Protein Synthesis in Maize during Anaerobic and Heat Stress

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ABSTRACT

Protein accumulation and protein synthesis were investigated during anaerobic stress and heat shock in maize seedlings (Zea mays L.). Antibodies against alcohol dehydrogenase (ADH) and cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPC) were used to investigate the expression of the genes encoding these proteins during stress treatment. ADH protein accumulation is shown to increase about 10-fold in the root after 24 hours of anaerobic treatment. The Gpc gene products are separable into two size classes: the slow mobility GAPC1 and GAPC2 (GAPC1/2), and the faster GAPC3 and GAPC4 (GAPC3/4). The GAPC1/2 antigen did not increase at all, whereas the GAPC3/4 antigen increased less than fourfold. The proteins synthesized in the root during aerobic and anaerobic conditions were compared, and GAPC3/4 was identified as an anaerobic polypeptide. In vitro translations were used to estimate the levels of different mRNAs in roots following anaerobiosis, recovery from anaerobiosis, and heat shock. This was compared with the in vivo protein synthesis rates in roots labeled under identical conditions. In vivo labeling indicates that GAPC and ADH are not heat shock proteins. Although both GAPC3/4- and ADH1-translatable mRNA levels increase about 10-fold during anaerobiosis, in vivo labeling of these proteins (relative to total protein synthesis) is further enhanced, leading to a selective translation effect for ADH1 of threefold, and for GAPC3/4 of sixfold. In contrast, anoxia causes no change in GAPC1/2-translatable mRNA levels or in vivo labeling. As an additional comparison, β-glucosidase mRNA levels are found to be constant during anoxia, but in vivo synthesis decreases.

Maize (Zea mays L.) responds to anaerobic stress (the lack of oxygen, or anoxia) by redirecting the synthetic machinery toward at least some of the enzymes involved with glucose-phosphate metabolism, such as ADH¹, pyruvate decarboxylase, and fructose 1,6-diphosphate aldolase. Activities of these and other enzymes increase to varying degrees (9, 27). Increased levels of these proteins are synthesized (22). Messenger RNAs accumulate (7, 19) and this is at least partially due to selective transcription (17). Where tested, these mechanisms also seem to be operative in other plants (4, 16, 21).

Previous studies in maize defined four Gpc genes (13, 19, 20). During anoxia, Gpc3 transcripts accumulate in parallel with Adh1 and aldolase transcripts, whereas Gpc1 and Gpc2 do not. Unique molecular probes are not available for Gpc4, but a Gpc3 probe cross-hybridizes to the Gpc4 gene coding region (20). It was also shown that heat shock causes an accumulation of RNA from some of the anaerobically active genes, although the increase was not as great as that seen during anoxia (19). Heat shock-enhanced expression of genes encoding glyceraldehyde-3-phosphate dehydrogenase and other enzymes related to glucose-phosphate metabolism has also been noted in yeast (12) and Xenopus (15).

In this report, gene expression during stress was studied at the level of translatable mRNA accumulation, protein synthesis, and protein accumulation. Although in vivo labeling indicates that GAPC3/4 and ADH1 are ANPs, none were found to encode heat shock proteins in maize. To assess the contribution of selective translation to gene expression during stress, the in vivo synthesis pattern was compared with that seen by in vitro translation of RNA from similarly treated seedlings. This comparison indicates that GAPC3/4 and ADH1 transcripts not only accumulate during anoxia, but also are selectively translated. Transcripts that do not increase in accumulation during anoxia, such as GAPC1/2 and β-glucosidase, are not selectively translated.

MATERIALS AND METHODS

Materials

Ultrapure Tris base, ultrapure SDS, and 4-nitro blue tetrazolium chloride were from U.S. Biochemicals. Acrylamide was from Boehringer-Mannheim. bis-Acrylamide was from Fisher. Guanidine HCl was from BRL. Ethanol was from Midwest Grain Products Co, (Pekin, IL). Other organic solvents were from Fisher. All other chemicals were from Sigma. The rabbit anti-maize ADH sera was a gift of M. Vayda and J. Strommer. The rabbit anti-horseradish peroxidase sera was a gift of D. Weil.

Seed Germination

Seeds from the inbred maize (Zea mays L.) line B73 were grown for 4 to 5 d under aseptic conditions at 27°C, 90% RH,
in the dark. The seeds were surface sterilized in household bleach (5.25% sodium hypochlorite) for 5 min, then rinsed in sterile water. After a 1-min incubation in germination buffer (2 mg/mL Capton 50 DP [DuPont], 0.2 mg/mL ampicillin), the seeds were placed on paper towels in a foam-stoppered flask. The treatment was repeated 20 to 30 h later, but with 10% household bleach and a small amount of Alconox detergent. The seedlings used had primary roots of 5 to 7 cm.

**Seedling Treatment**

For examining protein accumulation, the seedlings were analyzed after growth in air or after submersion in treatment buffer (5 mM Tris-Cl, pH 8.0, 0.2 mg/mL ampicillin). The polypropylene container was sealed and kept in the dark at 27°C. At least 25 mL of buffer was used per seedling.

For examining protein synthesis in the seedlings, a modification of previous methods was employed (21, 22). For aerobic labeling, two seedlings were placed in a sterile 50-mL polypropylene tube at 27°C with continuous water-saturated air flowing, and at a 30° angle from horizontal. The terminal 1 cm of a pair of roots was immersed in 0.5 mL treatment buffer containing 0.1 mCi Tran 35S-label (ICN) methionine for 1.5 h. For pulse/chase experiments, this buffer was removed and replaced with treatment buffer containing 5 mM methionine and 1 mM cysteine. Separate experiments with seedlings incubated in this buffer plus 0.1 mCi label showed that TCA cpm/total cpm was reduced by at least 90%. After treatment, the tubes were immersed in dry ice, causing the distal end of the root to be frozen in buffer. The 2-cm subterminal root segment not frozen in the buffer was excised, rinsed in cold rinse buffer (5 mM Tris-Cl, pH 8.0, 1 mM methionine, 0.2 mM cysteine), and extracted for protein as outlined below.

Labeling during heat shock was performed as for aerobic labeling, except during the last 1.5 h of a 2-h incubation at 42°C. For labeling during anoxia, two seedlings were inserted into a 1-cc syringe with a sealed end, containing 0.9 mL treatment buffer. A hole at the midpoint of the syringe that aligned with a septum in the side of the 50-mL polypropylene tube allowed introduction of buffers without contaminating oxygen. Water-saturated argon flowed continuously. For pulse/chase experiments, 0.5 mL of the buffer was removed and replaced with 0.5 mL of treatment buffer plus 10 mM methionine and 2 mM cysteine. After treatment, seedlings were frozen as above, and the 2-cm subterminal segment was saved for analysis.

**Protein Extraction**

Extracts of unlabeled samples were made in extraction buffer (50 mM Tris-Cl, pH 6.8, 0.5% 2-mercaptoethanol, 0.1 mM NAD+, 0.5 mM PMSF, 10 µM leupeptin, and 15% glycerol) using a cold mortar and pestle. Approximately 2 mL of buffer was used per g material. Insoluble material was removed by centrifugation for 5 min at 10,000g. For extracts of labeled roots, 1 mM methionine and 0.2 mM cysteine were included in the extraction buffer, and the roots were pulverized in a microfuge tube. The samples were extracted with 0.1 mL per pair of 1-cm sections, or 0.15 mL per pair of 2-cm sections. The extraction was repeated, the two extractions were pooled and then recentrifuged. The Bio-Rad Bradford assay kit was used to estimate protein concentration, with BSA as a standard.

Amino acid incorporation into protein was estimated by TCA precipitation, similar to the method outlined for the Promega reticulocyte lysate system. Because of the low efficiency of incorporation during anaerobiosis, background counts were subtracted from all samples. Background counts were determined by mixing the labeling buffer with root extract in a range of concentrations, generating samples with similar total cpm and protein concentrations, as seen in the different labeled root samples. Approximately 0.1 to 0.2% of the total cpm in these samples was precipitated.

**RNA Analysis**

Plants were treated as for labeling, but without radioactive amino acids. RNA was collected from 2-cm subterminal sections of three seedlings as outlined by Russell and Sachs (19), but without the high salt precipitation. In vitro transcriptions were performed in rabbit reticulocyte lysate as outlined by Russell and Sachs (19).

**Immune Precipitation**

Immune precipitations using the anti-ADH sera were performed with non-denatured samples essentially as described previously (21), but preclearing was only with protein-A Sepharose. For the anti-GAPC and anti-β-glucosidase sera, the denaturing method of Anderson and Blobel (1) was used with root extracts treated similarly to wheat germ lysates. Protein concentrations were kept constant by adding unlabeled root extract to the labeled extract and holding total volume constant by adding extraction buffer. Preliminary experiments defined a condition of saturating levels of anti-ADH sera that would be responsive to the amount of antigen. Similar experiments with the antibody to β-glucosidase and GAPC found conditions that were responsive to the antigen concentration but were not saturating for antibody.

**Protein Electrophoresis**

Nondenaturing 6% PAGE included 0.1 mM NAD+ in the gel and running buffer, and the resolving gel pH was 8.4, as outlined by Russell and Sachs (20). Gels were 0.75 × 130 × 100 mm.

For denaturing PAGE, 10% SDS-PAGE and fluorography was performed essentially as per Russell and Sachs (19) on 1.5 × 130 × 100 mm resolving gels. For western blots and in vivo-labeled samples, electrophoresis was continued at 25 mA for 1 h after the tracking dye had migrated off in an attempt to better separate GPC1 and GPC2. Band intensities were compared on multiple exposures with a Joyce-Loebl densitometer.

Two-dimensional gel analysis was performed essentially as per Russell and Sachs (20), with each dimension run as above.

**Activity Gel Analysis**

GAPC activity was assessed in the nondenaturing gel by a modification of the procedure of McAlister and Holland (14).
as per Russell and Sachs (20). ADH activity was assayed as per Russell et al. (21).

**Western Analysis**

Western blotting to nitrocellulose membranes (Schleicher and Schuell) and analysis with antibodies was performed as described previously (20, 26).

**RESULTS AND DISCUSSION**

**Oxygen Deprivation and Protein Accumulation**

Previously, it was shown that submersion of maize seedlings in a sealed container led to changes in mRNA accumulation. This treatment greatly reduces the level of oxygen available to the seedling, but initial conditions may not be fully anoxic. An 11-fold increase in ADH1 RNA in the roots was observed after 6 h of treatment, as well as a 6-fold increase in GAPC3 mRNA. GAPC1 and GAPC2 mRNA levels did not increase during this treatment (19). Similar increases are seen during true anoxic conditions (our unpublished observations).

To see how the changes in RNA accumulation are related to changes in GAPC isozyme expression, a native gel electrophoresis method (22) was modified, and the resultant gel was stained for GAPC activity. As shown in Figure 1A, untreated roots show eight activity bands (lane 1). The slowest mobility band, not detectable under these assay conditions, corresponds to homotetramers of GAPC3 (21). The slower mobility bands become more intense after a 24-h submersion (compare lanes 1 and 2). This increase is more obvious in the shoot samples (lanes 3 and 4). The fastest activity band, due to combinations of GAPC1 and GAPC2, does not change in intensity after treatment. GAPC4 homotetramers have an intermediate mobility and are not resolved from various heterotetramers.

To relate activity accumulation to protein accumulation, the different samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed with antibodies to maize ADH (a gift of M. Vaday and J. Strommer). Figure 1B shows ADH antigen levels in the different samples. The minor, diffuse band migrating faster than ADH is due to nonspecific antibody interactions. As expected from previous activity data (9, 27), the level increases in the roots after 24 h of submersion (lanes 1 and 2) as well as in the shoots (lanes 3 and 4).

In Figure 1C, western analysis was performed with antibodies to mustard GAPC (5). The slower mobility band in this figure does not change after submersion in the roots (lanes 1 and 2) or shoots (lanes 3 and 4). This band represents GAPC1 and GAPC2. They are barely resolvable by SDS-PAGE (19), and will be referred to as GAPC1/2. The faster mobility band increases accumulation in both organs after treatment. This band represents the unresolved GAPC3 and GAPC4 monomers (19, 20), and will be referred to as GAPC3/4.

Because others have shown that sucrose synthase isozymes can be expressed differently in the root cap than in the rest of the root (18), the antigen levels of GAPC and ADH were tested in the terminal 1 cm of the root tip, as well as the 2-cm section beyond the tip (subterminal section). Whereas the pretreatment levels of the different antigens are similar in the
activity bands detected in the native PAGE separation (Fig. 2B, lane 2), and have the expected SDS-PAGE mobility.

The two-dimensional profile of Figure 2C was also examined for a spot that aligns with GAPC activity, shown in lane 1 of Figure 2B. An elongated spot, indicated with a large arrow, is seen at the position that comigrates with the slower activity bands, and has an SDS-PAGE mobility identical to GAPC3/4 (19). Faint labeling can also be seen comigrating with the faster activity bands, and with the SDS-PAGE mobility of GAPC1/2 (indicated with a small arrow). These two elongated spots are also indicated on the profile of aerobically labeled material (Fig. 2A). Because the spot corresponding to GAPC3/4 is more intense under anaerobic conditions, GAPC3/4 is identified as an ANP (ANP31.5; 22). This identification could be confirmed with western blots of two-dimensional PAGE samples (20).

**Stress Affects mRNA Accumulation and Protein Synthesis**

The relative contributions of differential mRNA accumulation and protein synthesis to gene expression during stress were investigated in maize seedlings. *In vivo*-labeled protein products were compared with those seen by *in vitro* translations of RNA collected from similarly treated seedlings. Samples were loaded on the basis of equal amino acid incorporation. The *in vitro* translation pattern is shown in Figure 3A, lanes 1 through 4, whereas the *in vivo* labeling pattern is shown in lanes 5 through 10.

As shown in Figure 3A, anaerobic conditions tend to cause a greater change in the *in vivo* protein synthesis pattern (compare lanes 5 and 7) than seen by *in vitro* translation (lanes 1 and 2). The band that is least affected by the different treatments migrates at 26 kD. It should be noted that *in vivo* labeling patterns represent root proteins that are soluble, whereas *in vitro* patterns represent all translatable mRNA products without any native posttranslational modifications. Comparisons of individual bands can be misleading.

The profile seen when the seedlings are labeled after the reintroduction of air (recovery, lane 9) is a hybrid of the aerobic and anaerobic patterns. The pattern seen by *in vitro* translation of RNA from seedlings treated in parallel (lane 3) is similar to the *in vivo* pattern in lane 9, but the ANPs are less pronounced. This is most likely because the labeling is performed while levels of the anoxically induced mRNAs are decreasing (7). The RNA used for *in vitro* translation, however, was collected at the end of this recovery period.

The profiles of material from heat-shocked seedlings, shown in lane 10, indicate increased labeling of the heat shock proteins (10, 11) and relatively diminished labeling of bands seen during control conditions. These differences are more obvious *in vivo* (lane 10) than *in vitro* (lane 4).

Immune precipitations were performed to compare the synthesis of ADH and GAPC during the different treatments. Immune precipitations were also done with antibody to maize β-glucosidase, a protein that is highly expressed in roots (6). In Figure 3B, the immune precipitations of *in vitro* translation products (lanes 1–4) indicate that β-glucosidase mRNA levels were fairly constant during anoxia, but decreased during recovery and heat shock. *In vivo* labeling (lanes 5–10) shows that both anoxia and heat shock cause a large decrease in the level of β-glucosidase protein synthesis in the root; this indicates control at the level of translation during anoxia, but primarily at the level of transcript accumulation during heat shock.

A summary of the scan data from two separate experiments is shown in Table I. The first column indicates the change in specific mRNA levels due to anoxia, as determined by *in vitro* translation and immune precipitation. The second column indicates the change in specific protein synthesis *in vivo*, as...
The ratio in the third column normalizes the change in specific protein synthesis to the change in mRNA accumulation. Because the ratio is less than 1 for \( \beta \)-glucosidase, its in vivo translation rate is reduced during anoxia relative to total protein synthesis.

Protein synthesis versus mRNA accumulation during the different treatments was also analyzed with the anti-ADH sera, and is shown in Figure 3C. The fastest migrating band seen in lanes 1 through 4 is due to nonspecific antibody interactions, as previously seen in the western blot of Figure 1B. Labeled ADH1 product is detectable under aerobic conditions (lanes 1 and 5), but the slower migrating ADH2 is not. Anoxia leads to an increase in ADH mRNA (compare lane 2 to 1) and in vivo synthesis (compare lane 7 to 5). As summarized in Table I, the increase seen in vivo is greater than that seen by in vitro translation, indicating the selective translation of ADH1 during anoxia.

During heat shock, ADH1 mRNA (lane 4) and in vivo-synthesized protein (lane 10) are barely detected. Previously, northern analysis indicated both GAPC3 and ADH1 RNA increased two- to threefold during heat shock (19). Northern analysis of the RNA used in this study confirmed the increase in GAPC3 RNA during heat shock (data not shown). One possible reason for the discrepancy between northern and translation data is the mode of normalization. Northern blots are normalized to the level of ribosomal RNA, whereas labeling data are normalized to incorporated \(^{35}\)S amino acids.

Anti-GAPC sera was used as in the analyses with anti-ADH and anti-\( \beta \)-glucosidase, and the results are shown in Figure 3D and Table I. GAPC1/2, the band of slower mobility in Figure 3D, has fairly constant levels of translatable mRNA during the different treatments (lanes 1–4). Previously, northern analysis of total RNA from whole roots indicated that GAPC1 RNA levels are fairly stable during the first 6 h of hypoxia, whereas GAPC2 decreases. Heat shock has little effect on the accumulation of either transcript (19). The in vivo labeling intensity of GAPC1/2 during anoxia (lane 7) is similar to that during control treatment (lane 5), indicating that the translation of this mRNA in the root is unaffected by anoxia. Heat shock is inhibitory to translation, with no product detected (lane 10).

The faster mobility band in Figure 3D has the mobility of GAPC3/4. Anoxia leads to an increase in GAPC3/4 mRNA (compare lane 2 to 1) and in vivo synthesis (compare lane 7 to 5). As summarized in Table I, the in vivo synthesis increase during anoxia is greater than that seen by in vitro translation. This indicates that selective translation is involved in the anaerobic expression of GAPC3/4. In rice, anoxia also leads to a large increase in translatable mRNA accumulation and in vivo translation for one of two size classes of GAPC (16). It has not yet been determined whether GAPC RNA is

**Figure 3.** Comparison of proteins labeled in vitro and in vivo after separation by SDS-PAGE. Lanes 1 through 4 are in vitro translation products, and lanes 5 through 10 are in vivo-labeled products. The in vitro translations used RNA from subterminal root sections of control seedlings (lane 1), anoxic seedlings (lane 2), after a 2-h recovery from anoxia (lane 3), and after a 2-h heat shock (lane 4). The in vivo-labeled products were from subterminal root sections of seedlings labeled during control conditions (lane 5), during control conditions followed by a 6-h chase (lane 6), during anoxia (lane 7), during anoxia followed by a 6-h chase (lane 8), during recovery from anoxia for 2 h (lane 9), and during heat shock (lane 10). Equal TCA cpn were loaded in lanes 1 through 4 and lanes 5 through 10. A, Total protein synthesis profile. The molecular masses of the protein standards indicated on the left and right are 66, 45, 36, 29, 24, and 20 kD. b, a, and g indicate the migrations of \( \beta \)-glucosidase, ADH, and GAPC3/4, respectively, as determined by immune precipitation. B, Immune precipitations with anti-\( \beta \)-glucosidase sera. C, Immune precipitations with anti-ADH sera. D, Immune precipitations with anti-GAPC sera.

| Table I. Anaerobic Induction of mRNA in Roots Does Not Parallel Translation in Vivo |
|-------------------------------------------------|---------------|-----------|
| mRNA Induction | In Vivo Induction | Average Ratio |
| \( \beta \)-Glucosidase | 1.0, 0.29 | 0.28, 0.17 | 0.35 |
| ADH | 13.4, 4.3 | 36, 17.5 | 3.0 |
| GAPC1/2 | 1.7, 1.1 | 1.4, 1.4 | 1.0 |
| GAPC3/4 | 17.4, 6.3 | 24, 108 | 5.6 |

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selectively translated in anaerobic rice. During recovery from anoxia, significant GAPC3/4 mRNA is present (Fig. 3D, lane 3) as is protein synthesis (lane 9). GAPC3/4 synthesis is not detected during heat shock.

To determine if the relative protein synthesis rates under control and anoxic conditions were distorted by differences in protein turnover rates, seedlings were labeled, then chased with an excess of unlabeled amino acids for 6 h. Lane 6 in Figure 3A shows the aerobically labeled proteins after a chase under the same conditions. When compared with the profile in lane 5, most bands are of similar intensity, indicating similar decay rates; bands that decrease in intensity have a decay rate faster than the average protein. Similar results are seen when comparing the anaerobic pulse and pulse/chase profiles (lanes 7 and 8). When specific products are examined by immune precipitation, ADH and GAPC3/4 seem to have a slightly slower decay rate than the average protein during anoxic labeling. The apparent decrease in GAPC1/2 and GAPC3/4 stability under control conditions (lanes 5 and 6) is not consistently seen.

As summarized in Table 1, the different genes under study behave differently at the level of transcription accumulation and translation during anoxia. Transcripts that accumulate during anoxia also show selective translation. Speculation that the observed enhancement in translation is due to changes in charged tRNA pools during anoxia can be ruled out, because then all mRNAs would be affected in parallel. The variability in GAPC3/4 and ADH1 anaerobic induction may be related to observations in Xenopus by Nickells and Browder (15). They found the highest heat shock inductions of glyceraldehyde-3-phosphate dehydrogenase activity when pretreat enzyme levels were initially low. The magnitude of the translational control effect described here compares favorably with other systems once the data are normalized for any changes in mRNA levels (3, 8, 24, 25). Future studies of the translational machinery (2) and mRNA structure in anaerobic maize roots may elucidate this mechanism of gene control.

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LITERATURE CITED