Characterization and Intraorganellar Distribution of Protein Kinases in Amyloplasts Isolated from Cultured Cells of Sycamore (Acer pseudoplatanus L.)

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ABSTRACT

Incubation of amyloplasts isolated from cultured cells of sycamore (Acer pseudoplatanus L.) with [γ-32P]ATP resulted in the rapid phosphorylation (half-time of 40 seconds at 25 degrees Celcius) of organellar polypeptides. The preferred substrate for amyloplast protein kinases was Mg2+-ATP, and recovery of only [32P]serine after partial acid hydrolysis indicated the predominance of protein serine kinases in the organelle. These activities were located in the envelope and stromal fractions of the plastid, which showed different specificities toward exogenous protein substrates and distinct patterns of phosphorylation of endogenous polypeptides. A 66-kilodalton polypeptide, inaccessible to an exogenously added protease, was one of the major phosphorylated products found in intact amyloplasts at low [γ-32P] adenosine triphosphate concentrations. This polypeptide represented the major phosphoprotein observed with the isolated envelope fraction. The patterns of polypeptide phosphorylation found in intact amyloplasts and chloroplasts from cultured cell lines of sycamore were clearly distinguishable. The overall results indicate the presence of protein phosphorylation systems unique to this reserve plastid present in nonphotosynthetic tissues.

The amyloplast is a uniquely differentiated plastid present in nonphotosynthetic plant tissues. An active gluconeogenic pathway leading to starch formation when sucrose supply is plenty and the degradation of this reserve material in starvation conditions are major metabolic pathways occurring in this organelle (5). Although amyloplasts and chloroplasts are probably ontogenically related, their functions and metabolism are clearly distinct (5, 10). Chloroplasts are photosynthetic, ATP-generating "source" organelles, whereas amyloplasts must import the ATP needed for starch synthesis and other processes and can be categorized as "sink" plastids (19).

Reversible protein phosphorylation is a widely occurring posttranslational modification that regulates biological processes such as glycogen synthesis/degradation and other metabolic pathways, hormonal responses, cell growth and differentiation, etc. (3, 6, 7, 24–26). Among plant organelles, chloroplast polypeptides located in the thylakoids, stroma, and envelope have been shown to undergo reversible phosphorylation (3, 6, 7, 9, 12, 14, 17, 24, 27, 28).

We (18) previously communicated that an active system of protein phosphorylation operates in amyloplasts isolated from cultured cells of sycamore (Acer pseudoplatanus L.). In the present work we studied the distribution of the protein kinases in the amyloplast compartments (envelope, stroma, and starch), the phosphopolypeptide pattern obtained in these fractions and the intact organelle, and their comparison to those obtained with chloroplasts from a green cell line of sycamore.

MATERIALS AND METHODS

Materials

[γ-32P]ATP (3000 Ci/mol) was purchased from Amersham. Partially hydrolyzed casein, phosvitin, histones, phosphorylase b, phosphorylase kinase, trypsin, and soybean trypsin inhibitor were from Sigma Chemical Co. Other chemicals were of analytical grade. Purified amyloplast phosphorylase was a generous gift of Dr. T. Takabe.

Methods

Plastid Isolation and Preparation of Suborganellar Fractions

Isolation of intact amyloplasts and chloroplasts from protoplasts obtained from suspension-cultured white and green cells of sycamore (Acer pseudoplatanus L.), respectively, was carried out using discontinuous Percoll gradients as described previously (18). Isolation of suborganellar fractions from hypotonically lysed plastids was performed as described previously (12, 13, 19, 21, 22). The purity of the isolated fractions was assessed by comparison with the polypeptide patterns obtained after SDS-PAGE, which was reported previously (12, 19, 22), and by measuring the activity of the marker enzymes 6-PG4 dehydrogenase (stroma) and Mg2+-ATPase (envelope) (see below).

Abbreviation: 6-PG, 6-phosphogluconate.
Phosphorylation of Endogenous and Exogenous Substrates

The reaction was carried out at 25°C in a medium containing amyloplasts, chloroplasts, or the isolated fraction (25–50 μg protein), 50 mM Tricine-NaOH (pH 7.5), 10 mM MgCl2, 1 mM EDTA, 0.5 M mannitol, 50 μM [γ-32P]ATP (containing 500–1000 cpm/pmol), and, where indicated, 50 μg of the exogenous substrate or the additions indicated in the legends of the table and figures, in a final volume of 50 μL. The reaction was started by the addition of labeled ATP, and 10-μL aliquots were taken at 15, 30, 45, and 60 s for the determination of protein kinase activity or at the indicated times for time-course reactions. The aliquots were spotted on phosphocellulose paper (Whatman P-81, 2 × 2 cm), and the reaction was terminated by adding the pieces of paper to 10% (w/v) TCA containing 10 mM PPI and washing repeatedly with the same solution. The paper pieces were finally washed with ethanol and ethanol:diethyl ether (1:1) and were dried in air before Cerenkov counting with a liquid scintillation counter.

Trypsin Pretreatment of Intact Amyloplasts

Trypsin pretreatment of intact amyloplasts was performed as described before (19) to determine the intactness of isolated amyloplasts as well as to evaluate the phosphorylation pattern obtained after trypsin digestion of the intact plastids. Based on the retention of intraorganellar 6-PG dehydrogenase in the presence of trypsin, i.e. a latency assay (19), the intactness of the plastids used in the experiments reported in this work varied between 80 and 90%.

To determine the effects of the protease pretreatment on the phosphorylation pattern of intact amyloplasts, plastids were isolated in the absence of protease inhibitors. Incubation with protease was conducted at 25°C in 50 mM Tricine-NaOH (pH 7.5), 0.5 M mannitol, 1 mM EDTA, amyloplasts (equivalent to 1.5 mg protein), and 0.1 mg trypsin, in a final volume of 0.1 mL. At the times indicated in the legends to figures, 0.3 mg of trypsin inhibitor was added, and the phosphorylation reaction was carried out as described above.

Gel Electrophoresis

For the determination of the phosphorylation pattern of endogenous polypeptides, the phosphorylation reaction was carried out as described above for 1 min at the [γ-32P]ATP concentrations indicated in the legends to the figures. The reaction was terminated by the addition of ice-cold TCA (final concentration, 20%, w/v), and the samples were processed for SDS-PAGE essentially as described previously (23), except that they were incubated at 50°C for 15 min in SDS-sample buffer before electrophoresis. Incubation at higher temperatures was found to increase the amount of phosphorylated protein aggregates that did not enter the gel. Previously described procedures were used for SDS-PAGE (23). After electrophoresis, gels were immersed in 20% (w/v) TCA for 12 h at room temperature to fix polypeptides and to remove nonspecifically bound 32P, stained with Cooomassie blue, and destained (23). After the gels were dried, the labeled polypeptides were detected by autoradiography using Fuji RX Safety x-ray films plus intensifying screens.

Phosphopeptide Mapping

Phosphorylated samples were resolved by one-dimensional SDS-PAGE (23), and the gel was stained and destained as described above. After the phosphopeptide of interest was located by autoradiography, the band was excised from the gel and processed for Staphylococcus V8 protease treatment in a second polyacrylamide gel essentially as described (8).

Phosphoamino Acid Analysis

Intact amyloplasts were incubated with [γ-32P]ATP in the conditions described above, and the reaction was terminated by addition of TCA (final concentration, 50%, w/v) after 1 min. The protein pellet was collected by centrifugation and washed three times with 0.5 ml of 25% (w/v) TCA to remove nonbound radioactivity, once with ethanol and once with ethanol:diethyl ether (1:1). After evaporation of the solvent, the samples were resuspended in 0.1 mL of 6 M HCl, heated at 110°C for 1.5 h, and processed as described previously (17), except that cellulose sheets were used for phosphoamino acid resolution by one-dimensional high-voltage electrophoresis. Spinach thylakoids, phosphorylated with [γ-32P]ATP in the light (11), were used to verify recovery of [32P]Ser and [32P]Thr.

Other Methods

Assay of the marker enzymes 6-PG dehydrogenase and Mg2+-ATPase was carried out as indicated previously (12, 13, 18, 19). The protein content of amyloplast and chloroplast preparations was determined as described before (12, 13), using BSA as standard.

RESULTS

Protein Phosphorylation/Dephosphorylation in Isolated Amyloplasts

We (18) previously demonstrated the phosphorylation of several polypeptides when amyloplasts isolated from cultured sycamore cells were incubated with [γ-32P]ATP. Since then we have studied, in more detail, some properties of these phosphorylation reactions, as well as the suborganellar location of the protein kinases and their endogenous substrates. In Figure 1, the time course of 32P incorporation from [γ-32P]ATP into amyloplast polypeptides is shown. As shown, phosphorylation of endogenous protein substrates in the intact plastids reaches completion within 2 to 3 min of incubation at 25°C (half-time of 32P incorporation, approximately 40 s). Addition of nonlabeled ATP (2 mM) to the reaction mixture induces a rapid decline of approximately 50% of the incorporated 32P in <2 min. These results indicate the presence of a reversible phosphorylation of endogenous polypeptides in intact amyloplasts (also see ref. 18).

Nucleotide and Cation Requirements for the Amyloplast Protein Phosphorylation System

The range of ATP concentrations at which this reaction occurs was evaluated. Phosphorylation reached a maximum at 50 μM [γ-32P]ATP and remained stable at higher ATP concentrations.
concentrations (Fig. 2A). Concerning other putative nucleotide substrate(s), no inhibition of the phosphorylation reaction using [γ-32P]ATP as donor was observed in the presence of 0.2 mm of other nucleotide triphosphates (GTP, CTP, UTP) except for dATP, which inhibited the reaction approximately 30% (not shown). The presence of cyclic nucleotides (cAMP or cGMP) in the concentration range of 5 to 200 μM did not alter the kinetics or extent of 32P incorporation into amyloplast polypeptides (not shown). Essentially the same results as those reported above were obtained in the presence of 2% Triton X-100 (not shown), thus indicating that these effects are independent of a preferential transport across the plastid envelope.

A bivalent cation was essential for protein kinase activity, because the phosphorylation reaction was almost completely inhibited in the presence of 10 mm EDTA (18). Mg2+, much less effectively Mn2+ and, interestingly Co2+, were used as cofactors in the reaction (Fig. 2B). Although Ca2+ could support phosphorylation up to 2.5 mm, higher concentrations were inhibitory (Fig. 2B). Other bivalent cations tested, such as Zn2+, Sr2+, and Cu2+, were not cofactors in the phosphorylation reaction (not shown). The same results were obtained with Triton X-100-disrupted amyloplasts (not shown).

The overall results indicate the existence of a cyclic nucleotide-independent protein phosphorylation system in amyloplasts, in which Mg2+-ATP is the preferred substrate. This system occurs well within the ATP concentrations found in plant cells and is sufficiently rapid to be physiologically relevant.

Phosphorylation of Exogenous Proteins

We described previously that amyloplast protein kinases can phosphorylate various artificial substrates, including casein, phosvitin, and histones (18). A relationship between starch metabolism and protein phosphorylation has been postulated from studies in which cultured cells of tobacco and carrot were used (4). Therefore, it was of interest to determine whether amyloplast protein kinases were able to phosphorylate enzymes involved in starch metabolism or to recognize the described domains present in enzymes of glycogen metabolism such as glycogen phosphorylase or phosphorylase kinase (25). Amyloplast phosphorylase was of particular interest because its reported molecular mass (approximately 100 kD; ref. 12) is coincident with that reported for a phosphorylated polypeptide found in disrupted amyloplasts (18). However, neither of the above described enzymes was phosphorylated to an appreciable extent when added exogenously to intact or disrupted amyloplasts (not shown). In addition, an antiserum against amyloplast phosphorylase (12) also failed to precipi-
In chloroplasts, several protein kinase activities have been described in the stromal fraction, as well as in the thylakoid and envelope membranes (3, 6, 7, 9, 11, 14, 17, 24, 27, 28). We thought it was of interest to determine the distribution of these activities within the different fractions (stroma, envelope, and starch granules) that constitute the amyloplast. The results obtained in two different experiments are shown in Table I. Amyloplast protein was found to be distributed between stromal (approximately 70%) and envelope (approximately 30%) fractions, being only 2 to 3% associated with starch granules. The purity of the mentioned fractions was assessed by analyzing the marker enzymes Mg$^{2+}$-ATPase (envelope) and 6-PG dehydrogenase (stroma) (12, 13, 18, 19). As judged by the recovery of these enzymes, the envelope and stromal fractions were highly purified (97–98%, columns 3 and 4, respectively).

Protein kinase activities were evaluated by measuring endogenous polypeptide phosphorylation as well as phosphorylation in the presence of an exogenous protein substrate, such as casein (experiment 1) or phosvitin (experiment 2). The experiments shown in Table I, column 7, indicate slightly higher phosphorylating activities acting on endogenous substrates in the stromal fraction in comparison to the envelope (61 versus 38% recoveries of total units, respectively, in experiment 1 and 59 versus 40%, respectively, in experiment 2). The reverse situation was found when phosphorylating activities were measured in the presence of casein or phosvitin (column 8 of experiments 1 and 2). The marked increase in total units in the presence of either casein or phosvitin and in the specific activities (column 6) in the envelope in comparison to the stroma indicates that the former compartment is relatively enriched in protein kinases able to phosphorylate exogenous substrates. On the other hand, the kinases present in the stroma phosphorylated endogenous substrates preferentially, indicating higher specificities in their substrate requirements. It is worth noting that the kinases present in chloroplast envelopes have been reported to be unable to phosphorylate exogenous proteins (28), in sharp contrast to those of the amyloplast envelopes (this work).

The phosphorylating activities recovered in starch granules are almost negligible (Table I, columns 7 and 8) and may well represent contamination from other fractions, especially the envelope, as judged by the distribution of marker enzymes (column 3).

### Effect of Varying ATP and Trypsin Treatment of Intact Plastids

The pattern of phosphorylation of chloroplast polypeptides varies with the [$\gamma$-$^{32}$P]ATP concentration used during the assay (27). We found a similar situation in the case of intact amyloplasts. In Figure 3, A to E, the phosphorylation pattern of intact amyloplasts at various ATP concentrations (from 3–50$\mu$M) can be observed. At 25 to 50$\mu$M [$\gamma$-$^{32}$P]ATP, numerous polypeptides are found to be labeled (Fig. 3, D and E). It is also observed that a 66-kD polypeptide appears as a prominent phosphorylation product at the lower [$\gamma$-$^{32}$P]ATP concentrations (Fig. 3, A–C). The same results (i.e., the 66-kD polypeptide as the major labeled product) are obtained at [$\gamma$-$^{32}$P]ATP concentrations as low as 50 nM (not shown). It is interesting to note that most of the major phosphorylated polypeptides labeled by 50$\mu$M [$\gamma$-$^{32}$P]ATP in disrupted organelles (18) are also observed in the intact plastids (Fig. 3), except for the conspicuous absence of a 100-kD phosphorylpeptide (18). Interestingly, phosphorylation of this polypeptide was found to be dependent in the presence of Ca$^{2+}$ and inhibited by calmodulin antagonists (18). Therefore, these results suggest a tight regulation of the phosphorylation of this 100-kD polypeptide in intact organelles. The overall results indicate

### Table I. Distribution of Protein Kinase Activities in the Different Amyloplast Fractions

<table>
<thead>
<tr>
<th>Recovered Fraction (1)</th>
<th>Protein (2)</th>
<th>Marker Enzyme</th>
<th>Protein Kinase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mg-ATPase (3)</td>
<td>6-PGDH (4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mg</td>
<td>total units$^a$</td>
</tr>
<tr>
<td><strong>Experiment 1</strong> (casein as substrate)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stromal</td>
<td>9.5 (70)</td>
<td>ND$^{c}$</td>
<td>6.9 (98)</td>
</tr>
<tr>
<td>Envelope</td>
<td>3.8 (28)</td>
<td>19.1 (98)</td>
<td>0.1 (1)</td>
</tr>
<tr>
<td>Starch</td>
<td>0.3 (2)</td>
<td>0.3 (2)</td>
<td>0.1 (1)</td>
</tr>
<tr>
<td><strong>Experiment 2</strong> (phosvitin as substrate)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stromal</td>
<td>8.6 (70)</td>
<td>ND</td>
<td>6.2 (98)</td>
</tr>
<tr>
<td>Envelope</td>
<td>3.3 (27)</td>
<td>17.4 (97)</td>
<td>0.1 (2)</td>
</tr>
<tr>
<td>Starch</td>
<td>0.4 (3)</td>
<td>0.8 (3)</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Defined as nmol·min$^{-1}$.  **Defined as pmol·min$^{-1}$.  *Non-detectable.

(Continued on next page)
the presence of protein kinases with different affinities and/or accessibilities to $\gamma^{32}P$ATP in the intact plastid.

To obtain information concerning the localization of the protein kinases and their polypeptide substrates shown in Figure 3, A to E, the intact plastids were pretreated with trypsin before the phosphorylation reaction. The results are shown in Figure 3, F to J. Trypsin treatment of the intact plastids did not alter the labeling of the 66-, 59-, 50-, or 38-kD polypeptides (closed arrowheads), indicating that neither these proteins nor their kinase(s) are accessible to the protease. On the other hand, phosphorylation of polypeptides of 43, 36, 33, and 18 kD was abolished (open arrowheads), thus suggesting their presence (or their kinase) in the outer membrane of the plastid. It is worth noting that retention of the internal marker 6-PG dehydrogenase (latency assay) was between 80 and 90% and that analysis of Coomassie-stained gels showed no detectable proteolysis of envelope polypeptides (not shown), thus indicating that the integrity of the plastids was preserved during the trypsin treatment.

**Polypeptide Phosphorylation in Amyloplast Fractions**

We analyzed the phosphorylation pattern of endogenous polypeptides in the different compartments of the amyloplast (Fig. 4). It is obvious that, other than some minor bands, the 66-kD polypeptide constitutes the major component in the envelope fraction (lane B). Evidence that this 66-kD phosphoprotein-polypeptide is identical with the one observed in total plastids (lane A) was obtained after phosphopeptide mapping using *Staphylococcus* V8 protease (Fig. 5). These results suggest that the 66-kD phosphoprotein and its kinase are located in the amyloplast envelope.

The stromal phosphorylation pattern is shown in Figure 4C. In this compartment, major labeled bands have apparent molecular masses of 66, 59, 54, 45, and 37 kD (indicated by asterisks). Longer exposure times revealed an extra 100-kD component (not shown). This pattern is clearly distinguishable from the one obtained in the envelope (Fig. 4B), thus indicating the presence of distinct protein kinases and/or phosphoprotein-polypeptides in each compartment.

The presence of the 66-kD phosphoprotein in the stroma (Fig. 4C) exceeded the level of cross-contamination from the envelopes detected in this fraction (see Table I, column 3). In fact, radioactivity measurements of the excised 66-kD bands shown in Figure 4 (lanes B and C) indicated 1172 and 166 cpm (approximately 14%), respectively. Treatment of the envelope fraction with 1 M NaCl before collecting the membranes did not result in a significant modification of the phosphorylation pattern shown in Figure 4B (not shown). Moreover, an analysis of phosphorylated envelope fractions or total amyloplasts by the detergent-blotting technique (15) indicated that the 66-kD phosphoprotein remained in the original gel or was completely retained by the nonionic detergent layer (not shown), suggesting hydrophobic properties.
characteristic of membrane proteins. Therefore, the presence of this band in the stroma may represent contamination from the envelope (detectable because of its particular affinity for ATP) or a partitioning of the 66-kD polypeptide/kinase complex between envelope and stroma as described for other protein kinase systems (3).

The phosphorylation pattern of the starch fraction showed a diffuse smear of radioactivity (Fig. 4D). Minor bands seem to represent contamination from the envelope (although note that the 66-kD band is missing, see below).

We did not observe additional prominent polypeptides when envelopes and stroma were mixed before the phosphorylation reaction, although we observed a reduction in the phosphorylation level of the stromal polypeptides (Fig. 4, E versus C). We also noted that the starch fraction, when present simultaneously in the assay mixture, caused a marked reduction in the phosphorylation pattern of the envelope and stroma fractions (Fig. 4, F and G, respectively). For example, the incorporation of radioactivity into the 66-kD envelope polypeptide was reduced from 2446 (lane E) to 90 cpm in the presence of the starch fraction (lane F). Although the physiological relevance of these effects observed in vitro in the intact organelle cannot be evaluated at present, it is worth mentioning that glucose polymers such as glycogen have been described as effectors of several protein kinases (25) and that protein phosphatases have been found to interact with protein-glycogen complexes in animal tissues (26).

Comparison of Phosphorylation Patterns of Amyloplasts and Chloroplasts

The polypeptide phosphorylation patterns obtained in intact sycamore amyloplasts and chloroplasts and their respective envelope fractions are shown in Figure 6. It is obvious that the pattern observed for amyloplasts is clearly distinguishable from that in chloroplasts (Fig. 6, A and B, respectively).

In the latter, major phosphopolypeptides are represented by the 25-kD light-harvesting Chl a/b protein (11, 24), as well as two other unidentified bands of apparent molecular masses of 59 and 65 kD. In the envelopes of amyloplasts, the 66-kD component described previously represents the major component (Fig. 6C). In chloroplast envelopes, 65- and 55-kD polypeptides were phosphorylated (Fig. 6D), and longer exposure times also revealed a 15-kD component (indicated by asterisks in the figure). Phosphopeptide mapping after Staphylococcus V8 protease treatment indicated that the 65- and 66-kD envelope phosphopolypeptides found in chloroplasts and amyloplasts, respectively, are different (Fig. 5, G and F/H). In contrast, the phosphopeptide pattern of the 65-kD chloroplast envelope phosphopolypeptide (Fig. 5 G) is similar to that reported for the 64-kD polypeptide located in the intermembrane space of pea chloroplast envelopes (27). The 55- and 15-kD phosphopolypeptides found in sycamore chloroplast envelopes (Fig. 6D) were identified as the large and small subunits of Rubisco by immunoblot analysis (not shown). The presence of Rubisco as a common contaminant in chloroplast envelope preparations has been widely reported (2, 16, 27), as well as the phosphorylation of its small subunit by envelope-bound protein kinases (24, 27, 28). Interestingly, we also found that the envelope-bound large subunit of Rubisco was phosphorylated by the isolated chloroplast envelope membranes (Fig. 6D), although the physiological significance of this modification of Rubisco is unclear at present (6).

Phosphoamino Acid Analysis

In chloroplasts, protein-serine kinases have been reported in stromal (9), thylakoid (11, 17), and envelope (27) fractions. This is not the case for protein-threonine kinases, which seem to be restricted to thylakoid membranes and catalyze a light-activated phosphorylation of several polypeptides, including
the light-harvesting Chl a/b protein and other PSII components (ref. 11 and references therein).

Therefore, it was of interest to determine the amino acid specificity of amyloplast protein kinases. Phosphorylation of intact amyloplasts at two different \textit{[γ-32P]}ATP concentrations (5 and 50 \(\mu\)M) followed by partial acid hydrolysis and phosphoamino acid detection indicated \textit{[32P]}Ser as the principal product of protein phosphorylation (Fig. 7). The same results were obtained with amyloplast envelope membranes (not shown), indicating that the major protein-phosphorylating activities localized in this organelle are serine kinases.

**DISCUSSION**

In this work we analyzed the protein phosphorylation system previously described in amyloplasts isolated from cultured cells of sycamore (18). This system operates in the range of ATP concentrations found in plant cells and which have been reported to be optimal for the activity of plant protein kinases (7). The optimal phosphoryl donor was \(\text{Mg}^{2+}\)-ATP, and the phosphorylation reaction was not affected by cyclic nucleotides, as reported for most plant protein kinases (7). Detection of only \textit{[32P]}Ser indicates predominance of protein serine kinases in the organelle.

An analysis of the suborganelar fractions that constitute the amyloplast showed that protein kinase activities are present in envelopes and stromal fractions but barely detected in starch granules. Distinct patterns of phosphorylation of endogenous polypeptides and different activities toward exogenous protein substrates like casein or phosvitin suggest the presence of different and partly independent protein phosphorylation systems in the envelope and stroma. Trypsin accessibility assays suggest that the amyloplast envelope is a site that primarily responds to ATP stimulus with a rapid phosphorylation of several membrane polypeptides (Fig. 3).

We observed that phosphorylation patterns can be affected when different fractions are mixed before addition of labeled ATP (Fig. 4). Moreover, the pattern shown by intact (or even disrupted (18)) organelles is not just the sum of the different isolated fractions. Amyloplasts, as “sink” plasts (10, 19), require externally generated energy for their metabolic activities. However, accessibility of intraorganelar protein kinases to ATP should not constitute a major problem: immunological as well as nucleotide exchange studies indicate the existence of an active ATP/ADP translocator in the inner envelope membrane (1, 22). The overall results suggest a fine regulation of protein kinase activities in the intact organelle.

The presence of protein kinases and some of their functions have been extensively documented in the case of chloroplasts (3, 6, 7, 9, 11, 14, 24, 27, 28). This is not the case for the amyloplast, for which almost no studies have been conducted, despite its major metabolic role in reserve plant tissues (5, 10). Amyloplasts and chloroplasts have been postulated to be ontogenically related, although their functions are clearly distinct (5, 10, 20). In this paper we show that the patterns of phosphorylation shown by these intact organelles or their envelope fractions are strikingly different. The comparative results (including phosphoamino acid analysis and phosphorylating activities toward exogenous substrates) suggest that these organelles house distinct protein phosphorylation systems.

A relevant question concerns the possible role of the amyloplast protein phosphorylation system described in this work. Protein phosphorylation has been shown to be related to starch metabolism (4), chloroplast polypeptide binding/import (14), signal transduction across membranes (3, 24, 26), and genome expression (26), among others (3, 6, 7, 24–26). All of these mentioned processes are putative targets of regu-

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**Figure 6.** Comparison between amyloplast and chloroplast phosphopolypeptide patterns. Phosphorylation by intact plastids or their isolated envelopes was conducted for 1 min at 5 \(\mu\)M \textit{[γ-32P]}-ATP. Further processing of samples was carried out as indicated in "Materials and Methods." Lane A, amyloplasts (50 \(\mu\)g); lane B, chloroplasts (50 \(\mu\)g); lane C, amyloplast envelope (10 \(\mu\)g); lane D, chloroplast envelope (10 \(\mu\)g). All quantities refer to protein content. Arrowheads and asterisk, see Figure 3.

**Figure 7.** Phosphoamino acid analysis of \textit{32P}-labeled amyloplasts. Intact amyloplasts were incubated for 1 min with 5 (A) or 50 (B) \(\mu\)M \textit{[γ-32P]}ATP, and samples were processed as described in "Materials and Methods." The final position of P-Ser and P-Thr standards is indicated in the figure. Arrow, loading zone.
loration by protein phosphorylation in amyloplasts (5, 10, 20, 29). In this sense, the results presented in this work indicate that neither the organellar phosphatase nor the phosphorylatable domains (25) present in enzymes of glycogen metabolism constitute substrates for amyloplast protein kinases. Therefore, purification of amyloplast protein kinases and characterization of their substrates will help us elucidate the role played by the network of phosphorylations present in this unique reserve plastid.

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LITERATURE CITED