Hormonal Regulation of α-Amylase Gene Transcription in Wild Oat (Avena fatua L.) Aleurone Protoplasts

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ABSTRACT

The time of appearance and relative amounts of α-amylase mRNA in wild oat (Avena fatua L.) aleurone protoplasts incubated with 1 micromolar gibberellin A4 (GA4) were closely correlated with the amounts of α-amylase enzyme secreted by the protoplasts. In the absence of GA4, or when protoplasts were incubated with 25 micromolar abscisic acid (ABA) together with 1 micromolar GA4, no α-amylase mRNA was detected and only very low levels of α-amylase were secreted. Nuclei were isolated in high yields (65–71%) from aleurone protoplasts and in an in vitro transcription system displayed characteristics of a faithful DNA-dependent RNA synthesizing system. The time course of incorporation of [3H]-UTP suggested that the RNA synthesis was mainly ‘run off’ transcription and therefore that the transcripts produced in vitro were those being synthesized in the protoplasts at the times when the nuclei were isolated. By hybridizing in vitro synthesized [32P]RNA to barley α-amylase cDNA and control filters we have estimated that 90 ± 10 ppm of the transcripts synthesized by nuclei isolated from GA4 treated protoplasts can be attributed to α-amylase sequences and that statistically insignificant amounts of these transcripts are obtained from control and GA4 plus ABA treatments. The results suggest that GA4 and ABA influence the transcription of α-amylase genes in aleurone protoplasts of wild oat.

MATERIALS AND METHODS

Plant Materials. Seeds of Avena fatua were collected and stored as previously described (13).

Isolation and Incubation of Aleurone Protoplasts. Aleurone protoplasts were isolated and incubated in medium either without GA4, with 1 μM GA4, or with 1 μM GA4 plus 25 μM ABA as described previously (13, 14). GA4 was preferred to GA3 because available evidence (1) suggested that in barley it is the gibberellin ultimately responsible for α-amylase induction and we felt this might also be the case in wild oats. Although this view has since been disputed (9) the α-amylase inducing activity of GA4 in wild oat protoplasts is equal to that of GA3 (4).

α-Amylase Assay. The control or hormone treated protoplasts were sedimented at 50 g for 1 min. The supernatant, which in inducing treatments contains most of the α-amylase activity (13) was collected, dialyzed exhaustively against 100 mM Na-acetate buffer at pH 5.0, and the α-amylase activity was measured by the method of McCleary (17). The data presented in Figure 1 are the increases in A590 which resulted from incubating supernatants from 10⁶ protoplasts with Alphachrome substrate (Koch-Light Ltd., U.K.) for 10 min at 37°C.

Preparation and Fractionation of RNA from Protoplasts. The protoplast pellet was suspended in 2 ml of NTE (100 mM NaCl, 10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1% SDS [w/v]), 1 ml of phenol was added, and the mixture was vortexed for 2 min. One ml of chloroform was added and the mixture vortexed 2 min. The phases were separated, the aqueous phase was retained, and the organic phase was reextracted with 1 ml NTE. The combined aqueous phases were extracted with 1 ml phenol-chloroform (1:1) and nucleic acids were ethanol precipitated from the aqueous phase after the addition of 0.1 x volume of 3 M sodium acetate (pH 5.8). RNA (10 μg per treatment) was size-fractionated on a formaldehyde denaturing agarose gel (19), blotted onto a nitrocellulose filter (25), and hybridized with a probe containing 32P labeled barley cDNA for α-amylase (7). The filter was autoradiographed for 5 d at −80°C.

Preparation of Nuclei. After 72 h incubation, protoplasts (typically 3–4 x 10⁶) were sedimented at 50 g for 1 min, the supernatant was collected for α-amylase assay, and the protoplasts washed once by centrifuging through 25 ml of fresh incubation medium. The pellet of washed protoplasts was lysed in ice cold NIB (10 mM Tris-HCl [pH 7.5], 10 mM KCl, 10 mM MgCl₂, 10 mM β-mercaptoethanol, 20% glycerol [v/v], and 0.02% [v/v] Triton X-100) at a concentration of 10⁶ protoplasts per 15 ml. All subsequent manipulations were carried out on ice at 4°C. The suspension was passed through a 20 μm nylon mesh and this was rinsed with an equal volume of NIB. Nuclei were pelleted at 1000g for 20 min, resuspended in NIB at a density of 10⁶ nuclei per 10 ml, and the suspension allowed to sediment at 1 g for 2 to 3 min. The supernatant containing the nuclei and only a small number of starch grains were transferred to another tube and the nuclei were pelleted at 500g for 15 min.

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3 Abbreviations: GA₄, gibberellin A₄; GA₃, gibberellic acid; NIB, nuclear isolation buffer.
The nuclei were resuspended in NIB without Triton X-100, transferred to a 1.5 ml Eppendorf tube, pelleted at 500g for 3 min, and frozen in liquid N2. Estimations of numbers of nuclei were made with a haemocytometer. Estimations of DNA were made fluorimetrically with Hoechst 33258 on samples of protoplasts and nuclei (15).

Microscopy. For light microscopy frozen nuclei were thawed and stained with 0.025% Azur C in NIB. For electron microscopy standard procedures of fixation, dehydration, and embedding were employed. Frozen nuclei were thawed, resuspended in NIB containing 6% glutaraldehyde, and pelleted at 500g for 3 min. After 30 min the pellet was washed twice with NIB and postfixed in NIB containing 2% OsO4 (w/v) for 16 h at 4°C. After dehydrating through a 10% (v/v) stepped ethanol series the pellet was infiltrated with Spurr’s resin and polymerized at 95°C for 2 h. Ultrathin sections were cut and stained for 5 min in uranyl acetate (saturated solution—50% ethanol) then for 5 min in Reynold’s led citrate stain.

**In Vitro Synthesis of Radioactive RNA.** The transcription medium was based on that of Gallagher and Ellis (8) and contained 10 mM Tris-HCl (pH 7.8), 75 mM (NH4)2SO4, 2 mM MnCl2, 5 mM MgCl2, 2 mM β-mercaptoethanol, 0.4 mM ATP, GTP, CTP, 2.6 units/μl human placental ribonuclease inhibitor (Amersham, U.K.), 0.3 μM phosphocreatine, and 0.03 μg/μl creatine phosphokinase. When the synthesis of total RNA was being investigated, 5 μCi [α−32P]UTP (40 Ci per mmol Amersham, U.K.) was included in a 60 μl incubation containing 4 × 105 or 1 × 106 nuclei at 26°C for 30 min. An equal volume of cold 10% (w/v) TCA containing 10 mM sodium pyrophosphate and 10 mM EDTA was added to stop the reaction and the RNA was precipitated on ice. The precipitates were collected on glass fiber discs and washed 20 times with 5% ice-cold TCA containing 10 mM pyrophosphate and 10 mM EDTA, dried, dissolved in 0.5 ml ‘Soluene’ (Packard Instruments), and counted after the addition of scintillation fluid.

**In vitro** 32P labeled RNA for hybridization experiments was synthesized in 40 μl incubations containing, besides nuclei and other compounds, 333 μCi [α−32P]UTP (~3000 Ci per mmol, Amersham, U.K.) and 3.3 units per μl of human placental ribonuclease inhibitor (Amersham, U.K.). The nuclei (1.3 × 106 in each case) were prepared from protoplasts which had been incubated for 72 h without GA4, with 1 μM GA4 or with 1 μM GA4 plus 25 μM ABA.

The nuclei used in each incubation were divided into two equal aliquots and one was incubated in the transcription mixture for 30 min after which they were sedimented at 670g for 5 min and placed on ice. The supernatant was transferred to the remaining nuclei which were sedimented at 670g for 5 min after a further 30 min incubation. Preliminary experiments suggested that this batch procedure maximized the incorporation of radiolabel into RNA at the densities of nuclei used. The combined nuclear pellets were vortexed for 5 s with 1 ml of 10 mM Tris-HCl (pH 7.8), 5 mM MgCl2, 2 mM β-mercaptoethanol, 20% glycerol (v/v), and 0.02% (v/v) Triton X-100 and pelleted at 1000g for 5 min. RNA was prepared, and total cpm incorporated were measured essentially as described by Beach et al. (3).

**Filter Preparation and Hybridization.** DNA was prepared from the DNA phage fd103 (11) and from the same phage carrying a barley aleurone α-amylase cDNA insert fd103-19E (6). DNA (10 μg) from control and insert-containing phage was attached to separate 6 mm diameter nitrocellulose filters (25) and radioactive RNA from each of the transcription incubations was hybridized to the discs for 72 h at 48°C (3). The filters were washed (8), dried, and counted in liquid scintillant. The differences between the counts hybridized to control and α-amylase filters were expressed as the range of counts in the hybridization reactions were calculated and plotted. An estimate of the ppm of total input RNA cpm hybridized at each input level was obtained by dividing the net cpm hybridized to the filter at that input level by the total cpm in the RNA at the same input level and multiplying by 106. The overall estimate of net ppm hybridized was the mean of the estimates for all four levels of RNA input. The number of cpm hybridized per 105 nuclei was calculated for each treatment by dividing the sum of the cpm hybridized to the filters by the number of nuclei in the appropriate transcription incubation.

**RESULTS AND DISCUSSION**

The plasmid pHV19 containing barley α-amylase cDNA (7) was used in a ‘Northern’ hybridization to detect sequences in RNA prepared from wild oat aleurone protoplasts (Fig. 1). After 48 and 60 h incubation with GA4, a faint band corresponding to an RNA species of approximately 1700 bases was observed and the intensity of the band increased with longer incubation times. In the absence of GA4 no band was detected after 72 and 96 h incubation and the same result was obtained for the GA4 + ABA treatment. Changes in the α-amylase levels in the incubation medium (Fig. 1) paralleled changes in the levels of hybridization. An identical result was obtained when the plasmid used contained a different but related α-amylase barley cDNA (23).

In aleurow layers of wheat and barley substantial amounts of α-amylase mRNA are present after 12 h incubation with gibberelin (2, 7, 20, 23) and enzyme levels increase correspondingly. The results of Figure 1 show that in RNA from wild oat protoplasts probed with barley α-amylase cDNA, a band which is approximately the same size as barley α-amylase mRNA, appears after a longer incubation and is correlated in time of appearance and amount with α-amylase. We conclude that the band is wild oat α-amylase mRNA.

The yield of nuclei from protoplasts (10 experiments) was 65 ± 7% when estimated by haemocytometry and 71 ± 9% when measured by DNA recovery. Nuclear preparations were contaminated with starch grains carried over from the endosperm (Fig. 2a) but were devoid of any contaminating cytoplasmic organelles when examined by electron microscopy. Although a fairly low concentration of Triton X-100 was used during isolation, the membrane had been almost completely stripped from the majority of the nuclei (Fig. 2b). The isolated nuclei were highly active in the *in vitro* transcription system.

**FIG. 1.** Northern blot analysis of α-amylase mRNA extracted from wild oat aleurone protoplasts at various times after the hormone treatments indicated, and the amounts of α-amylase enzyme secreted by the protoplasts into the medium. Mol wt markers are indicated by the arrow heads.
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Fig. 2. Light (a) and transmission electron (b) micrograph of isolated nuclei.

Table 1. Synthesis of Total [3H]RNA by Nuclei Isolated from Protoplasts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3H Incorporated</th>
<th>Complete Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete reaction</td>
<td>34,570</td>
<td>100</td>
</tr>
<tr>
<td>Minus nuclei</td>
<td>148</td>
<td>0.4</td>
</tr>
<tr>
<td>Minus ATP, CTP, GTP</td>
<td>4,564</td>
<td>13.2</td>
</tr>
<tr>
<td>Minus ATP-generating system</td>
<td>32,958</td>
<td>95.3</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete reaction</td>
<td>50,221</td>
<td>100</td>
</tr>
<tr>
<td>Plus Act D 40 μg/ml</td>
<td>1,281</td>
<td>2.6</td>
</tr>
<tr>
<td>Minus RNase inhibitor</td>
<td>42,487</td>
<td>84.6</td>
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Incorporation of [3H]UTP into cold TCA-insoluble products by isolated aleurone nuclei had the characteristics of a DNA-dependent RNA-synthesizing system. It was dependent on the presence of the nonradioactive nucleoside triphosphates but less so on the ATP-generating system (Table I). RNA synthesis was DNA-directed as shown by its sensitivity to actinomycin D. The slight reduction of counts accumulated in the absence of human placental ribonuclease inhibitor suggests that there may have been some ribonuclease activity in the nuclear preparation. Nevertheless, judging from the time course (Fig. 3), there was no extensive degradation of newly synthesized RNA. It is probable that the synthesis is the result of the completion of transcripts initiated in vivo rather than to new initiation in vitro, but no direct evidence on this point was obtained. Incorporation proceeded at an approximately linear rate of about 20 pmol [3H]UTP/mg DNA-min for 15 to 20 min and at a lower rate thereafter. α-Amanitin at 4 μg/ml inhibited the incorporation by about 80 per cent.

The amount of [3H]RNA synthesized in vitro was proportional to the number of nuclei in the reaction. When increasing numbers of nuclei from 0.5 × 10⁵ to 4 × 10⁵ were present in 60 μl reactions the incorporation of [3H]UTP increased from 14 × 10³ to 170 × 10³ cpm in an approximately linear fashion.

Of the [32P]RNA synthesized in vitro by nuclei isolated from protoplasts which had been incubated with GA₃, 90 ± 10 ppm hybridized to phage DNA containing the α-amylase cDNA insert.
indicating that the α-amylase genes were being transcribed (Fig. 4). In contrast, RNA synthesized by nuclei prepared from protoplasts which had been incubated either without GA₄ or with both GA₄ and ABA did not contain statistically significant amounts of any RNA species that hybridized with the α-amylase cDNA sequence. This suggests that the α-amylase genes are being transcribed at a level below the detection limit of the experimental system or not at all. The possibility that the α-amylase transcripts are being "run-off" and then degraded almost instantaneously cannot be excluded. However, the data of Figure 3 suggest that there is no extensive degradation of newly synthesized RNA. The total cpm hybridized by the α-amylase cDNA per 10⁶ nuclei were 1398 (plus GA₄), 61 (minus GA₄), and 199 (plus GA₄ + ABA).

Essentially the same results were obtained in a similar experiment (data not shown) in which in vitro synthesized [³²P]RNA was hybridized to plasmid DNA containing the α-amylase cDNA insert (pHV19) (7) over an input range of 2 × 10⁶ to 15 × 10⁶ cpm. The nuclei had been isolated from protoplasts incubated either with or without 1 μM GA₄. Approximately 50 ppm of the RNA from the 'plus GA₄' treatment hybridized to the cDNA sequences compared with no statistically significant hybridization of the RNA from the 'minus GA₄' treatment. The total cpm per 10⁶ nuclei hybridized by the α-amylase insert were 962 (plus GA₄) and 105 (minus GA₄). In this hybridization the cDNA sequences on the filters were apparently saturated at the highest inputs of RNA.

The GA₄-induced increase in the transcription of α-amylase genes does not appear to be the result of nonspecific stimulation of transcription. In fact, the incorporation of [α-³²P]UTP into total RNA by the nuclei was depressed as a consequence of treating the protoplasts with GA₄ when 22 × 10⁶ cpm were incorporated, compared with 33 × 10⁶ cpm for the minus GA₄ transcription and 46 × 10⁶ cpm for the GA₄ + plus ABA treatment. While we have not attempted to measure the transcription of ribosomal or other possible control genes we believe that our results indicate a selective stimulation of the transcription of α-amylase genes by GA₄ and that this is prevented by ABA.

We conclude that this transcriptional control of α-amylase gene expression can at least partly account for the levels of α-amylase mRNA observed when wild oat aleurone protoplasts are incubated with these plant hormones. We cannot, however, exclude the possibility of a simultaneous gibberellin-induced increase in the half-life of α-amylase mRNA like that seen in the effects of steroids on the accumulation of mRNAs for ovalbumin and conalbumin in chick oviduct (18). Hagen et al. (10) have recently reported that increased mRNA levels in auxin-treated soybean hypocotyls are at least partially the result of raised transcription of certain DNA sequences. In the present work evidence is presented that the hormonal control of the level of a known enzyme is mediated at least in part by the rate of transcription of its genes.

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LITERATURE CITED

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FIG. 4. Hybridization of in vitro synthesized RNA to α-amylase cDNA. Nuclei in the transcriptions were prepared from aleurone protoplasts which had been incubated for 72 h without hormones (minus GA₄) (○), with 1 μM GA₄ (plus GA₄) (●) or with 1 μM GA₄ + 25 μM ABA (plus GA₄ + ABA) (■).
and (1-4)-β-D-Glucanase. Carbohydr Res 86: 97-104