F  F  F  L  L  A  L  F  S  N  L  A  F  G  K  C  K  E  T

GAGGTAGAAGAAGAAGACCCTGAGCTCGTAACTTGCAAACACCAATGCCAACAGCAACAG   152
E  V  E  E  E  D  P  E  L  V  T  C  K  H  Q  C  Q  Q  Q  Q

CAATACACTGAGGGTGACAAGCGGGTATGCTTGCAAAGTTGTGACAGGTATCATCGTATG   212
Q  Y  T  E  G  D  K  R  V  C  L  Q  S  C  D  R  Y  H  R  M

AAGCAAGAGCGAGAGAAACAAATCCAAGAGGAAACTCGTGAGAAGAAAGAGGAGGAAAGC   272
K  Q  E  R  E  K  Q  I  Q  E  E  T  R  E  K  K  E  E  E  S

CGTGAAAGGGAAGAAGAACAACAAGAGCAGCATGAGGAACAAGATGAAAATCCCTACATT   332
R  E  R  E  E  E  Q  Q  E  Q  H  E  E  Q  D  E  N  P  Y  I

TTTGAGGAAGATAAGGATTTTGAGACCAGAGTCGAAACTGAAGGTGGCAGAATTCGGGTT   392
F  E  E  D  K  D  F  E  T  R  V  E  T  E  G  G  R  I  R  V

CTCAAGAAGTTCACTGAAAAATCCAAGCTTCTTCAGGGCATTGAGAACTTCCGTTTGGCC   452
L  K  K  F  T  E  K  S  K  L  L  Q  G  I  E  N  F  R  L  A

ATTTTGGAAGCTAGAGCACACACGTTCGTGTCTCCTCGCCACTTTGATTCCGAGGTTGTT   512
I  L  E  A  R  A  H  T  F  V  S  P  R  H  F  D  S  E  V  V

TTTTTCAACATCAAGGGGCGAGCCGTACTTGGGTTGGTGAGTGAAAGTGAAACAGAAAAA   572
F  F  N  I  K  G  R  A  V  L  G  L  V  S  E  S  E  T  E  K

ATCACCCTTGAACCTGGGGACATGATACACATACCAGCTGGCACCCCACTGTACATCGTT   632
I  T  L  E  P  G  D  M  I  H  I  P  A  G  T  P  L  Y  I  V

AACAGAGATGAGAATGATAAGCTCTTCCTTGCCATGCTCCATATACCTGTCTCTGTCTCT   692
N  R  D  E  N  D  K  L  F  L  A  M  L  H  I  P  V  S  V  S

ACTCCTGGAAAATTTGAGGAATTTTTCGCGCCTGGAGGACGAGACCCAGAATCGGTCCTC   752
T  P  G  K  F  E  E  F  F  A  P  G  G  R  D  P  E  S  V  L

TCAGCATTCAGCTGGAATGTGCTGCAAGCTGCGCTCCAAACCCCAAAAGGAAAGTTAGAA   812
S  A  F  S  W  N  V  L  Q  A  A  L  Q  T  P  K  G  K  L  E

AACGTTTTGATCAACAGAACGAGGGAAGTATTTTCCGAATAAGCAGAGAACAGGTGCGT   872
N  V  F  D  Q  Q  N  E  G  S  I  F  R  I  S  R  E  Q  V  R

GCGTTGGCCCCTACCAAGAAAAGCTCTTGGTGGCCATTCGGTGGCGAATCCAAGCCTCAA   932
A  L  A  P  T  K  K  S  S  W  W  P  F  G  G  E  S  K  P  Q

TTCAATATTTTCAGCAAGCGTCCCACTATCTCCAACGGATATGGTCGTTTAACTGAAGTT   992
F  N  I  F  S  K  R  P  T  I  S  N  G  Y  G  R  L  T  E  V

GGTCCTGACGATGATGAAAAGAGTTGGCTTCAAAGACTCAATCTCATGCTTACCTTTACC  1052
G  P  D  D  D  E  K  S  W  L  Q  R  L  N  L  M  L  T  F  T

AACATCACCCAGAGATCTATGAGTACTATTCACTACAACTCACATGCAACGAAGATAGCA  1112
N  I  T  Q  R  S  M  S  T  I  H  Y  N  S  H  A  T  K  I  A

CTGGTGATCGATGGCAGAGGGCATCTTCAAATATCATGTCCACACATGTCATCAAGGTCG  1172
L  V  I  D  G  R  G  H  L  Q  I  S  C  P  H  M  S  S  R  S

TCACACTCGAAGCATGATAAGAGTAGCCCTTCATACCATAGAATCAGTTCCGATTTGAAG  1232
S  H  S  K  H  D  K  S  S  P  S  Y  H  R  I  S  S  D  L  K

CCTGGAATGGTGTTTGTTGTCCCTCCCGGTCATCCCTTCGTCACCATAGCTTCCAATAAA  1292
P  G  M  V  F  V  V  P  P  G  H  P  F  V  T  I  A  S  N  K

GAGAATCTCCTCATGATTTGCTTCGAGGTTAACGCTCGAGACAACAAGAAGTTTACATTT  1352
E  N  L  L  M  I  C  F  E  V  N  A  R  D  N  K  K  F  T  F

GCAGGGAAGGACAACATTGTGAGTTCTCTGGACAACGTTGCTAAGGAGCTTGCCTTTAAC  1412
A  G  K  D  N  I  V  S  S  L  D  N  V  A  K  E  L  A  F  N

TATCCTTCTGAGATGGTGAACGGAGTATTCCTGTTGCAACGATTCCTCGAACGGAAATTG  1472
Y  P  S  E  M  V  N  G  V  F  L  L  Q  R  F  L  E  R  K  L

ATAGGAAGACTCTACCACTTGCCTCATAAGGACCGAAAGGAGAGTTTCTTTTTCCCTTTT  1532
I  G  R  L  Y  H  L  P  H  K  D  R  K  E  S  F  F  F  P  F

GAGTTGCCGAGAGAGGAGCGTGGTCGTCGCGCTGATGCGTGAGAGGTTTAGAACAATCAA  1592
E  L  P  R  E  E  R  G  R  R  A  D  A

GAAAAGGTGTGCATGTGGCTGAAGATCACGGGGAATGTATTAAGCTTCAGAGACTCTTTA  1652

AATTAAATTTTCTGTATTTTGTGTTATATGTTACTAGTTCTTTAAATTAGCCAGATGGAG  1712
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Figure 1. Nucleotide and Deduced Amino Acid Sequence of the *sbp* Clone.

The nucleotide sequence was determined by dideoxy sequencing from the 5′ end beginning with a primer to the T3 promoter and continued using oligonucleotides based on the previous sequence as primers. The amino acid sequence from the *sbp* clone was deduced by using the GCG software for the VAX (Devereux et al., 1984) over a continuous open reading frame.

1985). The size of this sequence is within the 20- to 40-amino acid length of other known signal sequences, and it contains a hydrophobic core (LAIFFFFLLALF) approximately in the middle of the sequence. There is a charged residue (K at −2) within the first five amino acids in the amino-terminal direction from the cleavage site, a C at the −1 position (replacing the more common A), and an A residue at the −5 position (again, replacing the more common A at −3 position). The G residue at −3 has the potential to disrupt the α-helical secondary structure. All of these characteristics are consistent with known signal sequence structures (von Heijne, 1985).

Analysis of the *sbp* nucleotide sequence indicated that there is no significant homology to any other nucleotide sequence or amino acid sequence in the GenBank data base. One potential role for the 62-kD SBP is that it may function as an invertase. We therefore attempted to align the *sbp* cDNA nucleotide sequence and its deduced amino acid sequence to known

A

MGMRTKLSLAIFFFFLLALFSNLAFGKCKETEVEEEDPELVTCKHQCQQ
ETEVEEEDPELVTCKHQCQQ

B

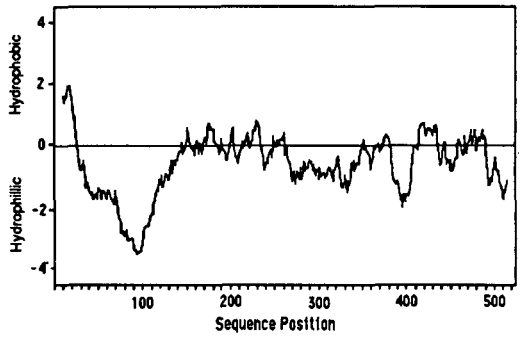

Figure 2. Leader Peptide Analysis and Hydropathic Plot of the 62-kD SBP Deduced Amino Acid Sequence.

(A) Mature 62-kD SBP was isolated (Ripp et al., 1988) and subjected to automated Edman degradation. The N-terminal sequence from this mature protein is shown in italics on the bottom line. The top line is the deduced N-terminal sequence starting from the 5′ end of the *sbp* open reading frame. The first 29 amino acids are apparently cleaved off the mature protein, indicating that these 29 amino acids are a leader peptide.

(B) A Kyte-Doolittle hydropathic plot (Kyte and Doolittle, 1982; Devereux et al., 1984) from the complete (i.e., including the 29-amino acid leader peptide) deduced amino acid sequence demonstrates that the 62-kD SBP is not an integral membrane protein with traditional transmembrane-spanning domains. Because the 29-amino acid leader peptide is not present on the mature protein, it is likely that this sequence does not serve as a membrane anchor.

invertase sequences (Sturm and Chrispeels, 1990) but found no homology. Furthermore, we compared two other invertase consensus sequences (MNDPNG and WECVDF) from tomato (Klann et al., 1991), and they are also absent from the 62-kD SBP. Apparently, the role of the 62-kD SBP is not merely to cleave sucrose into hexose sugars.

The 62-kD SBP does not appear to be glycosylated. Treatment of the 62-kD SBP with EndoH followed by SDS-PAGE indicated that the EndoH treatment did not cause any alteration of the M_r. Affinity blotting with concanavalin A did not stain the 62-kD SBP, indicating that the 62-kD probably does not contain high amounts of mannose or glucose.

Because we postulated that the 62-kD SBP was involved in sucrose transport across the plasma membrane, we determined whether this protein contained hydrophobic regions. Figure 2B shows that the 62-kD SBP is not a hydrophobic protein and does not contain conventional transmembrane-spanning domains, as expected from a Kyte and Doolittle (1982) hydropathy plot. The only distinctly hydrophobic region of this protein is the 29-amino acid leader peptide. This region does not appear to function as a membrane anchor, however, because it is absent from the mature protein. Extensive computer-assisted analysis of the deduced amino acid sequence indicated that other mechanisms for membrane linkage, such as amphipathic α-helices, are also absent. These results suggest that the 62-kD SBP is not an integral membrane protein.

If the 62-kD SBP is not an integral membrane protein, is it tightly associated with the membrane? It is possible that the 62-kD SBP is a soluble protein that is trapped inside membrane vesicles during homogenization. If this is the case, then it should be removed from these vesicles by repeated disruption with hypotonic buffer washes. Figure 3 shows that the 62-kD SBP was not washed out of vesicles even after four hypotonic washes, suggesting that the 62-kD SBP is tightly associated with the membrane fraction.

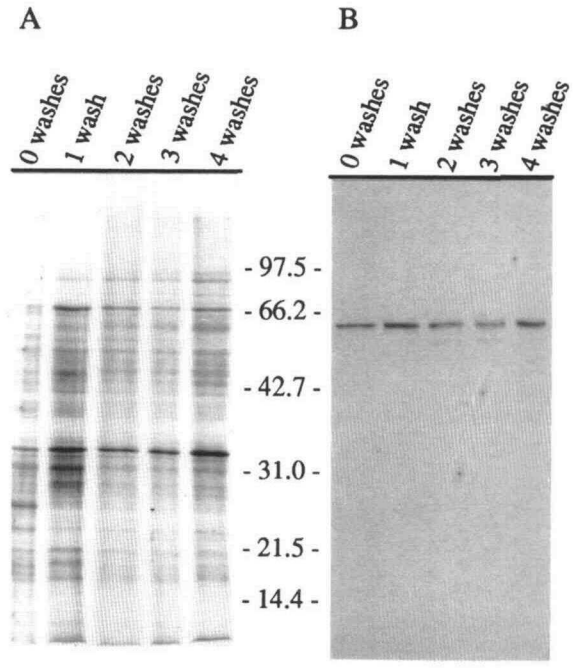

Figure 3. Hypotonic Washing of the Membranes Does Not Remove the 62-kD SBP.

Repeated hypotonic washes deplete membrane vesicles of any entrapped soluble protein by repeatedly bursting the vesicles followed by spontaneous revesiculation. These experiments indicate that the 62-kD SBP is not a soluble protein that is trapped within membrane vesicles during homogenization.

(A) Commassie blue-stained SDS-PAGE gel showing soybean cotyledon microsome before and after up to four hypotonic washes.

(B) Same as in **(A)**, but an immunoblot using the affinity-purified, anti–62-kD SBP IgG.

Developmental Regulation of the 62-kD SBP mRNA and Its Association with Active Sucrose Transport

The maternal (seed coat) and embryonic (embryonic axis and cotyledons) tissues of seeds are symplastically isolated; consequently, transfer of sugars into these tissues requires an apoplastic step. As the major organ in the developing embryo, the soybean cotyledon is highly specialized for sucrose uptake from the apoplast. During the formation of these cotyledons, sucrose is unloaded from the seed coat into the apoplastic space, engaging virtually all of the cotyledon cells in active uptake of this sucrose across the plasma membrane. The rate of sucrose uptake by cotyledon cells is developmentally regulated and reaches a maximum at ~15 to 17 days after fertilization (DAF; Ripp et al., 1988). If the 62-kD SBP has a role in sucrose transport, then we might expect its mRNA to be up-regulated just preceding accumulation of the protein and maximal sucrose uptake.

Figure 4A demonstrates that the *sbp* transcript was barely detectable before 10 DAF when sucrose transport was very low and there were very low levels of the 62-kD SBP; at 10 to 19 DAF, the transcript level increased dramatically. This increase in *sbp* transcript closely paralleled increases in sucrose transport and the level of 62-kD SBP in the cotyledons. After 20 DAF, the *sbp* transcript level declined again, coincident with a decrease in active sucrose transport in these cells. After 30 DAF, when no sucrose transport was occurring, there was no detectable *sbp* message (Figure 4A). We aligned this RNA gel blot with plots indicating the rate of sucrose transport and the relative amount of the 62-kD SBP, as shown in Figure 4B. Visually aligning the *sbp* transcript level with the amount of 62-kD SBP and with the rate of sucrose transport showed a very strong correlation (compare Figure 4A with 4B).

Most of the cells in newly forming soybean leaves (i.e., <1/8 expanded) actively transport sucrose because these leaves are primarily sink tissues and obtain most of their energy from imported sucrose. If the 62-kD SBP is associated with sucrose

transport in general, as opposed to a specialized function in the cotyledon, we would expect to see the *sbp* mRNA present in young leaves as well. Figure 5 demonstrates that <1/8 expanded leaves contained significant levels of *sbp* mRNA and that older leaves (1/4 and fully expanded), which have made the transition to source tissues, did not contain detectable levels of the *sbp* mRNA. The pattern of *sbp* gene expression as the leaf matures is consistent with the changing cellular function during the sink to source transition (Turgeon, 1989). While the leaf functions as a sink, photosynthesis is low and virtually all of the cells need to import sucrose. Hence, all of the cells express the *sbp* gene and the level is high enough that it is detectable on an RNA gel blot. As the leaf becomes a source organ, most of the cells are photosynthetically active and produce excess sugars for export. The need for uptake from the apoplast is low (only retrieval of leaked sucrose) and, consequently, the level of expression of *sbp* is not significant in the mesophyll cells. Only the companion cells of the phloem engage in active uptake of sucrose in the mature source leaf. Because these cells comprise only a very small amount of the total cell population, the mRNA for the *sbp* gene is essentially diluted out, making it undetectable by RNA gel blot analysis. Together, these experiments indicated that the *sbp* mRNA is detected in a manner consistent with the 62-kD SBP playing a role in sucrose transport in both cotyledons and young sink leaves.

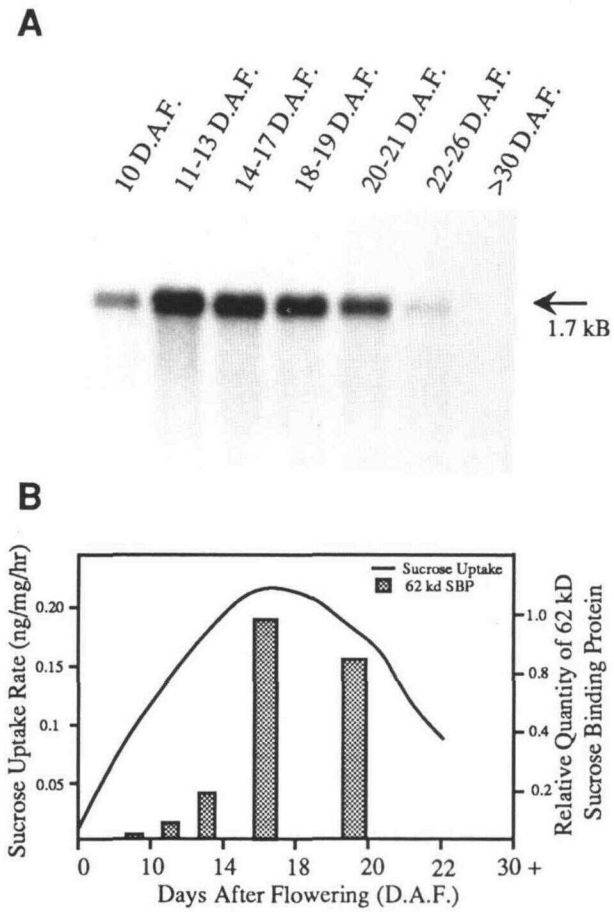

Figure 4. The 62-kD SBP Transcript Is Up-Regulated in the Soybean Cotyledon Just Preceding Increases in the Level of 62-kD SBP and Sucrose Transport.

(A) Poly(A)$^+$ RNA was isolated from soybean cotyledons at the indicated times after anthesis, and 1 μg/lane was subjected to RNA gel blot analysis using the *sbp* clone as a probe. By visually aligning these data with those shown in **(B)**, it is possible to see the tight correlation between an increase in the 62-kD SBP transcript, followed by an increase in the level of 62-kD SBP, and an increase in the rate of sucrose transport.

(B) The rate of sucrose uptake is plotted as a function of the DAF (data modeled after Ripp et al., 1988) on the solid line, and the relative level of 62-kD SBP is shown as a histogram.

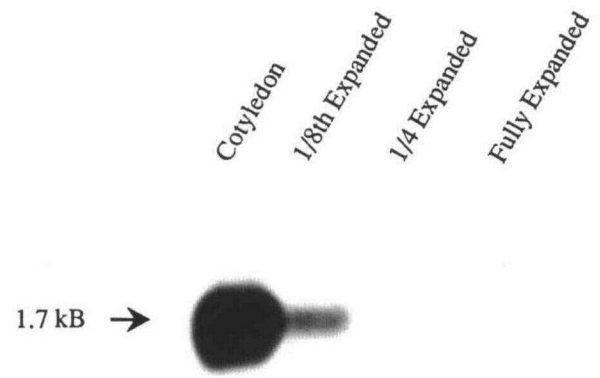

Figure 5. The 62-kD SBP Transcript Is Present in Young Soybean Leaves while They Function as Sucrose Sinks.

Poly(A)$^+$ RNA was isolated from the indicated stages of leaf development, and 1 μg/lane was subjected to RNA gel blot analysis using the *sbp* clone as a probe.

Cellular and Subcellular Localization of the 62-kD SBP in Cotyledons and Leaves

We examined the subcellular localization of the 62-kD SBP in cotyledonary tissues to determine whether it is associated with the plasma membrane. Soybean cotyledons contain cells that are highly specialized for transport. One indication of this is the presence of plasma membrane invaginations, an example of which is shown in Figure 6A. These regions of invaginated plasma membrane are called "plasmalemmasomes" or "paramural bodies" by various investigators (Marchant and

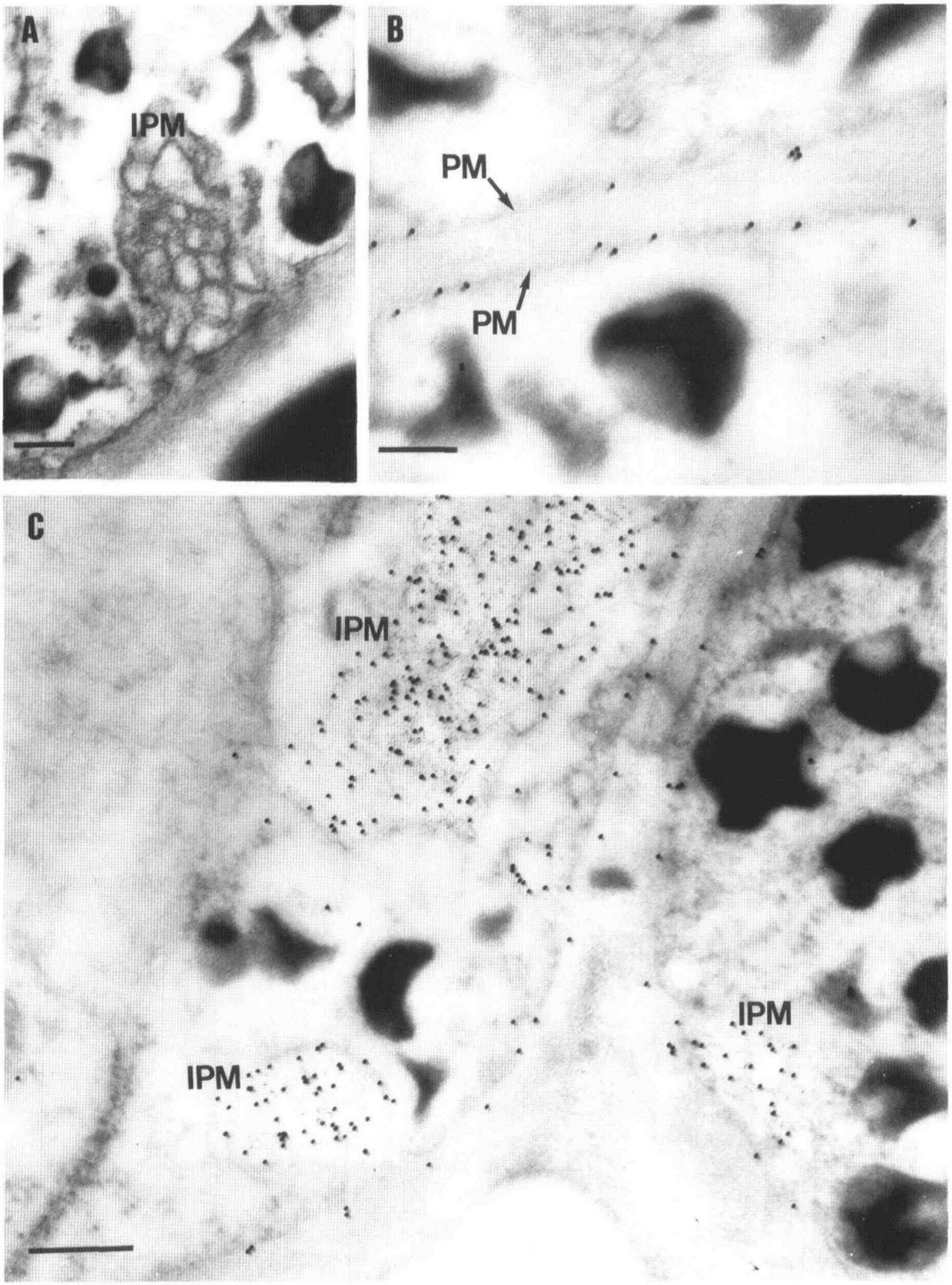

Figure 6. The 62-kD SBP Is Associated with the Plasma Membrane and Transport-Specialized Plasma Membrane Invaginations of the Cotyledon Cells.

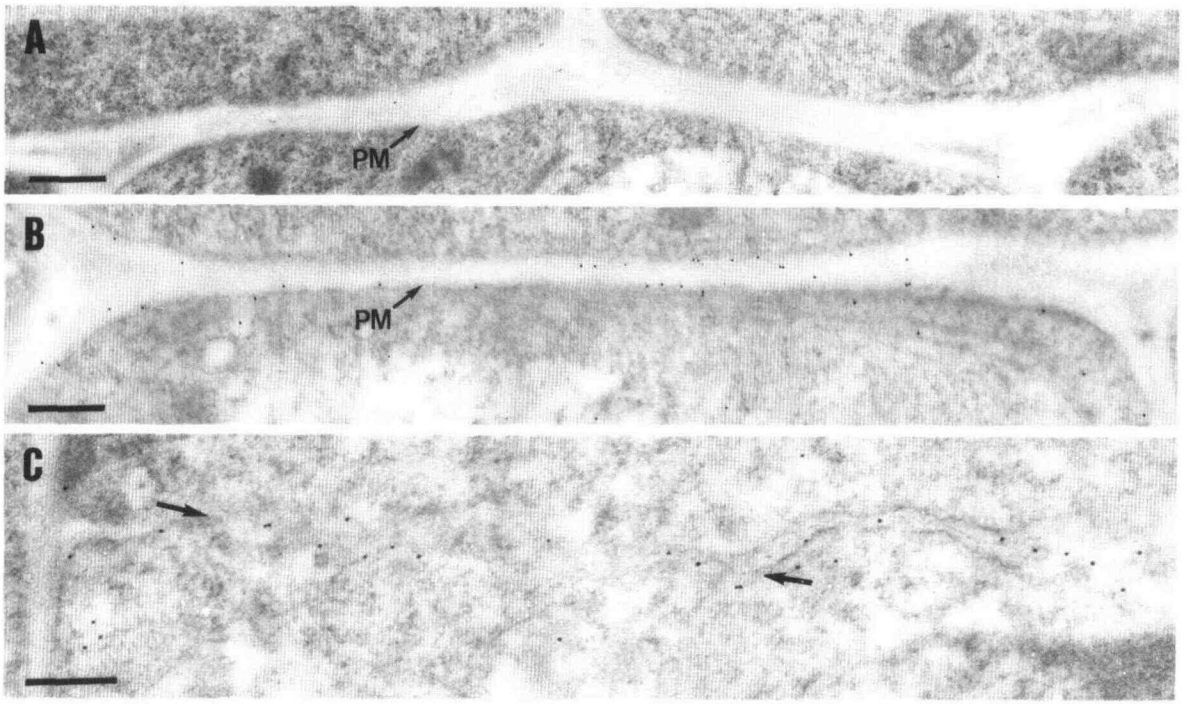

Figure 7. The 62-kD SBP Is Associated with the Plasma Membrane of Young Leaf Mesophyll Cells.

(A) Transmission electron microscopy of three mesophyll cells in a young (<1/8 expanded) leaf. Preimmune serum was used as a control, and no label was found associated with the plasma membrane (PM). Bar = 0.2 μm.
(B) Plasma membrane (PM) region of mesophyll cells from a young (<1/8 expanded) leaf. Gold label resulting from immunolabeling with affinity-purified, anti–62-kD SBP IgG is clearly associated with the plasma membrane. Bar = 0.3 μm.
(C) Section through a newly forming cell plate (indicated by arrows) of a young (<1/8 expanded) leaf. Localization of the 62-kD SBP is already seen during biosynthesis of the new plasma membrane. Bar = 0.3 μm.

Robards, 1968; Pate and Gunning, 1972). Pate and Gunning (1972) reported that many different types of transfer cells in higher plants are characterized by these plasma membrane invaginations. As shown in Figure 6B, our immunocytochemical investigations indicated that the 62-kD SBP is localized at the plasma membrane of soybean cotyledon cells. Furthermore, the 62-kD SBP is associated with the regions of invaginated plasma membrane (Figure 6C). This location is consistent with the pattern one would expect for a protein involved in sucrose transport.

Figure 7A shows a cross-section of the plasma membrane and cell wall area of three mesophyll cells from <1/8 expanded leaves treated with preimmune serum. As expected, no staining is observed. Figure 7B shows a similar cross-section of mesophyll cells from <1/8 expanded leaves reacted with a 62-kD SBP antibody and demonstrates clearly that the 62-kD SBP is associated with the plasma membrane of mesophyll cells which are actively accumulating sucrose. We also observed that the 62-kD SBP is associated with newly forming cell plates in young sink leaves (Figure 7C), indicating that the 62-kD SBP is rapidly transferred to the plasma membrane in newly divided sink cells.

As the leaf matures and begins to produce photoassimilated carbon as sucrose, it needs to load this sucrose into the phloem

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Figure 6. (continued).

(A) Transmission electron microscopy of a phosphotungstic acid–stained invaginated plasma membrane (IPM). Bar = 0.25 μm.
(B) Plasma membrane region of soybean cotyledon cells immunostained with affinity-purified, anti–62-kD SBP IgG. Gold label is localized exclusively on the plasma membrane (PM) region. Bar = 1 μm.
(C) A section through the plasma membrane and three transport-specialized regions of invaginated plasma membrane (IPM). Gold label is found to be heavily associated with these plasma membrane invaginations. Bar = 1 μm.

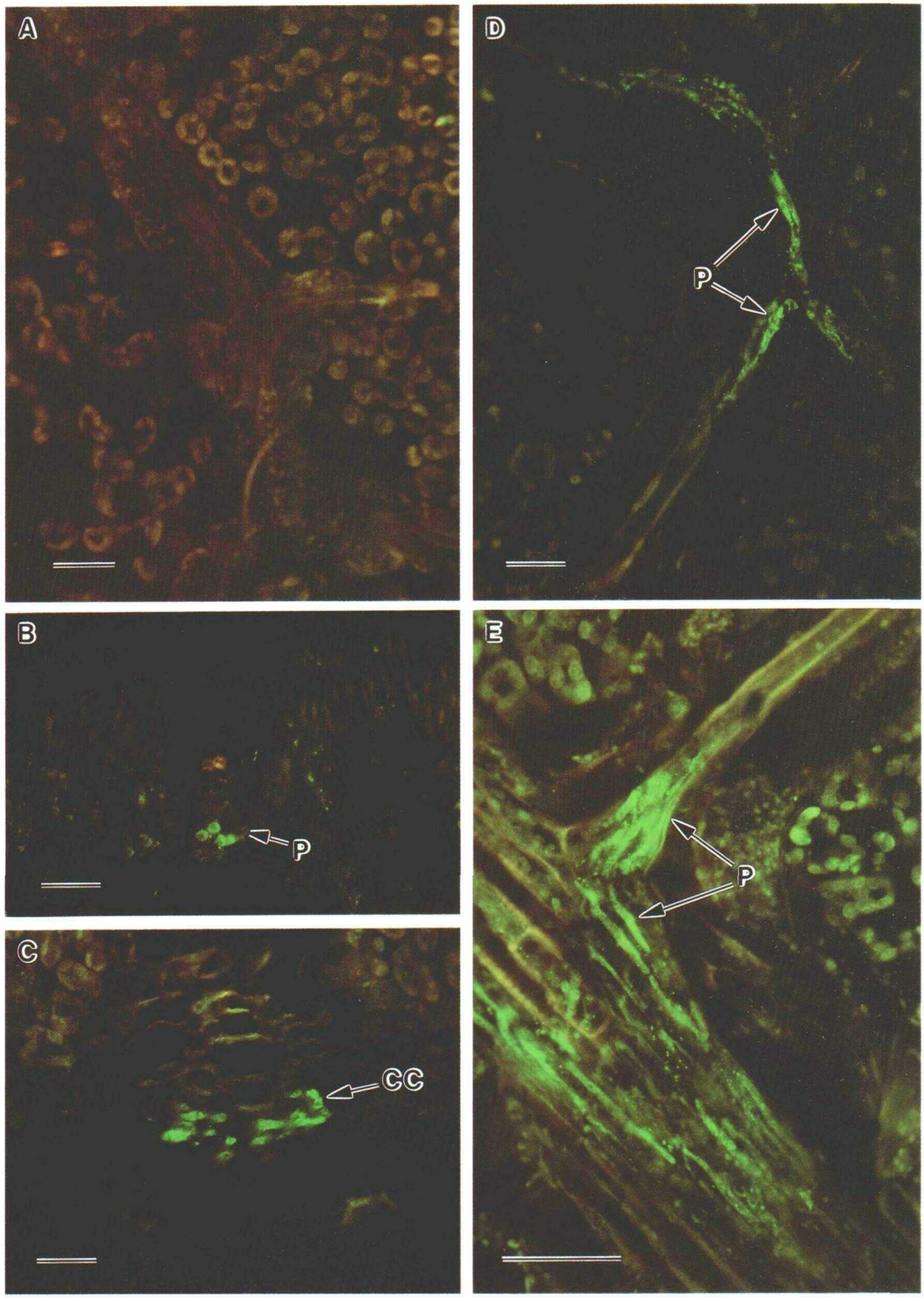

Figure 8. The 62-kD SBP Is Associated with the Phloem in Mature Source Leaves.

for long-distance transport. If the 62-kD SBP plays a role in phloem loading of sucrose, we would expect to find the 62-kD SBP associated with the phloem in mature leaves. To address this question, we used immunofluorescence techniques employing affinity-purified anti–62-kD SBP IgG followed by fluorescein isothiocyanate (FITC)–conjugated secondary antibody to localize the 62-kD SBP in mature leaf tissue.

Figure 8 shows that when a preimmune serum was used in the immunolabeling procedure, only a pale yellow autofluorescence is observed (Figure 8A). Figures 8B and 8C show two different leaf cross-sections in which vascular bundles are evident. In Figure 8B, the entire cross-section of the leaf is evident, and the bright-green fluorescence indicates that the only cells that are strongly labeled by the 62-kD antibody are the phloem cells in the bottom of the vascular bundle. The size and shape of the labeled cells indicate that they are companion cells. A higher magnification of the vascular bundle area is shown in Figure 8C, again demonstrating that the only cells with demonstrable labeling are the phloem cells. Figures 8D and 8E show two different phloem regions sectioned paradermally, one through minor veins and the other at a junction of veins of two different orders; both clearly demonstrate strong labeling of the phloem cells with the 62-kD antibody and only autofluorescence of surrounding mesophyll cells.

Figure 9 shows an electron micrograph of companion cells and adjacent sieve tube members labeled with the 62-kD antibody and demonstrates that the 62-kD SBP is localized primarily on the plasma membrane of the companion cells. The association of the 62-kD SBP with phloem cells suggests that it plays a fairly ubiquitous role in sucrose transport.

DISCUSSION

Our results demonstrate that the 62-kD SBP has several features implicating its role in sucrose transport. Expression of the *sbp* gene encoding this protein precedes the onset of active sucrose transport in the cotyledonary cells and precedes the accumulation of the protein in the cotyledon (Ripp et al., 1988). The *sbp* gene is also expressed in young leaves while they are a sink tissue and dependent upon sucrose uptake for growth and expansion. The protein is strongly associated with the plasma membrane of several cell types that are actively transporting sucrose, including the sieve element–companion cell complexes of mature phloem, the mesophyll cells of young sink leaves, and the cotyledon sink cells.

Immunocytochemistry of the phloem indicated that the companion cells are more strongly labeled with 62-kD SBP antibody than are the sieve elements. In the immunofluorescence studies, the sieve tube members are smaller than the companion cells, and if sieve tube members were labeled, they would appear as a punctate fluorescence. We observed a labeling pattern characteristic of the much larger companion cells. The companion cells, through the operation of an active transport mechanism, accumulate sucrose against a concentration gradient in plant species that use an apoplastic mode of phloem loading. Hence, the fact that the companion cells are labeled is consistent with known parameters of sucrose transport. The occurrence of the 62-kD SBP on mature sieve element–companion cell complexes and young leaf mesophyll cells (sink tissue) in soybeans indicates that the 62-kD SBP is involved in sucrose transport in a number of different cell types and is not a specialized protein associated only with the soybean cotyledon.

Analysis of the deduced amino acid sequence of the 62-kD SBP demonstrated that it is not a typical integral membrane protein. Rather, the protein is relatively hydrophilic and does not contain transmembrane-spanning domains like those observed in the H^+-ATPase (Harper et al., 1989) or the glucose transporter (Sauer et al., 1990). Extensive computer-assisted modeling of the secondary structure of the 62-kD SBP suggests that it contains no amphipathic α-helices large enough to span the bilayer (Eisenberg et al., 1982). Hypotonic wash experiments, however, indicated that the 62-kD SBP is a membrane protein and further experimentation with various chaotropic agents demonstrated that the 62-kD SBP is tightly associated with the membrane (P. J. Overvoorde and H. D. Grimes, unpublished results).

Because the hydrophobic 29-amino acid leader peptide is cleaved during protein maturation, the 62-kD SBP cannot be anchored to the membrane by this hydrophobic sequence. This

Figure 8. (continued).

Immunofluorescence studies using the 62-kD SBP antiserum demonstrated that the 62-kD SBP is associated with the phloem in source leaves.
(A) Paradermal section through a minor vein stained with preimmune serum. The slight yellow fluorescence is due to autofluorescence of the tissue. Bar = 30 μm.
(B) Cross-section of a mature soybean leaf showing both epidermal surfaces and a vascular bundle. Staining was performed with 62-kD SBP antiserum. The only cells that show green (FITC secondary antibody) fluorescence are the circular companion cells of the phloem (P) in the vascular bundle. Bar = 30 μm.
(C) Cross-section of a larger vascular bundle showing distinct labeling of the companion cells (CC) of the phloem. Bar = 30 μm.
(D) Paradermal section through a minor vein showing the association of the 62-kD SBP with the phloem (P). Bar = 30 μm.
(E) High magnification of a minor vein shown in paradermal section. Again, clear labeling of the phloem (P) is evident after staining with the 62-kD SBP antiserum. Bar = 30 μm.

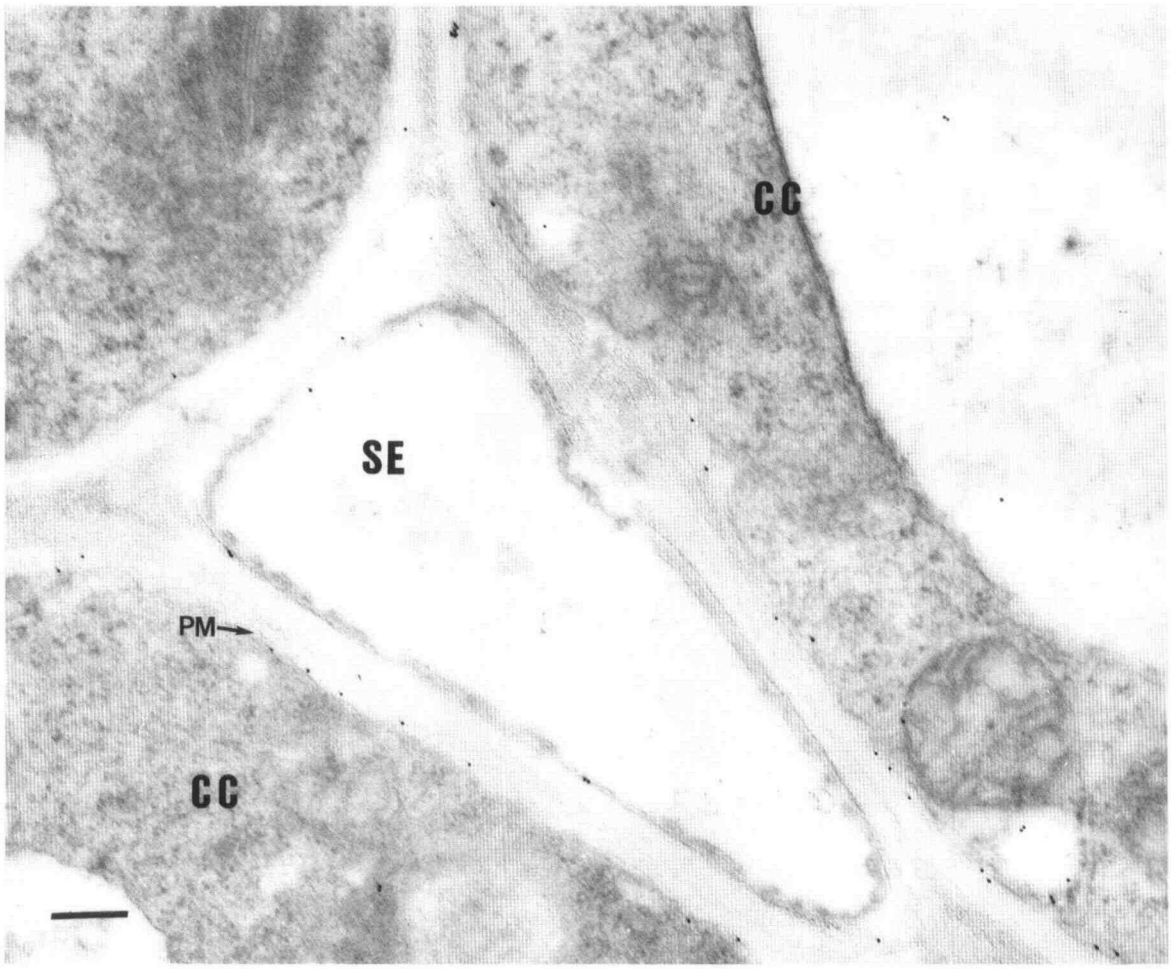

Figure 9. The 62-kD SBP Is Associated with the Plasma Membrane of Mature Phloem.

A section showing two companion cells (CC) and one sieve element (SE) immunolabeled with affinity-purified, anti–62-kD SBP IgG. The gold label is evident on the plasma membranes (PM) of both the companion cells and the sieve elements, but is much more dense on the companion cells. Bar = 0.4 μm.

leader peptide may be involved in targeting the protein to the external leaflet of the plasma membrane. This type of leader peptide would normally target proteins for secretion (Chrispeels, 1991) unless a carboxy-terminal signal was present to keep the protein in the endoplasmic reticulum (Bednarek and Raikhel, 1992). There are no KDEL or HDEL signals in the 62-kD SBP; thus, this protein probably enters the secretory pathway and is processed during maturation such that the 62-kD SBP is destined for the external leaflet of the plasma membrane.

Analysis of both the nucleotide sequence and the deduced amino acid sequence demonstrated no significant homology to any other gene or protein sequence that has been published. Although the function of the 62-kD SBP is unknown at this time, our results provide very strong evidence that it does play a role in sucrose transport. How can we reconcile the evidence that the 62-kD SBP is not an integral membrane protein with the evidence that it is involved in sucrose transport? Although speculative at this time, we present two views that can reconcile all available data on the 62-kD SBP. Perhaps the 62-kD SBP is located on the external leaflet of the plasma membrane and is one subunit of a multimeric protein complex that transports sucrose. If this is the case, then the 62-kD SBP may play an important regulatory role in sucrose transport. Regarding the possibility that sucrose transport in higher plants may be the result of a multimeric carrier, Delrot and his colleagues have independently reached the same conclusion (Li et al., 1991). A second possibility is that the 62-kD SBP may function as an external "monitor" of sucrose concentration in the apoplast for cells that are engaged in sucrose uptake. In this

model, the protein is not involved in transport per se but still may play an important role in regulating other aspects of transport physiology. Regardless of which of these models, or an as yet undetermined model, for the function of the 62-kD SBP proves to be correct, the collective evidence builds a powerful case that this protein is involved in active sucrose uptake and phloem loading.

METHODS

Preparation of Affinity-Purified Antibody against the 62-kD Sucrose Binding Protein

IgG against the 62-kD sucrose binding protein (SBP) was purified by affinity chromatography with highly purified 62-kD protein coupled to CNBr-activated Sepharose 4. The coupling procedure was performed according to the manufacturer's (Pharmacia) protocol, starting with antigen that had been purified by the method of Ripp et al. (1988). Approximately 2 mg of the 62-kD SBP was coupled to 2 mL of gel. The serum (2 to 5 mL) was typically equilibrated with 10 mM potassium phosphate, pH 7.8, 300 mM NaCl by gel filtration. The serum and gel were incubated in this buffer for 1 hr at 25°C, followed by 1 hr at 4°C. A small column was prepared, washed with four volumes of the above buffer, and the IgG was eluted with 100 mM glycine, pH 2.5. Fractions were collected directly into tubes containing 2 M Tris, pH 7.8. Fractions were checked by immunoblotting, and the desired fractions were combined and stored at 4°C with 0.02% Thimerosal.

Construction and Screening of a cDNA Library from Developing Soybean Embryos

Soybean embryos (18 to 20 days after flowering [DAF]) were removed from the pods and frozen in liquid nitrogen. The frozen embryos were ground to a fine powder in the presence of liquid nitrogen, and total RNA was isolated as described below. The nucleic acid fraction was enriched for poly(A)$^+$ RNA by passing total RNA through an oligo(dT)-cellulose column (Sambrook et al., 1989). The synthesis of cDNA was performed according to the manufacturer's instructions (Bethesda Research Laboratory). The resultant double-stranded DNA was methylated by EcoRI methylase (Promega) prior to filling in the ends with T4 DNA polymerase (Bethesda Research Laboratory) and blunt-end ligation to the phosphorylated EcoRI linkers using T4 DNA ligase (Pharmacia). The double-stranded DNA was digested with EcoRI, size selected on Sepharose CL-4B, and ligated to λZAP vector (Stratagene). Ligated DNA was packaged into phage using Gigapack packaging extract (Stratagene), and the resultant cDNA library was amplified and stored at −80°C. An *Escherichia coli* lawn on 14-cm plates was infected with the plasmid containing phage to give ~3×10^4 plaques/plate. Expression of the cloned sequences was induced after plaque formation by overlaying nitrocellulose filters treated with isopropyl-thiogalactoside. Duplicate nitrocellulose lifts of the lysed plaques were taken, the lifts were blocked with 1% BSA, washed, and incubated with affinity-purified IgG from anti–62-kD serum. The secondary antibody was goat anti–rabbit conjugated to alkaline phosphatase. Screening of ~5×10^5 plaques gave 16 positives, which purified to homogeneous populations. The inserts from several positive clones were subcloned into pBluescript SK+ for subsequent analysis by restriction enzyme digests, hybridization, and sequencing.

DNA Sequencing Analysis

The dideoxy chain termination method was used for sequencing from the 5′ end beginning with a T3 promoter primer and continued using oligonucleotides based on the previous sequence as primers (Sambrook et al., 1989). The sequence was checked by sequencing from 3′ to 5′. The nucleotide sequence has been entered into the GenBank data base as accession number L06038.

Membrane Isolation and Electrophoresis

Pods were removed from plants 14 to 24 DAF, and cotyledons were removed from the pod. Microsomal proteins were obtained as previously described by Ripp et al. (1988), except that the grinding buffer to seed ratio was increased to 3:1. The microsomal pellet obtained in this manner was fully resuspended in rinse buffer (25 mM potassium phosphate, pH 6.8; 5 mM β-mercaptoethanol) and repelleted by centrifugation at 85,000*g* for 1 hr. This washing was repeated zero to four times.

Membrane pellets obtained after zero to four hypotonic washes were resuspended in 25 mM Tris-HCl, pH 6.8, and 0.5% SDS to a protein concentration of 10 to 20 mg/mL. Protein was assayed with the bicinchoninic acid reagent according to the manufacturer's directions (Pierce Chemical Co., Rockford, IL). Proteins in 25 mM Tris-HCl, pH 6.8, and 0.5% SDS were heated at 80°C for 5 min and incubated on ice for 20 sec. Samples were mixed (1:1) with twice-strength Laemmli (1970) sample buffer with 8 M urea included. The samples were then centrifuged at 10,000*g* for 2 min at room temperature. Polyacrylamide gels were run according to Laemmli (1970), except that 7.5 to 15% acrylamide gradient was used with an accompanying 7.5 to 15% glycerol gradient. Gels were stained with 0.2% Coomassie Brilliant Blue R-250.

Membrane proteins resolved by SDS-PAGE were electroblotted to nitrocellulose according to Towbin et al. (1979) at 100 mA for 12 hr. The blot was blocked for 1 hr with 5% nonfat Carnation instant milk in Tris-buffered saline (TBS buffer: 20 mM Tris-HCl, 50 mM NaCl, pH 7.5) with the pH readjusted to 7.5. After blocking, the blots were incubated with affinity-purified soybean 62-kD SBP antiserum for 1 hr (1:5000 dilution) in the blocking solution. Secondary antibody was goat anti–rabbit IgG peroxidase (Pierce Chemical Co.) in the blocking solution at a concentration of 1:5000 for 1 hr. Color was developed by immersing the blot in 30 mg of 4-chloro-1-napthol (first dissolved in 10 mL of cold [−20°C] methanol) and 30 μL of 30% H_2O_2 in 50 mL of TBS.

RNA Isolation and RNA Gel Blot Analysis

Cotyledons were removed from the pod walls, frozen in liquid nitrogen, and stored at −80°C. For leaf RNA gel blot analysis, 0.5- to 1-cm (<1/8 expanded), 2.5- to 3.25-cm (~1/4 expanded), and 11- to 13-cm (fully expanded) leaves were collected and immediately frozen in liquid nitrogen. RNA was isolated by grinding 0.5 g of tissue to a fine powder with a mortar and pestle prechilled with liquid nitrogen. The powder was transferred to a warm mortar containing 9.5 mL of extraction buffer (810 mM Tris-HCl, pH 9.0; 1% SDS, 20 mM EDTA, pH 8.0)

and 0.5 mL of β-mercaptoethanol, and ground for 30 to 45 sec to thoroughly mix the material. The homogenate was centrifuged at 31,000*g* for 15 min. The supernatant was collected and extracted with an equal volume of phenol/chloroform (1:1), pH 8.0. The two layers were separated by centrifugation at 31,000*g* for 20 min at 4°C. The aqueous phase was collected and the nucleic acids were precipitated with an equal volume of isopropanol at −20°C for 1 hr. The nucleic acids were collected by centrifugation at 14,000*g* for 10 min at 4°C and then resuspended in 600 μL TE/SDS buffer (10 mM Tris-HCl, pH 7.0; 1 mM EDTA, pH 8.0, 0.1% SDS). This suspension was extracted twice with equal volumes of phenol/chloroform (1:1), pH 8.0. Nucleic acids were precipitated with 0.1 volume of 3 M sodium acetate and two volumes of 95% ethanol and stored at −20°C for 1 hr. Nucleic acids were collected by spinning at 10,000*g* for 10 min at 4°C, washed with 70% ethanol, and dried. The pellets were then resuspended in 1 mL TE/SDS buffer, 250 μL of 10 M lithium chloride was added, and the RNA was precipitated overnight at 4°C. RNA was collected by centrifugation at 10,000*g* for 10 min at 4°C, washed with 70% ethanol, and dried. Pellets were resuspended in 100 μL of TE/SDS and analyzed spectrophotometrically. Polyadenylated RNA was purified by oligo(dT)-cellulose chromatography, as described by Sambrook et al. (1989).

For RNA gel blot analysis, poly(A)$^+$ or total RNA was separated on a formaldehyde gel (1% agarose; Sambrook et al., 1989) and transferred to DuPont GeneScreenPlus membrane, according to the manufacturer's recommendations. The probe was a 1.7-kb NcoI/SpeI fragment of the 62-kD SBP cDNA labeled by random priming, according to the manufacturer's directions (Amersham). Membranes were prehybridized at 65°C for 1 hr in 2.5 mL of 1% SDS, 1 M sodium chloride, and 10% dextran sulfate. Hybridizations were carried out overnight at 65°C in the same buffer containing 1 to 5 ng/mL of denatured probe and 100 μg/mL denatured salmon sperm DNA. The blots were washed twice with 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) for 5 min each with constant agitation at room temperature, twice with 2 × SSC/1% SDS for 30 min each at 65°C, and twice with 0.1 × SSC for 30 min each at room temperature. After drying, the blot was exposed to x-ray film.

Determination of Sucrose Uptake Rate and Quantification of the 62-kD SBP

Soybean cotyledons were collected at various ages (5 to 35 DAF), and the rate of sucrose uptake was determined as described by Ripp et al. (1988). The amount of 62-kD SBP present in the cotyledons was quantitated by collecting cotyledons at various ages (5 to 35 DAF), isolating the membrane fraction, and resolving the proteins via SDS-PAGE. After transfer to nitrocellulose, the blot was reacted with anti–62-kD SBP, affinity-purified antiserum, and the level of 62-kD SBP was determined by densitometry. The three data sets (RNA blot analysis, densitometry of the 62-kD SBP, and rate of sucrose uptake) were obtained from different sets of cotyledons. These assays were repeated several times, however, and were consistent between different plants and sets of cotyledons.

Transmission Electron Microscopy Immunocytochemistry

Leaf and cotyledon tissue were sliced into pieces ~2 mm^2 and placed immediately into a vessel containing a freshly prepared fixative with the following components: 2% (v/v) paraformaldehyde, 1.25% (v/v) glutaraldehyde, 50 mM Pipes, pH 7.2, and 2 mM $CaCl_2$. Tissue was allowed to fix for 6 hr at room temperature with gentle agitation before being rinsed with 50 mM Pipes, pH 7.2, three times for 15 min and dehydrated with an ethanol series. Tissue was infiltrated with L.R. White resin and polymerized in gelatin capsules at 60°C for 24 hr. Thin sections were picked up onto nickel grids and incubated in TTBS + BSA for 1 hr at room temperature. The grids were then incubated for 4 hr in soybean 62-kD SBP antiserum at a dilution of 1:100 with TTBS + BSA. Grids were rinsed four times by immersion (5 min) in TTBS + BSA and then incubated for 1 hr in protein A–gold (15 nm; Janssen) diluted 1:50 with TTBS + BSA. After extensive washes by immersion in TTBS + BSA, TTBS, and then water, the sections were poststained with uranyl acetate, potassium permanganate, and lead citrate. Sections were examined and photographed with a Hitachi H-300 transmission electron microscope (Mountain View, CA).

Fluorescence Microscopy

Mature soybean leaves (just at 100% expansion) were cut into 5 × 10-mm pieces and immediately immersed in a fixative solution consisting (v/v) of 4% formaldehyde, 5% acetic acid, and 45% ethanol. The tissue was fixed for 16 hr, at 4°C, dehydrated with an ethanol series, infiltrated with xylene, and then 60°C melting point paraffin. The embedded tissue was sectioned at 12-μm thickness, dried onto gelatin-coated slides and heated at 55°C for 6 hr. The sections were treated with TTBS + BSA for 1 hr, then incubated for 6 hr with a 1:100 dilution of the affinity-purified, anti–62-kD SBP IgG. After extensive washing with TTBS + BSA, the sections were incubated for 1 hr with goat anti–rabbit IgG conjugated to fluorescein isothiocyanate. The sections were washed three times with TTBS + BSA, twice with TTBS, then covered with TBS, coverslipped, and examined and photographed with a Leitz Aristoplan (Rockleigh, NJ) fluorescent microscope with appropriate filters. Slides using preimmune serum, no primary serum, and no chromaphore-labeled secondary antibody were used as controls for nonspecific binding of rabbit IgG and the goat anti–rabbit secondary antibody, and for identifying and characterizing the position, color, and intensity of autofluorescence.

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