Organellar Protein Synthesis Controls Amyloplast Formation Independent of Starch Synthesis Gene Expression

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Accepted October 26, 2000

Summary The transfer of stationary-phase cultured tobacco (*Nicotiana tabacum* L.) BY-2 cells into auxin-depleted culture medium induces amyloplast formation. To investigate the timing and requirement for *de novo* protein synthesis in organelles during amyloplast development and the enhancement of starch synthesis gene expression, we added chloramphenicol, at various times, to cells grown in amyloplast-inducing medium. Changes in cell growth, starch accumulation, and the mRNA levels of the ADP-glucose pyrophosphorylase small subunit (AgpS) gene were monitored. Chloramphenicol inhibited starch accumulation, but had no significant effect on cell growth, irrespective of the time of addition. RNA gel-blot analyses revealed that chloramphenicol treatment did not reduce the accumulation of mRNA from the AgpS gene, irrespective of addition time. These results suggest that organellar protein synthesis affects starch accumulation independently of starch synthesis gene expression. The necessity of organellar gene expression for starch accumulation is also discussed.

Key words Amyloplast, Differentiation, Chloramphenicol, Starch synthesis.

Amyloplasts are mature starch-containing plastids found in the differentiated cells of the root cap, as well as in storage tissues, such as endosperm, cotyledons, and tubers. They play an important role in the synthesis and accumulation of starch as a carbohydrate reserve in storage tissue, and in graviperception in the root cap cells (Kirk 1978). In addition, amyloplasts also play an important role in human nutrition, since the starch they contain provides the major source of calories in the human diet. For these reasons, detailed studies have revealed much information on the enzymatic properties of those enzymes directly responsible for starch synthesis, such as ADP-Glc pyrophosphorylase (AGPase), granule-bound starch synthase (GBSS), and starch branching enzyme (SBE) (reviewed in Smith *et al.* 1997). Accumulation patterns of AGPase, GBSS, and SBE transcripts have also been analyzed during storage tissue differentiation in several species (Dry *et al.* 1992, Visser *et al.* 1994, Ainsworth *et al.* 1995, Burton *et al.* 1995, Bachem *et al.* 1996). However, the pattern of starch synthesis gene expression, as related to amyloplast development, is unknown.

In storage organs, transported carbohydrates are converted not only into starch in plastids, but also into many other substances, such as cell wall polysaccharides, and into energy by respiration in mitochondria. The highly organized nature of storage organs makes it difficult to investigate the relationships between organellar protein synthesis and amyloplast development, so we have used a simpler *in vitro* culture system that mimics amyloplast development during the growth of tobacco BY-2 cells. High levels of transcripts for AGPase small subunit (AgpS), GBSS, and SBE accumulate under these conditions (Miyazawa *et al.* 1999). Experiments with transcription/translation in-

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hibitors have revealed that BY-2 cells require nuclear and organellar (plastid and mitochondrial) gene expressions during amyloplast formation (Sakai *et al.* 1997). Thus, at least in this *in vitro* amyloplast inducing system, organellar gene expression might have important roles in amyloplast development, although all known starch synthesis genes are encoded in the nuclear genome. Furthermore, immunoblot analysis has revealed that GBSS begins to accumulate between 6 and 12 h after transferring cells to the amyloplast-inducing medium (Sakai *et al.* 1999).

The main aim of this study was to elucidate the relationships between organellar protein synthesis and amyloplast development, especially starch synthesis and the expression of starch synthesis genes. We examined the effects of chloramphenicol treatments on cell growth, amyloplast formation, and the accumulation of *AgpS* mRNA, which encodes a key enzyme of starch biosynthesis, by adding the compound to amyloplast-inducing cultures at various times. Chloramphenicol application did not affect cell proliferation, but it did reduce starch accumulation. However, the inhibitory effect of chloramphenicol treatment became weaker as amyloplast development proceeded. RNA gel-blot analyses revealed that chloramphenicol treatment did not affect the expression of starch synthesis genes. These results suggest that protein synthesis in organelles affects starch synthesis independent of the expression of starch synthesis genes.

Materials and methods

Cell culture and induction of amyloplast formation

Tobacco (*Nicotiana tabacum*) Bright Yellow-2 cell suspension cultures were grown in a modified Murashige and Skoog's medium enriched with 0.2 mg/l 2,4-D, and were maintained as described by Nagata *et al.* (1992). Amyloplast formation was induced by transferring 5 ml aliquots of stationary-phase cells into 95 ml of F-medium (modified Murashige and Skoog's medium without 2,4-D), and the cells were cultured as described previously (Miyazawa *et al.* 1999).

Treatment with inhibitors

Chloramphenicol was dissolved in dimethyl sulfoxide (DMSO) at 50 mg/ml. After filter-sterilization, this stock solution was added to cultures to a final concentration of 10 mg/l chloramphenicol. For control cultures, an equivalent volume of filter-sterilized DMSO was added to the culture medium.

Cell counts and measuring starch content

The number of cells per milliliter of culture was counted microscopically. For starch estimation, cells derived from 1 ml of culture were collected and treated with a solution of 0.4 M mannitol, 1% cellulase YC (Kikkoman Co. Ltd., Tokyo, Japan), and 0.1% pectolyase Y23 (Kikkoman Co. Ltd., Tokyo, Japan), pH 5.5, for 90 min at 30°C to make protoplasts. SDS was added to a final concentration of 1% to lyse the protoplasts, and the starch granules were pelleted by centrifugation at 18500 g for 15 min. After extraction with hot water and perchloric acid as described previously, the starch was quantified by the phenol-sulfuric method (Dubois *et al.* 1956).

Microscopic observations

The cell suspensions were observed by bright-field microscopy immediately after sampling. For phase-contrast microscopic observations, cells were treated with a solution of 0.4 M mannitol, 1% (w/v) cellulase YC, and 0.1% pectolyase Y23 for 90 min to make protoplasts. These were then fixed with 1% (w/v) glutaraldehyde dissolved in buffer (20 mM Tris-HCl pH 7.6, 0.5 mM EDTA, 1.2 mM spermidine, 7 mM 2-mercaptoethanol, and 1.4 mM PMSF), and observed under a microscope equipped with phase-contrast optics.

RNA isolation and RNA gel blot analysis

Cells were collected by centrifugation, frozen in liquid nitrogen, and stored at -80°C until RNA extraction. Approximately 1 ml of frozen pelleted cells was ground to powder in liquid nitrogen and homogenized in 5 ml of extraction buffer (300 mM NaCl, 50 mM Tris-HCl pH 7.6, 100 mM EDTA, 2% Sarkosyl, 4% sodium dodecyl sulfate) that was pre-warmed to 65°C . Following extractions with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1), the nucleic acids were precipitated by adding an equal volume of 2-propanol and were then dissolved in 1.0 ml of sterile water. Finally, 0.33 ml of 10 M LiCl was added to precipitate the RNA. After incubation at 4°C for 2 h, the RNA was collected by a 15 min centrifugation at 18500 g.

The RNA was denatured with glyoxal and subjected to RNA gel blot analysis (Sambrook *et al.* 1989). The RNA was transferred to a Hybond-XL membrane (Amersham Pharmacia Biotech, UK). Cloned fragments of *AgpS* cDNA were used as probes to detect transcripts as described in Miyazawa *et al.* (1999), and hybridized in Rapid Hyb buffer (Amersham Pharmacia Biotech, UK). The blots were washed twice in $2\times\text{SSC}$ ($20\times\text{SSC}=3\text{M NaCl}$, 300 mM trisodium citrate), 0.1% SDS for 15 min at room temperature, once in $1\times\text{SSC}$, 0.1% SDS for 15 min at 65°C , and once in $0.1\times\text{SSC}$, 0.1% SDS for 15 min at 65°C . Autoradiography was performed at -80°C with Kodak X-Omat film (Kodak, Rochester, NY) with an intensifying filter.

Results

Effect of the addition of chloramphenicol to BY-2 cells on cell proliferation and starch accumulation

Previously, we reported that chloramphenicol and cycloheximide, which inhibit protein synthesis in organelles (i.e., plastids and mitochondria) and in the cytosol, respectively, inhibit amyloplast formation (Sakai *et al.* 1997). To assess the timing of protein synthesis responsible for amyloplast formation in organelles, we treated BY-2 cells with 10 mg/l chloramphenicol at 0, 6, 12, 18, and 24 h after transfer to F-medium, and monitored cell proliferation and starch accumulation. As a control, DMSO alone was added (see Materials and methods). A slight but significant ($p>0.05$) reduction in cell multiplication was observed only when chloramphenicol was added immediately after transfer to F-medium (Fig. 1, left panel). When chloramphenicol was added after 6 h, no obvious reduction of cell proliferation was observed. Since cell growth is repressed in F-medium (Miyazawa *et al.* 1999), this indicates that the inhibition of protein synthesis in organelles has little further effect on repressing cell growth. In contrast, starch accumulation was repressed by chloramphenicol application, irrespective of the time of addition (Fig. 1, right panel). Strong inhibition of starch accumulation was observed in the cells when chloramphenicol was added up to 18 h after transfer. However, when chloramphenicol was added 24 h after transfer, a less inhibitory effect on starch accumulation was observed. This indicates that *de novo* protein synthesis in organelles is necessary at the early stages (i.e. within 18 h after transfer) of amyloplast formation.

Since amyloplast formation in BY-2 cells involves drastic changes in plastid morphology (Miyazawa *et al.* 1999), we observed chloramphenicol-treated BY-2 cells to examine the effect on plastid morphology and growth of starch granules. After 48 h, cultured control cells treated with DMSO contained many amyloplasts, and each amyloplast contained several starch granules (Fig. 2a, g). When chloramphenicol was added to amyloplast-induced BY-2 cells, the treated cells contained fewer amyloplasts than control cells (compare Fig. 2a to 2b–f). The inhibitory effect was reduced when chloramphenicol was added at 18 or 24 h after transferring to F-medium. This coincides with the biochemical measurement of starch accumulation (Fig. 1). The overall morphology of the chloramphenicol-treated plastids was similar to that of control cells (i.e., round or ellipsoidal shape); however, starch granule enlargement was inhibited. We measured the diameters of starch granules in the chloramphenicol-treated BY-2 cells and compared them to those of control cells (Table 1). The mean diameter of the starch granules was always significantly smaller in chloram-

Fig. 1. Effect of chloramphenicol application on growth and starch accumulation during amyloplast formation in BY-2 cells. The panels on the left show the change in cell number, while the panels on the right show the changes in starch content during the culture. Arrowheads indicate the time of chloramphenicol addition (final concentration 10 mg/l). Data are the means of 3 independent experiments. Vertical bars represent the S.D. ○: control cells (DMSO added immediately after transfer), ●: sample cells (chloramphenicol added during culture). BY-2 cells ceased starch accumulation whenever chloramphenicol was added to the culture, whereas the effect of inhibitor addition on cell proliferation was relatively small.

phenicol-treated cells than in control cells ($p < 0.05$). Moreover, the earlier the chloramphenicol treatment, the greater the inhibition of starch granule enlargement. Therefore, chloramphenicol inhibits the enlargement of starch granules, rather than morphological changes of plastids.

Fig. 2. Photomicrographs of chloramphenicol-treated plastids during amyloplast formation. Chloramphenicol was added 0 (b, h), 6 (c, i), 12 (d, j), 18 (e, k), and 24 (f, l) h after cells were transferred to F-medium. The cells were cultured for a total of 48 h and compared with cells cultured in F-medium for 48 h (a, g). Arrows indicate amyloplasts. Arrowheads show starch granules. The bars in f and l represent 50 μm and 5 μm , respectively.

Inhibition of protein synthesis in organelles does not affect AgpS gene expression

Since all known starch synthesis genes are encoded in the cell nucleus and chloramphenicol inhibits starch biosynthesis during amyloplast formation, we examined the relationship between the expression of starch synthesis genes and organellar proteins synthesized *de novo* by RNA gel-blot analyses. We chose *AgpS* cDNA as a probe to detect transcripts, because AGPase is widely believed to catalyze the first rate-limiting step in starch biosynthesis (Preiss 1991). Since the inhibitory effect of chloramphenicol on starch accumulation varied with the timing of inhibitor addition, we added chloramphenicol to cultures growing in amyloplast-inducing medium at 6 h intervals and analyzed the change in the amount of *AgpS* transcripts. As shown in Fig. 3, *AgpS* mRNA levels were not affected by chloramphenicol addition, irrespective of the timing of addition. Therefore, the reduction in starch accumulation by chloramphenicol is not caused by a reduction in *AgpS* expression, but rather by unknown *de novo*-synthesized or-

Table 1. Mean diameter of chloramphenicol-treated starch granules

Samples (n)	Mean diameter (μm)
Control (55)	1.30 ± 0.36
Addition at 0 h (47)	0.65 ± 0.42
Addition at 6 h (79)	0.66 ± 0.30
Addition at 12 h (108)	0.69 ± 0.32
Addition at 18 h (97)	0.89 ± 0.22
Addition at 24 h (140)	1.00 ± 0.30

The mean diameters of randomly chosen starch granules from chloramphenicol-treated BY-2 cells, which were cultured for 48 h, were measured. The number of starch granules measured is shown in parentheses. Statistical significance was determined using Student's two-tailed *t* test ($p < 0.05$).

Fig. 3. Response of *AgpS* gene expression to chloramphenicol addition during amyloplast formation in BY-2 cells. Chloramphenicol was added to the culture 0, 6, 12, 18, and 24 h after transferring stationary-phase cells to F-medium. Total RNA was extracted from the cells 0, 6, 12, 18, 24, and 48 h after transfer and subjected to RNA gel blot analyses using a fragment of the *AgpS* cDNA as a probe. For detection, each lane was loaded with 10 μg of total RNA. Arrowheads indicate the time of inhibitor addition.

ganellar protein(s). Since the expression of the *AgpS* gene was neither lowered nor stimulated by chloramphenicol treatment, we concluded that *de novo*-synthesized organellar protein(s) affect starch accumulation in a manner other than the regulation of *AgpS* gene expression during amyloplast formation.

Discussion

Amyloplast formation is inhibited by the inhibition of de novo protein synthesis in organelles

Chloramphenicol was used to examine the contribution of *de novo* protein synthesis in organelles during amyloplast formation. Chloramphenicol treatment did not significantly affect cell multiplication, whereas it did inhibit starch accumulation. It is not clear why chloramphenicol did not inhibit cell proliferation, because the role of protein synthesis in organelles during cell proliferation cannot be examined in F-medium culture, in which cell proliferation is repressed. However, it is likely that under such growth-repressing conditions, the energy obtained from glycolysis alone (i.e., not from respiration) is sufficient for cells to maintain their state. Microscopic observation revealed that the overall morphology of chloramphenicol-treated plastids was similar, irrespective of the timing of inhibitor addition, although the starch granules varied in size (Table 1). There are two possible explanations for the effect of chloramphenicol treatment on the inhibition on starch accumulation. First, chloramphenicol might inhibit mitochondrial protein synthesis, so that BY-2 cells can only obtain energy from glycolysis instead of respiration. Therefore, the carbohydrate resource for starch synthesis is exhausted, and thus starch accumulation is inhibited. In this case, the reduced inhibitory effect of chloramphenicol observed when the compound was added at later stages of amyloplast formation can be explained as follows: a certain amount of resources for starch synthesis, such as ADP-Glc, has already accumulated before inhibitor addition, and starch accumulated until these resources were exhausted. Alternatively, chloramphenicol might inhibit protein syntheses, which is encoded by the plastid or mitochondrial genome, necessary during the early stages of amyloplast formation. This protein might be encoded in one of the many open reading frames in the plastid or mitochondrial genomes that have no defined function. The measurement of metabolites resulting from glycolysis and respiration and the study of specific open reading frames in the plastid and mitochondrial genomes will clarify this question.

Relationship between de novo protein synthesis and the expression of starch synthesis genes

Previously, we reported that the expression of starch synthesis genes (*AgpS*, *GBSS*, *SBE*) coincided with starch accumulation (Miyazawa *et al.* 1999). In this study, we analyzed the relationships between the synthesis of protein in organelles and the expression of starch synthesis genes by adding chloramphenicol at 6 h intervals to cultures of BY-2 cells in the early stages of amyloplast formation. When chloramphenicol was added to amyloplast-inducing BY-2 cells, the expression of starch synthesis genes was not affected. Since these transcripts are translated in the cytosol, the starch synthesis proteins are assumed to be synthesized normally, although it is not clear whether these proteins are transported into plastids in their active form. In contrast, expression of *AgpS* and *GBSS* genes is affected by cycloheximide addition (Miyazawa *et al.* submitted). Furthermore, expression of these genes required the *de novo* synthesis of protein in the cytosol. These results indicated that expression of the starch synthesis genes is regulated by cytosolic factors rather than by organellar protein. Since genetic modification of starch synthesis gene expressions alters the quality and quantity of starch, starch accumulation is responsible for starch synthesis gene expression to some extent (reveiwed in Slattery *et al.* 2000). However, our results indicated that starch accumulation is also regulated by *de novo*-synthesized organellar protein during amyloplast development. Moreover, this organellar factor affected starch content independent of starch synthesis gene expression.

With an increasing population, it would be beneficial to increase the yield of starch by modifying the gene expression responsible for starch accumulation. Several projects have been established to engineer increased quantity and quality of starch by genetic modification of the starch biosynthetic pathway (reviewed in Slattery *et al.* 2000). From our results, we believe that consideration of organellar function, along with the expression of starch synthesis genes, is critical to understanding starch storage in plants.

Acknowledgements

This work was supported by a research fellowship to Y. M. (no. 5122) from the Japanese Society for the Promotion of Science for Young Scientists and by a Grant-in-Aid to T. K. (no. 12440222) from the Ministry of Education, Science, Sports and Culture of Japan.

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