THE SITE OF STARCH SYNTHESIS IN NONPHOTOSYNTHETIC PLANT TISSUES: THE AMYLOPLAST

A hypothetic metabolic pathway must pass a crucial test to be accepted by the scientific community: Are the enzymes in the "right" compartment within the cell so that the product of one reaction can be used by the next enzyme in the pathway? Starch granules are large enough to be observed with a microscope (see Fig. 2 in the chapter, "Physicochemical Structure of the Starch Granule"). With good techniques, it is possible to see that the starch granule is enclosed within a defined structure within the cell. Are the ADPGlc PPase, the starch synthase, and the branching enzyme also there?

A large part of the biosynthetic capacity of a plant cell is localized in plastids, which are self-replicating organelles surrounded by a double-membrane envelope. Plastids are present in most cells of photosynthetic eukaryotes. In most angiosperms, however, sperm cells lack plastids, a fact that makes plastid inheritance solely maternal. The envelope is composed of an outer and inner membranes, which differ in their permeability, separated by a 10 to 20-nm gap. The plastids contain DNA that is concentrated in a section of the stroma, which is the background matrix of the plastid. The plastidial ribosomes are smaller that the cytoplasmic ribosomes.

The green chloroplasts are the site of photosynthesis; chromoplasts contain carotenoid pigments and can be found in flowers, fruits, senescing leaves, and sometimes in roots; etioplasts are present in seedlings grown in the dark. Storage plastids are designated according to the nature of the product they accumulate (i.e., protein in proteinoplasts, lipids in elaioplasts, and starch in amyloplasts). Plastids from different tissues can differ both in morphology and biochemistry, but the DNA they contain is identical (Dennis *et al.*, 1985). This indicates that all plastids arise from the same precursor plastids and later differentiate in response to the development of the tissue. For a proposal of how proplastids develop into the different types of plastids in a plastid cycle, see Whatley (1978).

Amyloplasts are characterized by the presence of one or more starch granules that grow in size as the storage organ develops, distending the plastid. The structure of the amyloplast can be studied using microscopy,

but preparation of the samples requires great care to avoid artifacts. Microscopy can also help in the localization of enzymes within the amyloplast. The study of amyloplast biochemistry and transport demands "good" amyloplasts (i.e., functional, whole plastids, clean of contaminating enzymes belonging to other cellular fractions, and with intact envelopes).

I. MICROSCOPY AND IMMUNOCYTOCHEMICAL STUDIES

Kim *et al.* (1989) employed immunocytochemistry at the light microscopy level. An antibody raised against the spinach leaf ADPGlc PPase (which specifically reacts with the potato ADPGlc PPase in immunoblotting experiments) was used on thin sections of the potato tuber, and the bound antibody was detected with either a secondary fluorescent antibody or with protein A tagged with gold particles (Fig. 1). The ADPGlc PPase was specifically localized within the amyloplast, confirming the results obtained by others (Mohabir and John, 1988) using a different methodology (see later).

Ultrathin sections for electron microscopy (thickness approximately 100 nm) must be able to withstand the electron beam and the vacuum in the microscope. For this, it is first necessary to stabilize the ultrastructure of the fresh tissue by fixation, then to dehydrate it with an organic solvent, and finally to embed it in a resin. The resulting hard block can be cut into ultrathin sections, which are then mounted on a grid and stained. For immunogold labeling, free-aldehyde groups and nonspecific binding sites

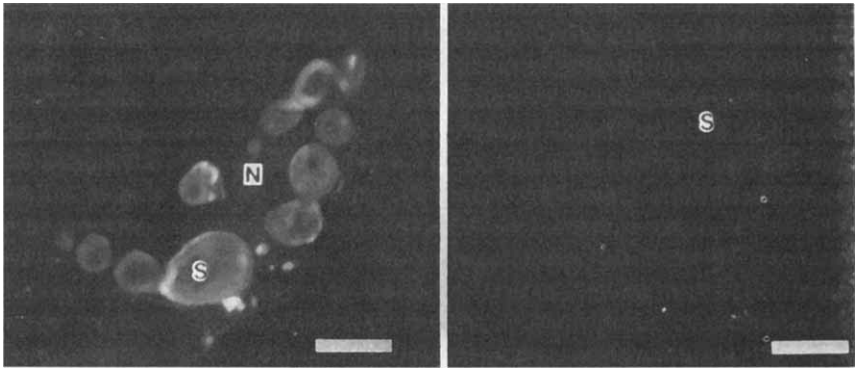

FIG. 1. *Left:* Immunofluorescence labeling for ADPGlc PPase demonstrating intense fluorescence only in the amyloplasts. Bar = 10 μm. *Right:* Preimmune control for immunofluorescence labeling. The background fluorescence is low throughout the cell. Figure reprinted with permission from Kim *et al.* (1989).

are saturated with buffers containing glycine and gelatin, and the grids are incubated with a suitable concentration of the antiserum. After washing excess serum, the sections are incubated with protein A-gold, then washed, dried, and stained with aqueous uranyl acetate (Kram, 1995).

Starch storage tissues are difficult to prepare for ultramicroscopy, because starch granules are often incompletely fixed, and as a consequence, they fold and detach from the rest of the section. Kram (1995) obtained good sections for ultrastructural research using conventional embedding of potato microtubers in Epon resin (Fig. 2). For immunolocalization, she found that slow cooling during dehydration and embedding in Lowicryl K4M (Norticon, Breda, The Netherlands) at −30°C gave the best results. Amyloplasts in potato microtubers are not identical to those present in normal tubers but are, in many respects, a good experimental system. Indeed, starch granules in potato tubers are very large, adding to the difficulty in preparing good-quality sections, but the granules in microtubers are smaller.

Electron micrographs of castor bean endosperm tissue showed the presence of proplastids with starch grains. Starch synthase was shown to be associated with the proplastid fraction of the endosperm tissue along with the starch granule (Reibach and Benedict, 1982).

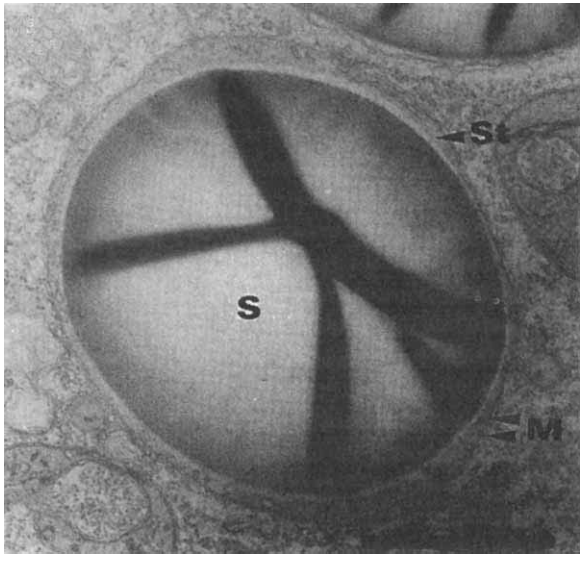

FIG. 2. Electron micrograph showing a potato microtuber amyloplast. S, starch; M, amyloplast membranes; St, amyloplast stroma. Bar = 1 μm. Figure reprinted with permission from Kram (1995).

II. CELL FRACTIONATION

Fractionation techniques relying on differential centrifugation, of the kind used for isolation of chloroplasts (see the chapter, "Starch Accumulation in Photosynthesis Cells), are inadequate because the amyloplast contains large starch granules that disrupt the integrity of the membranes even under mild centrifugal forces. Macdonald and ap Rees (1983) were successful in isolating intact amyloplasts not seriously contaminated by cytosol, from soybean cell protoplasts and showed that ADPGlc PPase and starch synthase were confined to the amyloplast. Echeverria *et al.* (1985, 1988) isolated amyloplasts from protoplasts prepared from maize endosperm harvested 14 to 17 days after pollination (DAP). Similarly, Journet and Douce (1985) isolated amyloplasts from cauliflower buds, and Macheral *et al.* (1985) and Journet *et al.* (1986) isolated them from sycamore (*Acer pseudoplatanus*) cells. Entwistle *et al.* (1988) and Entwistle and ap Rees (1988) were able to isolate amyloplasts from lysates of protoplasts obtained from the endosperm of developing grains of wheat, and Mohabir and John (1988) obtained a fraction enriched in intact potato tuber amyloplasts. The findings and conclusions reached by all these groups were similar to those of Macdonald and ap Rees (1983) (i.e., that the starch biosynthetic enzymes are primarily, if not exclusively, confined to the amyloplasts of those tissues).

In short, cell fractionation, which is composed of three steps—homogenization, fractionation, and analysis—can be an excellent way to locate an enzyme within the cell. The reader is referred to the excellent commentary by ap Rees (1995), in which the rigorous criteria to follow so that a cell fractionation provides good, reliable information is summarized. The author concludes that work done on soybean protoplasts (Macdonald and ap Rees, 1983), wheat endosperm protoplasts (Entwistle and ap Rees, 1988), wheat endosperm (Tetlow *et al.*, 1993), pea embryos (Denyer and Smith, 1988), and pea roots (Borchert *et al.*, 1993) provides further support for the view that ADPGlc PPase essentially is confined to the plastid.

In short, authors using a variety of methods and plant systems reported that in nonphotosynthetic tissues the enzymes of starch biosynthesis appear to be restricted to the amyloplast. However, two reports have proposed that a significant portion of the ADPGlc PPase activity may be present in the cytosol. Amyloplasts were isolated from wheat endosperm by Thornbjørnsen *et al.* (1996), with intactness ranging from 41 to 89%. The proportion of enzymatic activity recovered in the amyloplast fraction, in relation to total activity, was 13 to 17% for starch synthase and alkaline pyrophosphatase, and only 2.5% for the ADPGlc PPase. On this basis, the authors calculated that of ADPGlc PPase activity residing in the amyloplast was 15% of the total, and that the rest was in the cytosol. Immunologic studies

by the same authors detected two different isoforms of the ADPGlc PPase: one mainly cytosolic and the other mainly plastidial. The authors indicated that there is an excess of ADPGlc PPase activity in the amyloplast to account for the starch synthetic rate, and they were uncertain about the function of the putative cytosolic ADPGlc PPase.

In a report by Denyer *et al.*, 1996, preparations enriched in maize endosperm plastids contained 24 to 47% of the total activity of the plastid-marker enzymes, starch synthase and alkaline pyrophosphatase, but they contained only 3% of the total ADPGlc PPase activity. On this basis, the authors estimated that more than 95% of the ADPGlc PPase activity was nonplastidial. Using antibodies prepared against the *Bt* 2 subunit of the maize endosperm ADPGlc PPase, they showed that most of the *Bt* 2 protein was confined to the supernatant, and some was in the plastid. In *bt* 2 mutant kernels, the cytosolic protein that reacted with the *Bt* 2 antiserum was not detected, but there was a plastidial form of ADPGlc PPase. These data are somewhat different than what has been obtained by Miller and Chourey (1995) and by J. L. Prioul (personal communication, 1997) who, using immunogold labeling, detected the *Bt* 2 protein in the amyloplast. If the data from Denyer *et al.* (1996) are not artifactual, this would mean that there is more than one route for synthesis of ADPGlc in maize endosperm. Most authors believe that carbon translocated into the plastid via a glucose-6-P translocator is converted to ADPGlc by the action of the (plastidial) phosphoglucomutase and the ADPGlc PPase (Neuhaus *et al.*, 1993). Conversely, Denyer *et al.* (1996) believe that some ADPGlc synthesis goes on in the amyloplast, catalyzed via a plastidial ADPGlc PPase, but since in their model most of the ADPGlc is synthesized in the cytosol, it must be translocated into the plastid for starch synthesis; thus their model demands an ADPGlc transporter.

III. TRANSPORT OF CARBON INTO AMYLOPLASTS

As discussed previously, the model of Denyer *et al.* (1996), in which most of the ADPGlc is synthesized in the cytosol, demands an ADPGlc transporter if starch synthesis is to proceed (as it does) within the amyloplast. However, no protein with those properties has yet been identified. Although ADPGlc uptake by the *A. pseudoplatanus* amyloplasts has been reported (Pozueta-Romero *et al.*, 1991), Borchert *et al.* (1993) and Batz *et al.* (1994) showed that this ADPGlc transport may not be relevant physiologically. In vitro ADPGlc may be translocated via the ATP/ADP translocator, but since both ADP and ATP effectively inhibit ADPGlc uptake at concentrations lower than their physiologic concentrations (in pea-root

and cauliflower-bud amyloplasts), in vivo transport of ADPGlc by the ATP/ADP translocator is unlikely to be relevant.

It has been suggested that the *Bt* 1 gene product may be the ADPGlc transporter. The *Bt* 1 gene encodes a plastidial membrane-associated protein (Cao *et al.*, 1995; Sullivan and Kaneko, 1995), whose deduced amino-acid sequence shows similarity to known adenine nucleotide transporters (Sullivan *et al.*, 1991). The *bt* 1 mutant is starch deficient and shows a high level of ADPGlc concentration in the endosperm as compared with the normal endosperm (Shannon *et al.*, 1996). However, this is highly speculative and the *Bt* 1 protein remains to be studied and characterized, and its function remains to be determined. In conclusion, the hypothetic ADPGlc transporter required by the model used by Denyer *et al.* (1996) remains to be found.

Hypothetic models aside studies employing a variety of methods have shown that the starch biosynthetic enzymes in leaf tissue are localized in the chloroplast (see the chapter, "Starch Accumulation in Photosynthesis Cells"), and in nonphotosynthetic tissue they are localized in the amyloplast. However, an important question remains to be answered: What metabolite is transported into the amyloplast to provide carbon and energy for starch synthesis? For the chloroplast, it is clear that the main transport system for carbon is the triose-P/P_i translocator (Heber and Heldt, 1981). It had been assumed that a similar process would also be functional in the amyloplast envelope, based on the rationale that amyloplasts could develop into chloroplasts and vice versa, and some data seemed to support this view. For example, Echeverria *et al.* (1988) isolated amyloplasts from maize endosperm able to convert labeled triose-P into starch. Mohabir and Johns (1988) also suggested that potato tuber amyloplasts have a triose-P/P_i translocator when triose-P, generated by the addition of labeled fructose-1,6-bisP with aldolase, triose-P isomerase, and fructose-2,6-bisP to an intact amyloplast fraction, was converted into starch. The plastids from cauliflower buds were shown to contain all the enzymes necessary to convert triose-P to starch (Journet and Douce, 1985). Borchert *et al.* (1989) prepared amyloplasts from pea roots and identified a translocator that exchanged P_i with glucose-6-P, dihydroxyacetone-P, or 3PGA. The translocator had low affinity for 2PGA or glucose-1-P. The highest affinity was seen with dihydroxyacetone-P and P_i, and then with 3PGA and glucose-6-P. It is possible, however, that contamination from other cellular fractions may have led to artifacts.

Other evidence, however, supports the presence of a different transport system in the amyloplast envelope. Keeling *et al.* (1988) studied starch synthesis in isolated wheat endosperm tissue or in the intact plant by incubation with [1-^{13}C]- and [6-^{13}C]-glucose and by looking at the extent of the

redistribution in the glucose moieties of the starch formed. The starch was isolated and the distribution of the ^{13}C isotope was determined. If carbon flow into starch were via the triose-P isomerase, the redistribution would have been extensive. However, there was very little increase of the incidence of ^{13}C in carbons 2–5. A redistribution of 15 to 20% of label, between carbons 1 and 6 of glucose recovered, is consistent with some conversion of glucose into triose-P, resynthesis of the hexose, and its conversion into starch. Since the same redistribution was observed for sucrose, it was concluded that the redistribution occurred in the cytosol and not in the amyloplast. These data did not support a triose-P/P_i translocator as a major transport system of carbon into the amyloplast for starch synthesis, indicating that the major carbon transport system involves a sugar-P: glucose-1-P, glucose-6-P, or fructose-6-P.

Entwistle and ap Rees (1988) found that wheat endosperm lacked significant amyloplastic fructose-1,6-bisphosphatase, an enzyme that would be required if a triose-P/P_i transport system were involved in starch synthesis. In the search for a transport system to supply carbon for starch synthesis in the wheat endosperm, Tyson and ap Rees (1988) incubated intact amyloplasts with different ^{14}C-labeled compounds (i.e., glucose, glucose-1-P, glucose-6-P, fructose-6-P, fructose-1,6-bisP, dihydroxyacetone-P, and glycerol-P). Only glucose-1-P was incorporated into starch, and this incorporation was dependent on the integrity of the amyloplast. These results are consistent with the results of Keeling *et al.* (1988). Direct import of C-6 compounds has been reported for amyloplasts of potato tubers, fava beans (Viola *et al.*, 1991), maize endosperm, and suspension cells of *Chenopodium rubrum* (Hatzfeldt and Stitt, 1990). Labeled hexose monophosphates can be converted into starch by plastids isolated from wheat endosperm (Tyson and ap Rees, 1988), soybean suspension cultures (Coates and ap Rees, 1994), pea cotyledons (Hill and Smith, 1991), and cauliflower florets (Batz *et al.*, 1994).

Is there an explanation for the contradictory results obtained by different research groups? An easy explanation is the existence of very different transport systems in amyloplasts of different tissues. Another explanation is that some workers studied amyloplasts that had been damaged and/or contaminated during isolation. The permeability properties of the isolated amyloplasts depend on the degree of intactness, and the present methodology to evaluate plastid intactness measures enzyme latency (ap Rees and Entwistle, 1989) or relies on microscopic examination (Pozueta Romero *et al.*, 1991). None of these methods are sufficient to evaluate the extent of the damage suffered by the plastid envelopes or the degree of contamination of the amyloplast by other cellular components. Although intact amyloplasts are far more difficult to isolate than spinach or pea chloroplasts, it is hoped

that the more recently developed methodology will be further improved, resulting in more reliable experimental results.

Although it appears that the major carbon transport system for the wheat grain amyloplast does not involve triose-P, and most likely involves hexose-P (Fig. 3), the major carbon transport systems for other amyloplasts are not certain. More recent evidence does indicate that what is true for wheat is also true for pea embryo amyloplasts, for maize, and for potato. Thus, it may be that the major transport system for most reserve, nonphotosynthetic plant systems is at the hexose-P level and not at the triose-P level. The glycolytic scheme in the amyloplast may then take on a more important function than is observed in the chloroplast, in that the scheme aids in the production of amyloplastic ATP. Amyloplastic 3PGA may then also be an indicator of the state of ATP level in the amyloplast and, on its accumula-

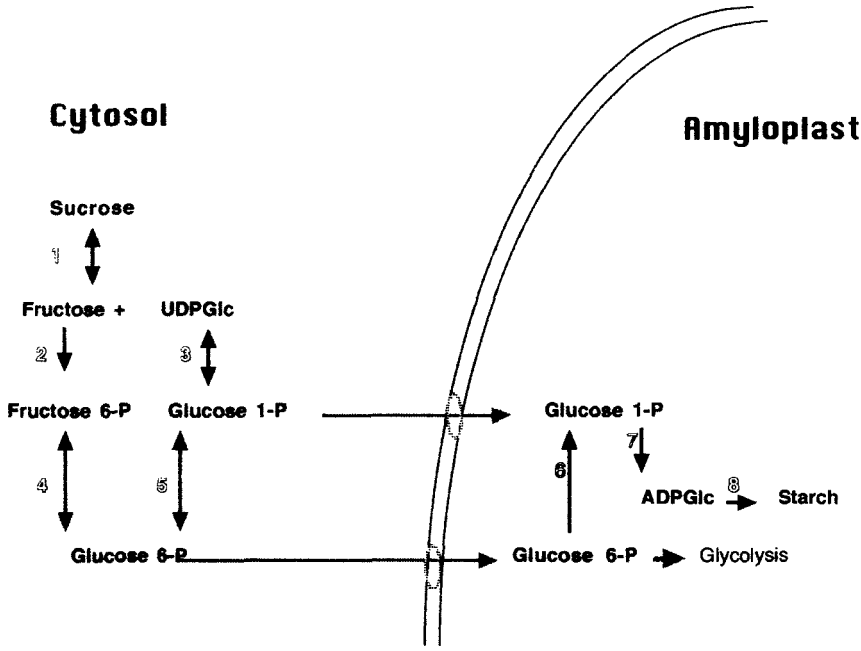

FIG. 3. Transport of carbohydrates into amyloplasts and possible routes they follow after they are inside the plastid. In the cytosol, sucrose is metabolized into glucose-6-P and glucose-1-P, which are then translocated into the amyloplast via specific translocators (ovals). The enzymatic reactions shown are as follows: 1, sucrose synthase; 2, fructokinase; 3, UDPGlc PPase; 4, cytosolic P-hexoseisomerase; 5, cytosolic P-glucomutase; 6, plastidial P-glucomutase; 7, ADPGlc PPase; and 8, starch synthase. A cytosolic ADPGlc PPase and the corresponding ADPGlc translocator proposed by some authors are not shown in this figure because the evidence supporting their existence is insufficient (see text).

tion, may be an indicator of high ATP concentration and/or carbon excess, thus stimulating starch synthesis by stimulating the ADPGlc PPase or reversing its P_i inhibition.

From the controversies that persist in the biochemistry and molecular biology of starch biosynthesis, that regarding the localization of ADPGlc PPase has been visited and revisited by researchers several times since the mid-1970s. Biochemical studies clearly indicate that the enzyme is located in the chloroplasts of leaves (see the chapter, "Starch Accumulation in Photosynthesis Cells"). Conversely (as discussed in this chapter), there is less agreement concerning the localization of the enzyme in nonphotosynthetic tissue; several methods have been used in the cereals, including immunolocalization at the electron microscopy level (Miller and Chourey, 1995) and cell fractionation followed by enzyme assay (Echeverria *et al.*, 1988); contradictory results have been published (Villand and Kleczkowski, 1994). The data obtained by Brangeon *et al.* (1997) confirm the earlier results of Miller and Chourey (1995) and provide detailed information on the correlation between the expression of ADPGlc PPase and starch accumulation within the endosperm, showing how the tissue- and cell-specific expression varies throughout the grain-filling period, as the endosperm and amyloplasts mature.

Brangeon and his collaborators (1997) studied maize kernels using light microscopy to determine the citologic structure of the fruit wall (pericarp and nucellus) and endosperm during development and filling of the grain. The authors chose four different stages representing late cellularization [8–9 days after pollination (DAP)], cell differentiation and enlargement (15 DAP and 23 DAP, respectively), and maturation of the endosperm tissue (35 DAP). For immunolocalization of the ADPGlc PPase, they used antibodies against the small or the large subunit, and they also did in situ hybridization of the corresponding mRNA transcripts.

In very young kernels, immunolabel was observed exclusively in cells of the pericarp layer, with no staining visible in the endosperm. At this early stage, there were no starch-bearing plastids in the young developing endosperm cells. By the next stage of development examined (14 DAP), the endosperm had expanded to some extent. The outer meristematic layer of cells underwent tangential divisions to produce cells to the inside, and radial divisions to extend its surface. The meristematic cells ceased to divide by 16 or 17 DAP; the outer layer cells gave raise to aleurone and subaleurone cell layers, which contained protein and lipid globules. Conversely, the endosperm contained starch, displaying a gradient of maturation in which the outer layers containing small vacuoles and storage bodies but no starch-bearing plastids; the cells toward the center were larger, with denser cytoplasm and larger starch granules within the amyloplasts. Throughout the endosperm, the strength of immunolabeling was correlated

with the number and size of the starch granules. Under high magnification, a circular pattern was seen in young amyloplasts (Kim *et al.*, 1989, in potato) interpreted by Brangeon *et al.* (1997) as reflecting a higher enzyme concentration in zones active in starch synthesis.

Cytoplasmic strands were clearly immunonegative, and the immunolabel was associated exclusively with the amyloplasts. When using cDNA probes for mRNA coding for the small or large subunits of ADPGlc PPase, the label was dispersed throughout the cytosol, as expected for an enzyme encoded by nuclear genes.

By 23 DAP, cell division had ceased, as increase in endosperm volume was by cell expansion only. The pericarp had collapsed and the aleurone and subaleurone cell layers were fully differentiated. The outer layers of the starchy endosperm contained small, rounded granules embedded in cytosol, and the more central cells were filled with starch and had very little cytosol.

At 35 DAP, the endosperm had reached its maximum size and had started drying—the cells eventually dying, with starch grains filling them completely. The pericarp was crushed by expanding endosperm, becoming the final outer fruit coat composed of thick-walled dead cells.

In short, the data obtained by Brangeon *et al.* (1997) confirm the previous work of Miller and Chourey (1995) concerning the localization of the ADPGlc PPase within the amyloplasts. It is worth noting that these two groups came to similar conclusions after working with different lines, using different methodology and different antibodies against ADPGlc PPase. Conversely, the data of Denyer *et al.* (1996), proposing that most of the ADPGlc PPase in maize endosperm is associated with the cytosol, are still to be confirmed by other laboratories. Although Brangeon and collaborators (1997) suggests a number of explanations for the results of Denyer and colleagues (1996), in our view the most likely problem was the inability of Denyer *et al.* (1996) to obtain intact amyloplasts and to take into account this lack of intactness in their interpretation of the data. Until good amyloplast preparations displaying intact envelopes are obtained, immunolocalization data such as those obtained by Brangeon *et al.* (1997) and Miller and Chourey (1995) should be preferred to data such as that from Denyer and colleagues, especially in the context of all the other evidence available.

To conclude: despite proposals presented by some authors and discussed in this chapter, the experimental evidence available supports mechanisms for the transport of carbon into the amyloplast and its conversion into starch, as depicted in Fig. 3.