HORMONAL REGULATION OF NON-α-AMYLASE GENES IN BARLEY ALEURONE CELLS

by

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B.A., Tung-Hai University, 1982

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Genetics

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1987

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ACKNOWLEDGMENTS

I would like to express my appreciation to my major advisor Dr. Subbaratnam Muthukrishnan for his support and guidance. I thank Dr. George H. Liang and Dr. Lawrence C. Davis for their assistant and reviewing the manuscript. Appreciation also goes to members in laboratories for their stimulating discussions and friendships.

I would also like to thank my family for their support and encouragement; and to my husband, Hsing-Chung Lee, for his support and inspiration.

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INTRODUCTION

I. The influence of gibberellic acid on expression of proteins in barley aleurone cells

Modification of barley kernels during malting is a complex process. After rehydration of the desiccated grain synthesizes and secretes large quantities of hydrolases which mobilize the endosperm reserves for use by the growing plant. Gibberellins produced by the embryo during germination have been shown to be responsible for this induction (Jones and Jacobsen, 1978).

After the discovery that α-amylase production in embryoless cereal half seeds was shown to be induced by exogenous gibberellic acid (GA) (Yomo, 1962. Paleg, 1960). Varner et al (1964) used aleurone layers dissected from embryoless half seeds and obtained the same response reported by Paleg and Yomo using half seeds. Since then, the *in vitro* response of dissected barley aleurone layers to external addition of GA has been studied as a model system for hormonal control of protein synthesis and gene expression in plants.

In addition to α-amylase, GA treated aleurone layers produce a variety of hydrolytic enzymes such as protease, phosphatase and β-1,3-1,4-glucanase (Briggs, 1963). The synthesis and secretion of α-amylase and protease is greatly enhanced by GA. In the absence of GA very little protease or α-amylase activities were detected in the aleurone layers (Varner, 1964). α-amylase is the major GA-induced enzyme. After 24 hour of GA treatment, more than

50% of the newly synthesized protein is α-amylase (Mozer, 1980). GA also causes a several fold increase in protease in barley aleurone layers.

Some other enzymes are present at low levels prior to GA treatment and show a considerable increase in level during imbibition of the seeds. The addition of GA to the aleurone layers causes a further increase in the total amount of enzyme activity. However release of some of these enzymes such as glucanase, RNase, acid phosphatase, and carboxypeptidase to the medium is strictly dependent on GA. For example, the activity of the (1-3,1-4)-β -glucanase isoenzyme in aleurone layers increases during imbibition and is enhanced by GA and calcium ions. The increase in glucanase activity after GA treatment is the result of new protein synthesis (Stuart, et al. 1986). Acid phosphatase is not a GA-inducible enzyme since addition of GA causes only a small increase in the activity of this enzyme. However, release of acid phosphatase from aleurone layer is under GA control (Ashford and Jacobsen, 1974). The GA response of carboxypeptidase is similar to that described for acid phosphatase. GA is not required for synthesis but affects the release of the enzyme (Hammerton and Ho, 1986). GA-treatment also enhances the activities of several other enzymes such as catalase and malate synthetase (Jones, 1972), and phosphoryl choline cytidyl transferase and phosphorylcholine glyceride transferases (Johnson and Kende, 1971), but these do not appear to represent new synthesis and may be due to enzyme activation.

Density-labelling experiments have shown that in response to

the addition of GA, α-amylase, protease, ribonuclease and 1,3-β-glucanase are synthesized *de novo* (Varner, 1964, Jacobsen and Varner, 1967. Bennett and Chrispeels, 1972). But it has not been established that all GA-inducible enzymes are synthesized *de novo*.

II. Effect of hormone on α-amylase mRNA synthesis in barley aleurone cells

A. Effect of GA

Inhibitor studies have shown that the effect of GA on the expression of α-amylase in barley aleurone cells requires protein synthesis and RNA synthesis. Hydrolase synthesis in aleurone cells is inhibited by the protein synthesis inhibitor cycloheximide (Varner and Chandra, 1964., Muthukrishnan et al. 1979). RNA synthesis inhibitors, notably actinomycin D, also inhibit the production of α-amylase and other hydrolases (Varner and Chandra, 1964). Using cDNA prepared from enriched α-amylase mRNA, it has been demonstrated that GA addition results in an increase in the amount of complementary mRNA sequences (Bernal-Lugo et al, 1981). The lag period of 2-6 hours between GA addition and release of α-amylase into the medium (Chrispeels and Varner, 1967) is consistent with the observations that the response of barley aleurone cells to GA requires new RNA and protein synthesis.

In 1964 Varner and Chandra reported that in barley aleurone

cells, GA_3 treatment causes enhanced synthesis of RNA. Other experiments using incorporation of radio-labeled adenosine into poly(A)$^+$RNA showed that synthesis of RNA containing poly(A)$^+$ was enhanced by 3-4 hours and reached a maximum at 10-12 hours after GA treatment (Ho and Varner, 1974; Jacobsen et al. 1974). However, the production of α-amylase was not sensitive to cordycepin, a RNA synthesis inhibitor, if cordycepin was added 12 hours or more after GA treatment, indicating that α-amylase was translated from a stable mRNA (Ho, Varner, 1974). In vitro translation of RNA recovered from aleurones treated with GA showed that GA stimulates protein synthesis by increasing the amount of translatable mRNA for several proteins including α-amylase (Higgin, et al. 1976; Muthukrishnan, et al. 1979). Cordycepin, added prior to 12 hours after GA treatment decreased the amount of translatable mRNA that can be extracted, proving that GA-induced protein synthesis required the continuing synthesis of mRNA (Muthukrishnan, et al. 1979). mRNA for α-amylase is the predominant poly(A)$^+$RNA present in the aleurone tissues with 50% of in vitro translated synthesized protein being α-amylase (Mozer, 1980). In addition, cDNA sequences prepared from poly(A)$^+$RNA isolated from GA-treated aleurone layers have been cloned into pBR 322. From this library α-amylase clones have been identified by in vitro translation of hybrid-selected RNA and by immunoprecipitation of the translation products using antiserum specific for α-amylase (Muthukrishnan et al, 1983; Rogers and Milliman, 1984; Deikman and Jones, 1985). Such cDNA clones have been used to detect and quantitate mRNA and their precursors.

Hybridization of an α-amylase cDNA to aleurone mRNA demonstrated GA induced increase of two classes of α-amylase from 10-30 fold in mRNA after incubation for 12 hours.

B. Effects of abscisic acid (ABA) and cycloheximide

The GA-mediated synthesis of α-amylase in barley aleurones is sensitive to the presence of the negative growth regulator, abscisic acid (ABA), in the induction medium (Chrispeels and Varner, 1967; Ho and Varner, 1976). Addition of ABA to aleurone layers inhibited synthesis of α-amylase within 2 to 3 hours (Chrispeels and Varner, 1967). After 12 hours of GA treatment, the synthesis of α-amylase mRNA approached its maximums, and α-amylase synthesis at this point was no longer sensitive to inhibitors of transcription such as cordycepin (Higgins et al, 1976). Unlike cordycepin, ABA was shown to decrease the accumulation of α-amylase activity after 12 hours of GA incubation (Ho, 1976). In another study, simultaneous addition of GA and ABA resulted in the inhibition of the synthesis of α-amylase in vivo, but RNA isolated from ABA and GA treated aleurone layers could direct the in vitro synthesis of α-amylase as efficiently as RNA from aleurone treated with GA only (Mozer, 1980).

Using gel blot hybridization of [^{32}P] labeled cDNA to α-amylase mRNA, Bernal-Lugo (1981) reported that the levels of α-amylase mRNA in cells treated with GA+ABA are lower than in cells treated with GA only and suggested ABA acted at the transcriptional level. Higgins et al (1982) compared in vivo labeling mRNA levels

by cell-free translation of aleurone RNA at different times after GA and ABA treatment. They reported that when ABA was added at 20 hours, by which time mRNA had reached its maximum level, the hormone had relatively little effect on α-amylase synthesis. They concluded that regulation of the level of α-amylase mRNA plays a major role in the ABA reversal of GA-promoted α-amylase synthesis. Muthukrishnan et al (1983a) reported that in the presence of ABA and GA, the level of α-amylase mRNA in aleurone cells decrease 16 fold after 12 hours of GA-treatment as measured by *in vitro* translation assays and by analysis of RNA blots with α-amylase cDNA probes. They concluded that regulation of α-amylase gene expression by ABA is at the level of transcription and not at the level of translation.

Addition of cycloheximide, an inhibitor of protein synthesis, resulted in an immediate inhibition of enzyme synthesis (Chrispeels and Varner, 1967). The analysis of translation products of RNA extracted from aleurone treated with GA and cycloheximide (Muthukrishnan et al.,1979) and experiments using RNA blots probed with ^{32}P-labelled α-amylase cDNA indicated that cycloheximide blocked the appearance of α-amylase mRNA when added along with GA. A delay in the addition of cycloheximide of only a few hours resulted in a synthesis of less α-amylase mRNA. The results suggested the induction of synthesis of α-amylase mRNA by GA requires new protein synthesis after hormone addition (Muthukrishnan et al., 1983a). Similar results were found by using the lysine analog aminoethylcysteine (AEC), a translational inhibitor (Muthukrishnan et al., 1983b). The results confirmed

that a protein factor appears to be required in addition to GA for transcription of α-amylase genes.

III. Hormone effects on the synthesis of non-α-amylase mRNA

A. Barley Aleurone

Hormone effects on expression of non-α-amylase genes of barley have also been investigated. Using an apparently full-length cDNA clone for protease from barley aleurone cells, Rogers et al (1985) reported that mRNA for a protease is present in untreated aleurone cells, but mRNA for the protease increased 7 fold after incubation with GA for 18 hours. Immunoprecipitation of *in vitro* translation products with antiserum specific for (1-3,1-4)-β-D-glucanase 4-glucanohydrolase showed that low levels of translatable mRNA for this enzyme can be detected in aleurone cells in the absence of GA, but GA caused a 10-fold increase in levels of translatable mRNA (Mundy and Fincher, 1986).

The thiol protease and glucanase genes are like α-amylase genes in their response to GA and ABA in aleurone cells (Rogers et al, 1985). In the presence of ABA, the mRNA for thiol protease decreases. ABA treatment also suppresses the relative abundance of translatable (1-3,1-4)-β-D-glucanase mRNA (Mundy and Fincher, 1986).

Other studies have shown that the synthesis of a number of functionally uncharacterized polypeptides are stimulated in barley aleurone layers by ABA.

The synthesis of the α-amylase/subtilisin inhibitor (ASI) mRNA

is reduced by GA. Additionally, synthesis and accumulation of the ASI mRNA is induced upon incubation with ABA (Mundy, 1984). Probable amylase/protease inhibitor (PAPI) mRNA is present at high levels in aleurone tissue and its expression in these layers is not affected by either ABA or GA (Mundy and Rogers, 1986).

B. Wheat aleurone

In vitro translation of polyadenylated RNA indicates that six mRNA species increase in relative concentration in GA-treated wheat aleurone layers, and these multiple poly(A)$^+$RNA species are regulated in a manner similar to α-amylase mRNA. At least one mRNA species declines in relative level in response to GA (Baulcombe, Buffard, 1983). GA may increase poly(A)+RNA synthesis or may increase the stability of the mRNA, or GA may have both effects.

Intermediate GA-dependent events in the production of hydrolytic enzymes include: 1) vesiculation of endoplasmic reticulum, presumably to support transport of the hydrolytic enzymes, 2) increased rate of synthesis of polyadenylated mRNA with a specific rise in the level of translatable mRNA, 3) *de novo* synthesis of some of the secreted enzymes including α-amylase, protease, β-glucosidase, 4) secretion and release of these enzymes and also others, e.g. β-glucosidase and acid phosphatase, which are synthesized even in the absence of GA (Baulcombe et al., 1983). It is not yet known whether GA influences these diverse processes.

It is not known whether there is a common mechanism for hor-

monal control of specific genes in aleurone tissue of barley. My approach to understand this problem is to study the effects of GA and ABA at the RNA level. This has been done by using cDNA clones as probes for studying the pattern of expression of some GA-induced mRNAs.

Materials

Antibiotics

Ampicillin: Sigma.

Chloramphenicol: Sigma.

Tetracycline: Sigma.

Bacteria

E.coli LE392: American Type Culture Collection (ATCC).

Barley seeds

The barley(*Hordeum Vulgare* L. cv *Himalaya*) seeds, harvested in 1981, were obtain from Agronomy club, University of Washington, Pullman, Washington. The seeds were stored at -20^0.

Chemicals and other Materials

Acrylamide: Bio-Rad.

Agarose: Bio-Rad.

Ammonium persulfate: Sigma.

Bis-acrylamide: Bio-Rad.

Bromophenol blue: Fisher.

Cesium chloride, prep. grade: Pharmacia.

Cetyltrimthylammonium bromide (CTAB): Sigma.

Cobalt chloride: Fisher.

Coomassie Brilliant Blue R-250: Bio-Rad.

Creatine phosphate: Sigma.

Cronex intensifying screen: E.I. Du Pont de Nemours and Co Inc.

Dextran sulfate (sodium salt): Pharmacia.

Dextrose: Fisher.

Developer: Eastman Kodak, Inc.

D-Glycogen: Aldrich Chemical Co, Inc.

Dimethyl sulfate: Aldrich Chemical Co, Inc.

Ethidium bromide: Sigma.

Ficoll: Sigma.

Fixer: Eastman Kodak Co.

Formic Acid, 88% : Fisher.

Formaldehyde, solution 30% w/w: Fisher.

Gibberellic acid (90% GA^3): Sigma.

Phenol: MCB Manufacturing Chemist, Inc.

Polyvinylpolyprrolidone (PVP): Sigma.

Pyridine: MCB Manufacturing Chemist, Inc.

Sand: J.T. Baker Chemical Co.

Sodium dodecyl sulfate (SDS): Polysciences, Inc.

Sodium hypochlorite (4-6%): Fisher.

Trishydroxymethylamino methane (Tris): Sigma.

Urea: Fisher.

X-ray film XAR-5: Eastman Kodak Co.

Column medium and Resins

AG 501-X8 mixed bed-resin: Bio-Rad.

DEAE-cellulose, DE-52: Whatman.

Oligo(dT)-cellulose: Collaborative Research, Inc.

Sephadex G-50: Pharmacia.

Enzymes

Alkaline Phosphatase: P-L Biochemical, Inc.

Creatime Phosphokinase: Calbiochem, Inc.

DNA ligase (E. Coli): New England Bio-Labs.

DNA polymerase I (E. Coli): New England Bio-Labs.

Hinf I: Bethesda Research Laboratory (BRL).

Proteinase K: Boehringer Mannheim.

Polynucleotide Kinase: New England Bio-Labs.

Pst I: New England Bio-labs.

Reverse transcriptase: Life Science, Inc.

Terminal Deoxynucleotidyl transferase: Washington Diagnostic System, Inc.

Medium components

Agar: MCB manufacturing Chemists, Inc.

Bacto-Trypton: Difco Laboratories.

Thiamine HCl: Sigma Chemical Co.

Yeast Extract: Difco Laboratories.

Membranes

Dialysis Tubing: Spectrum Medical Industries Inc.

Nitrocellulose membrane (BA85): Schleicher & Schuell.

Nucleic Acids,Nucleotides and Nucleosides

ATP: Calbiochem.

dNTP: Sigma Chemical Co.

E. coli DNA, Type VIII: Sigma Chemical Co.

GTP: Sigma Chemical Co.

Oligo(dG)12-18: P-L Biochemicals, Inc.

Oligo(dT)12-18: P-L Biochemicals, Inc.

Herring Sperm DNA, type III: Sigma chemical Co.

Proteins

Bovine Serum Albumin: BRL.

Bovine Serum Albumin (fatty acid free): Sigma Chemical Co.

Radioactive Reagents

(^{32}P) dNTPs: New England Nuclear.

(^{32}P) ATP: New England Nuclear.

METHODS

RNA isolation

The procedures used to isolate RNA were as described by Higgins et al (1976) with some modification by Muthukrishnan et al (1979). The procedures were described as follows:

Preparation of barley half seeds

Seeds were prepared as described by Chrispeels and Varner (1967). Seeds of barley were cut in half transversely and the halves without the embryo were used. The half seeds were sterilized with 1% sodium hypochlorite for 15 minutes, rinsed twice with sterile water, degassed for 5 minutes by aspiration vacuum, rinsed twice with water again. Then 100-150 seeds were placed on moistened sterile sand in 100mm petri dishes and wrapped in aluminium foil and stored at 4^oC for 3 days.

Obtain aleurone layers

Barley half seeds, after 3 days imbibition in sand were washed with washing solution (chloramphenicol 10 µg/ml H_2O). Aleurone layers were then removed from the half seeds by dissection and washed twice with 3 ml of ice-cold washing solution. Twenty aleurone layers were placed into 2 ml of incubation buffer (1mM NaOAC, pH 4.8, 10mM $CaCl_2$, 10 µg/ml chloramphenicol) with or without GA. Incubation was at 25^oC in a Dubnoff metabolic shaker at 60 oscillations per minute for a period of 2 to 24 hours. The concentration of GA was 1 uM unless otherwise indicated. After incubation, aleurone layers were collected and stored at -20^oC

until use.

Grinding

For every 180 aleurone layers, 0.2 g of polyvinylpolypyrrolidone (PVP) and 2.0 g of sterile sand were placed into a chilled mortar ($-20^{o}C$). The aleurone layers were removed from the freezer, placed in the mortar and ground for 3 minutes. Four ml of grinding buffer (100 mM sodium glycinate, pH 8.0, 10mM EDTA., 100mM NaCl, 1% SDS, 50 μg proteinase per ml) were added and ground for an additional 3 minutes. Another 6 ml of grinding buffer was added, mixed well and the homogenate was transferred into 40 ml sterile centrifuge tubes. The mortar was rinsed with 7.5 ml more of grinding buffer and added to centrifuge tubes. The homogenate was centrifuged at 9750 x g for 10 minutes at $4^{o}C$. The supernatant was transferred into a sterile 500 ml flask and discarded the pellet.

Extraction

Seventeen and one half ml of phenol/chloroform (1/1=v/v) was added to the flask and shaken at 50 RPM for 5 minutes at room temperature. The content of the flask was poured into centrifuge tubes and centrifuged at 12,000 x g for 10 minutes. The organic phase was back extracted once with 15 ml of grinding buffer, and the aqueous phase was separated from organic by centrifugation. The two aqueous phases were combined.

To the combined aqueous phases an equal volume (30 ml) of the phenol/chloroform mixture and 0.2% SDS was added, shaken for 5 minutes, and centrifuged for 10 minutes at 12,000 x g. The aqueous phase was again extracted with an equal volume of

phenol/chloroform solution. The aqueous layer from the final extraction was transferred into a 250 ml plastic centrifuge bottle, two volumes of absolute ethanol were added, mixed thoroughly and kept at -20°C overnight to precipitate nucleic acids. At this point the preparation is largely free of protein and carbohydrate.

Cetyltrimethylammonium bromide (CTAB) precipitation

The centrifuge bottle was removed from the freezer and centrifuged at 4°C for 30 minutes at 13,200 x g. The pellets were transferred to a Corex centrifuge tube. NaOAc concentration was adjusted to 0.5 M by adding 3 ml of 1 M NaOAc (pH 5.8), kept on ice for 5 minutes and centrifuged at 13,200 x g for 10 minutes at 4°C to pellet insoluble material. The supernatant was transferred to a 50 ml beaker and 1 ml of 1% CTAB was added dropwise with stirring and stirred for 1 hour at 4°C. The RNA pellet was obtained by centrifuge at 4°C, 13,200 x g for 10 minutes. The supernatant was discarded and the RNA pellet was suspended in ice-cold 0.2 M NaOAc pH 5.8 in 70% ethanol. After second centriguga-tion the RNA pellet was dried under N_2 and the pellet was resuspended in 5 ml of 3 M NaOAc pH 5.8, 5mM EDTA, kept on ice with stirring for 1-2 hours to dissolve DNA and then centrifuged at 13,200 x g. The Pellet was dried and dissolved in 0.4 ml of 20mM Tris-Cl pH 8.0. OD_{260} and OD_{280} reading was taken after 1/60 dilution. RNA samples were stored at -20°C. RNA concentrations were calculated by assuming that an absorbance of 1 at 260 nm corresponds to 40 µg of RNA per ml.

Poly(A)$^+$RNA isolation

The procedure of isolating poly(A)$^+$RNA was according to Aviv et al (1972) with some modifications by Muthukrishnan et al (1979). Poly(A)$^+$RNA fractionation was accomplished by chromatography of total RNA on an oligo(dT)-cellulose column. Oligo(dT) and poly A tail of the RNA would anneal under high salt condition and would dissociate under low salt condition.

Total RNA was made up to 3 ml in 20 mM Tris-Cl pH 7.5 (low salt buffer), heated at 60°C for 10 minutes to denature RNA, followed by chilling on ice. NaCl concentration was adjusted to 0.5 M by adding 1 ml of 2 M NaCl, left on ice for 10 minutes, and centrifuged at 9,750 x g for 10 minutes at 4°C to remove any un-dissolved material before loading. An aliquot (0.5 ml) of super-natant was removed and placed in an Eppendorf tube, the RNA was precipitated with ethanol and saved. The rest of the solution was adjusted to 0.2% SDS, 0.5M NaCl, so the RNA was in high salt buffer.

The RNA was loaded onto a 1 ml oligo(dT)-cellulose column which had been equilibrated with high salt buffer. The flow through fraction was collected and ethanol used to precipitated it.

The column was washed with medium salt buffer (20mM Tris-Cl pH 7.5, 0.1 M NaCl) to remove the small amount of ribosomal RNA that bound to the column. Finally, the bound poly(A)$^+$RNA was eluted from the column with 10 ml of low salt buffer (20mM Tris-Cl pH 7.5). The poly(A)$^+$RNA fraction was collected in a polyallomer tube. NaCl concentration was adjusted to 0.2 M then ethanol

precipitated it.

Poly(A)$^+$ and poly(A)$^-$RNA were recovered by centrifugation at 25,000 for 30 minutes at 4°C using SW 27 rotor. The RNA pellets were dried under N2 and dissolved in 400 ul of 10 mM Tris-Cl pH 8.0. Aliquots of each were diluted with 10 mM Tris-Cl pH 8.0. A_{260} and A_{280} reading of each diluted samples was taken in order to determine the yield and the purity of the RNA. The samples were stored at -20°C.

Construction of cDNA clones

Unfractionated poly(A)+ RNA which was isolated from aleurone layers treated with 1 µM GA for 6 hr was used for the construction of recombinant cDNA clones. The procedure used was according to Gubler and Hoffman (1983).

cDNA synthesis

Synthesis of first-strand cDNA is carried out in a reaction volume of 20-40 µl containing 50 mM Tris-HCl, pH 8.3, 10mM DTT, 4mM Na.pyrophosphate. 1.25 mM dGTP, 1.25mM dATP, 1.25 mM TTP, 0.5 mM dCTP. 15-20 µCi of [o-32P]dCTP (±3000 Ci/mmol), 100µg/ml of oligo(dT12-18), 150 µg/ml poly(A)+RNA, and 3000 units of reverse transcriptase/ml for 30 min at 43°C. The reaction is stopped by adding EDTA to 20 mM. The products are extracted with phenol and precipitated with ethanol pit pf 2 M NH_4.acetate as described by Okayama and Berg (1982). The amounts of first strands synthesized are estimated by assaying TCA-insoluble reaioactivity. For second strand synthesis up to 500ng of single-stranded cDNA can be

processed in 100 µl of 20 mM Tris. HCl, pH 7.5. 5 mM $MgCl_2$, 10mM $(NH_4)_2SO_4$, 100 mM KCl, 0.15 mM -NAD, 50µg/ml BSA, 40 µM dNTPs, 8.5 units/ml of E.coli RNase H, 230 units/ml DNA polymerase I, 10 units/ml E. coli DNA ligase. Incubations are sequentially 60 min at 12°C and 60 min at 22°C. EDTA is added to 20 mM to stop the reaction. The products are extracted with phenol twice and then precipitated out of 2 M NH_4.acetate twice as described above. This procedure yields double-stranded cDNA that can be tailed and cloned without further sizing.

Addition of dCMP to the double-strand cDNA

The addition of dCMP to the double strand cDNA 3'-termini by terminal deoxynucleotidyl transferase was carried out according to Land et al (1981). The reaction was performed in 50 µl volume containing 0.1 M Hepes pH 7.0, 20 mM β-mercaptoethanol, 1 mM dCTP, 1 mM $CaCl_2$, 25 units of terminal deoxynucleotidyl transferase (TdT) and 25 µl of first strand cDNA. TdT was added to a pre-warmed reaction mixture and allowed to react at 37°C for 6 minutes. Preliminary data showed that under this condition about 20 dCMP residues are added onto 3'-OH end. Incubation was stopped by adding 1 µl of 0.5 M EDTA, pH 8.0 and quickly chilled on ice. Phenol/chloroform extraction was performed by adding 100 µl of 10 mM tris-Cl, 1 mM EDTA pH 8.0 and 150 µl phenol/chlorofrom mixture. The organic phase was back extracted with 150µl of 10 mM Tris-Cl, 1 mM EDTA pH 8.0. Aqueous phases were combined and passed through a Sephades G-50 column. dC-tailed cDNA was recovered by ethanol precipitation. C-tailed double strand cDNA

was suspended in 20 µl of 10 mM Tris-Cl pH 7.5, 1 mM EDTA and was used to anneal with G-tailed pBR 322 plasmid vector DNA.

Terminal addition of dGMP to linearized pBR 322

Plasmid pBR 322 DNA was isolated from E.Coli LE 392 that had been previously transformed with pBR 322. Purificaton was carried out through CsCl density gradient centrifugation. Twenty µg of purified pBR 322 DNA were digested with 50 units of Pst I at 37°C in 100 µl volume containing 6 mM Tris-Cl pH 7.6, 6 mM $MgCl_2$, 6 mM β-mercaptoethanol, 50 mM NaCl, 250 µg/ml of bovine serum albumin. Completeness of digestion was checked by running an aliquot of the reaction mixture on 1.0% agarose gel. After the digestion was completed, the mixture was extracted with phenol/chloroform and the plasmid DNA was precipitated with ethanol. DNA was recovered by centrifugation.

Terminal addition of dGMP was performed under conditions similar to that for dC-tailing of ds-cDNA except that dCTP was replaced by dGTP and the reaction was 6 minutes. The reaction was carried out in a final volume of 50 µl containing 1 mM dGTP, 0.1 M Hepes pH 7.0, 20 mM β-mercaptoethanol, 1 mM $CaCl_2$, 50 units of TdT and 10 µg of linerarized pBR 322. Approximately 20 dGMP residues were added to the 3'-OH end based on the amount of ^{32}P-dGTP incorporated into TCA precipitable material in a preliminary experiment. DNA was purified by phenol/chloroform extraction, gel filtration and ethanol precipitaiton as described above. The pellet was dissolved in 10 mM Tris-Cl pH 7.6, 1 mM EDTA.

Annealing

In order to find the maximum number of clones obtainable from the annealed products, several annealing ratios (mole ratio of vector to cDNA) were tested. Annealing was performed in 100 mM NaCl, 10 mM Tris-Cl pH 7.6, 1 mM EDTA and 2 ng/µl of DNA. The mixture was heated for 3 hours at 65°C and was cooled gradually to room temperature. The annealed DNA was used to transform *E coli* LE 392.

Transformation of E. coli by annealed DNA

E. coli LE 392 was used to prepare competent cells for transformation according to the procedure of Bolivar et at (1979). A loop of LE 392 from stock culture was streaked on an L-agar plate and incubated at 37°C overnight. Single colonies were picked and inoculated into a 3 ml of L broth. Half ml of this overnight culture was used to inoculate 50 ml of L-broth in a 500 ml Erlenmeyer flask. The culture was incubated with vigorous shaking until OD_{600} reached 0.6.

Competent cells were prepared by $CaCl_2$ treatment as follows. Twenty ml of the above E. coli culture was centrifuged at 4°C, 4,340 xg for 5 minutes. The cell pellet was washed with 10 ml of ice-cold 50 mM $CaCl_2$, 10 mM Tris-Cl pH 7.5 followed by centrifugation. The cells were resuspended in 10 ml of ice-cold 50 mM $CaCl^2$, 10 mM Tris-Cl pH 7.5 and kept on ice for 30 minutes. Cells were in competent state after this treatment and were collected by centrifugation and the cell pelldt was resuspended in 2

ml of ice-cold 50 mM $CaCl_2$, 10 mM Tris-Cl pH 7.5.

Transformation was carried out by mixing 100 µl of competent cells with 35 µl of annealed DNA. This was done in polypropylene tubes (Falcon 2057). After 30 minutes incubation on ice, the mixture was heat-shocked for 5 minutes at 37°C. 1.35 ml of L-broth was added to make a 10-fold dilution. The tube was incubated at 37°C with shaking for 60 minutes. Aliquots of 100 µl were plated on L-plate containing tetracycline 12.5 ug/ml). All plates were incubated at 37°C for 18 hours. Thousands of transformants were obtained. Transformants were screened for resistance to tetracycline (Tet^r) and sensitivity to ampicillin (Amp^s). 3900 clones were Tet^r and Amp^s.

Plasmid DNA isolation

Rapid isolation of plasmid DNA

The procedure of Birnboim and Doly (1979) was followed. A single colony was picked and inoculated into a 3 ml of L-broth containing tetracycline and incubated overnight at 37°C with shaking (300 rpm). A 50 µl aliquot was transferred into a 3 ml L-broth containing tetracycline and was incubated at 37°C with shaking until OD_{600} was about 0.8-1.0. At this point, chloramphenicol (85 mg/ml in 75% ethanol) was added to the culture medium to a final concentration of 170 µg/ml to amplify plasmid. Incubation was continued for 12 hours. One and half ml of bacteria were centrifuged for 10 seconds using a microcentrifuge. To the cell pellet, 100 µl of ice-cold 25 mM Tris-Cl pH 8.0, 10 mM

EDTA, 50 mM glucose and 2 mg/ml lysozyme were added. The mixture was vortexed to suspend the cell pellet and kept on ice for 10 minutes.

The cells were lysed by adding 200 µl of 1% SDS, 0.2 N NaOH solution. The tube was gently inverted several times and kept on ice for 5 minutes. The solution was neutralized by adding 150 µl of 3 M sodium acetate pH 4.8. The tube was inverted several times ,kept on ice for another 10 minutes and centrifuged for 5 minutes to remove the cell debris and most of the host DNA. The supernatant was transferred into another Eppendorf tube, two volumes of 100% ethanol were added to precipitate DNA by keeping at -70°C for 30 minutes and the tube was centrifuged for 2 minutes in a microcentrifuge. The last step was repeated once more and plasmid DNA pellet was dissolved in 10 mM Tris-Cl pH 8.0, 1 mM EDTA and stored at 4°C.

Large scale plasmid DNA isolation

The procedure of Maniatis et al (1982) was followed. A single colony was inoculated into a 3 ml of L-broth containing tetracycline at 37°C overnight with vigorous shaking. 3 ml of overnight culture was incubated into a 2-liter Erlenmeyer flask having 500 ml of L-broth containing tetracycline. The incubation was completed at 37°C with vigorous shaking until the culture reached OD_{600} of 0.8. Chloramphenicol was added to 170 µg/ml and the incubation was continued for at least 12 hours.

Cells were harvested by centrifugation at 6,000X g for 10 minutes at 4°C and the pellet was suspended in 100 ml of ice-cold

10 mM Tris-Cl pH 7.6, 1 mM EDTA, 0.1 M NaCl. Cells were collected again by centrifugation. Cells were lysed by alkali as follow. The cell pellets were suspended in 8 ml of ice-cold 25 mM Tris-Cl pH 8.0, 10 mM EDTA, 50 mM glucose, plus 40 mg lysozyme. The suspension was transferred into a SW 27 centrifuge tube and kept at room temperature for 5 minutes, followed by addition of 16 ml of 0.2 N NaOH, 1% SDS. The tube was inverted several times and was kept on ice for 10 minutes. Centrifugation was carried out at $4^{o}C$, 20,000 RPM for 20 minutes using SW 27 rotor.

Fifteen ml of supernatant was transferred into a Corex centrifuge tube and mixed with 9 ml of isopropanol. The tube was left at room temperature for 15 minutes and then centrifuged at 9,000 RPM for 30 minutes at room temperature. Plasmid DNA formed a pellet at the bottom, the pellet was washed by suspension in 70% ethanol and was centrifuged again at 9,000 RPM for 30 minutes at room temperature and the pellet was dried under N_2.

The pellet containing plasmid DNA was dissolved in 8 ml of ice-cold 10 mM Tris-Cl (pH 8.0), 1 mM EDTA and then 8 g CsCl and 0.8 ml of ethidium bromide (10 mg/ml in H2O) were added. The solution was transferred to an ultracentrifuge tube (Quick-seal, capacity 15 ml) and the remainder space was filled with mineral oil. Centrifugation was carried out at 48,000 RPM for 12 hours at $20^{o}C$ using Type-VTi 50 rotor. After the run, two DNA were visualized under a UV lamp. The upper band contained linear host DNA and nicked circular plasmid DNA. The lower band contained closed supercoiled plasmid DNA. The supercoiled plasmid DNA was collected by inserting a hyperdermic needle connected with a 3 ml

syringe into the centrifuge tube. The plasmid band was slowly drawn into the syringe. The collected solution containing plasmid was DNA transferred to a fresh vertical centrifuge tube and the tube was filled with a mixture of 1.55 g/ml CsCl and 600 µg/ml EtBr). The tube was sealed and centrifuged at 48,000 RPM for 12 hours at $20^{o}C$.

Plasmid DNA was collected as described above and transferred to Eppendorf tubes. The EtBr was removed by repeatedly extracting the collected medium with equal volume of CsCl saturated n-butanol. Finally CsCl was removed by dialyzing the aqueous phase against ice-cold 10 mM Tris-Cl (pH 8.0), 1 mM EDTA for 24 hours with several changes of buffer. When dialysis was complete, an aliquot of 20 µl was taken and diluted with 380 µl of 10 mM Tris-Cl (pH 8.0), 1 mM EDTA. The OD_{260} and $_{280}$ reading of the diluted DNA was taken so the purity and yield of plasmid could be determined. An absorbance of 1 at 260 nm corresponds to 50 µg of DNA per ml. The plasmid DNA was stored at $4^{o}C$.

Preparation of radioactive labeled hybridization probes

Labeling DNA by nick translation

The procedure used was according to Rigby et al (1977). The reaction mixture consisted of 0.5 µg DNA, 4 µl of nick translation buffer (50 mM Tris-Cl pH 7.5, 10 mM β-mercaptoethanol, 4 mM $MgCl_2$, 100 pmoles of cold dNTPs, 25 pmoles of all four [32-P] dNTPs, 1 µl of BSA (0.5 mg/ml), 1 µl of diluted DNase I (10 pg) and appropriate amount of sterile H_2O to make a volume of 19 µl.

The mixture was incubated at 37°C for 2 minutes in order to nick DNA. This was followed by addition of 1 µl of E. coli DNA polymerase I (10 units) and incubated at 14°C for 1 hour. The reaction was stopped by adding 2 µl of EDTA and incubated at 14°C for 1 hour. The labeled DNA was prified by chromatography through Saphadex G-50 spin column. Specific activities were in the range of 1.0-4.0 x 10^8 cpm/ug.

Colonies hybridization

The procedure was according to Maniatis et al (1982). Colony hybridization is accomplished by transferring bacteria from a master plate to a nitrocellulose filter. Using sterile tooth-picks, transfer the individual bacterial colonies to be screened onto 2 master agar plates that contain tetracycline. Make small streaks in a grid pattern. Invert the plates and incubate at 37°C until the bacteria have grown to 0.5-1.0 mm in width. Using a sterile nitrocellulose membrane lift colonies and the filter was transferred to an agar plate containing chloramphenicol (10 µg/ml). Incubate for a further 12 hours at 37°C. After incubation, peel filter from the plate and place it, colony side up, on the SDS-impregnated 3MM paper for 3 minutes. Transfer the filter to the second sheet of 3MM paper that had been saturated with denaturing solution (0.5 M NaOH, 1.5 M NaCl) and soak the filter for 5 minutes. Transfer the filter to the third sheet of 3MM paper that had been saturated with neutralizing solution (1.5 M NaCl, 0.5 M Tris.Cl, pH 8.0), leave for 5 minutes, and then transfer the filter to the fourth sheet of 3MM paper that was

saturated with 2xSSPE (3.6 M NaCl, 200mM NaH_2PO_4, pH 7.4, 20 mM EDTA, pH 7.4). Leave the filter, colony side up, on a sheet of dry 3MM paper. Sandwich the filter between two sheets of dry 3MM paper. Bake for 2 hour at 80°C in a vacuum oven. Hybridize the filter to a ^{32}p-labeled probe as described later.

Preparation of RNA slot blot

Total RNA (5 µg) from aleurone was treated with 20% deionized formaldehyde and 6xSSC. The samples were heated at 65°C for 15 minutes followed by chilling in ice in order to destroy the secondary structure of RNA. The RNA samples were immobilized on nitrocellulose membrane using a 75-well manifold filtration device. Blots were dried and baked as described.

Electrophoresis of DNA

The agarose gels were made in 10 mM sodium phosphate ph 7.0 and electrophoresis was carried out in horizontal gel apparatus (BRL) using 1xTBE (0.089 M Tris-borate, 0.089 M Boric acid, 2 mM EDTA pH 8.0) as electrophoresis buffer. DNA sample were prepared by adjusting DNA solution with 5x gel loading buffer to 1x concentration by dissolving DNA pellet directly into 1x gel buffer (10 mM sodium phosphate pH 7.2, 2 mM EDTA, 10% glycerol, 0.1% bromophenol blue, 2% ficoll). Electrophoresis was carried out at room temperature at 15 mA. The gel was stained in 1 µg/ml solution of ethidium bromide for 5-10 minutes and then photographed with a polaroid MP 4 camera, and polaroid coatless type 667 film, using a UV transilluminator.

Transfer of DNA from agarose gels to nitrocellulose membrane

After electrophoresis, the DNA was stained with ethidium bromide and the gel was soaked in 400 ml of 0.25 M HCl at room temperature for 30 minutes with gentle shaking. After rinsing the gel briefly with H_2O, the DNA was denatured by soaking the gel in 200 ml of 0.5 N NaOH, 1.5 N NaCl at room temperature for 15 minutes with gentle shaking. This was followed by neutralizing the gel by soaking in 200 ml of 1.0 M Tris-Cl pH 7.4, 3.0 N NaCl at room temperature for 30 minutes with gentle shaking. After these treatments, DNA fragments on gels are ready to be transferred. Two sheets of 3MM papers which were previously soaked with 20xSSC, 1 M NH_4Ac were placed in a tray. One nitrocellulose membrane (presoaked in 20xSSC, 1 M NH_4Ac) of the same size as the gel was placed on top of the gel and 2 sheets of wetted 3MM paper were placed on top of the nitrocellulose membrane. Finally a stack of paper towels was placed on the top of 3MM paper and a weight were placed on top of the stack. Transfer of DNA was allowed to proceed for about 18 hours.

Alternatively, gel can be sandwiched in between two sheets of nitrocellulose membrane, 3MM paper, stacks of paper towels in order to obtain duplicate nitrocellulose blots. When transfer was complete, nitrocellulose blot was removed and soaked in 1 M NH4Ac for 20 seconds. The blot was dried and baked as described.

Electrophoresis of RNA through gels containing formaldehyde

Total RNA (10 µg) was denatured with deionized formaldehyde and prepared for gel electrophoresis according to Maniatis et al

(1982). RNA was taken up in 4.5 µl sterile H2O. 2 ul of 10xMOPS buffer (0.4 M morpholinopropane sulfonic acid, pH 7.0, 0.1 M sodium acetate, 10 mM EDTA pH 8.0). 3.5 µl formaldehyde and 10 µl formamide were added and heated at 65°C for 15 minutes to denature RNA. 5 µl of 5x gel loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue) was added so the final concentration was 1x. The sample was loaded into a 1% agarose gel containing 1.1 M formaldehyde, 40 mM MOPS pH 7.0, 10 mM sodium acetate, 1 mM EDTA pH 8.0. The samples were separated by electrophoresis at room temperature until the blue dye had migrated 10 cm. The gel was stained with acridine orange (33µg/ml) in 10 mM sodium phosphate pH 7.0 and destained in the same buffer.

Transfer of Formaldehyde-denatured RNA to nitrocellulose membrane

Soak the gel for 1 hour in 20xSSC and placed nitrocellulose membrane (pre-soaked in 20xSSC) above the gel as described in DNA blotting. After transfer was complete, baked for 3 hours at 80oC under vacuum.

Hybridization of DNA and RNA immobolized on nitrocellulose membrane

The procedure used was according to Muthukrishnan et al (1983). The nitrocellulose membranes were prehybridized in a solution containing 5xSSC, 50 mM phosphate buffer pH 7.0, 10x Denhardt solution (0.2% of bovine serum albumin, ficoll and polyvinylpyrolidone), 250 µg/ml of salmon sperm DNA, and 10 µg/ml of E. coli DNA for 4 hours at 65°C. Nitocellulose membranes then were

incubated with heat denatured 32P-labelled probe in the same buffer containing 0.1% SDS and 10% dextran sulfate for 12-18 hours at 65°C. The nitrocellulose membranes were washed twice at room temperature followed by two 15 minutes washes at 65oC with washing buffer (2xSSC, 50 mM phosphate buffer pH 7.0, and 0.5% SDS) and then washed twice at 65°C with 0.1x SSC, 0.5% SDS for 30 minutes. The membranes were dried and exposed to X-ray film using intensifier screen for 2-5 days. For RNA slot blots 72% of formamide was used in the hybridization solution and they were incubated at 55°C for 14-18 hours.

Estimation of DNA and RNA size from Mobility

The method was according to Methods in Enzymology (volume 68). The size standard for DNA was pBR322 digested with HinfI. The size standard for RNA was an RNA ladder from BRL which contains a mixture six RNA components with different size.

Three points are chosen, corresponding to size standards L_1, L_2, L_3 with mobilities m_1, m_2, and m_3. The three values of L should span the range in which measurements are to be made. The value of m_o that determines that these three points are joined by a straight line is given by

$$L=k_1/(m-m_o)+k_2$$ L= size of unknow

m_o=correction factor

$$m_o=\frac{m_3(L_2-L_3)(m_2-m_1)-m_1(L_1-L_2)(m_3-m_2)}{(L_2-L_3)(m_2-m_1)-(L_1-L_2)(m_3-m_2)}$$

$$k_1 = \frac{L_1 - L_2}{1/(m_1 - m_o) - 1/(m_2 - m_o)}$$

$k_2 = L_1 - k_1/(m_1 - m_o,$

RESULTS

I. Construction of cDNA libraries and identification of α-amylase and non-α-amylase cDNA clones

The cDNA library that was available in our laboratory at the start of this work consisted of a small group of 400 clones. Further, this library construction involved the use of S1 nuclease which is known to remove sequences near the ends. In an attempt to get a longer library and longer inserts with intact end sequences, I started the construction of a new library using the improved techniques that have become available recently (Gubler and Hoffman, 1983).

In barley aleurone layers incubated with GA, translatable α-amylase mRNA can be detected within 3-4 hours, reaches maximum levels around 12 hours after GA addition (Higgins et al, 1976). Analysis of RNA using Northern blots had indicated that α-amylase mRNA levels increase as early as 1 hour after addition of GA (Muthukrishnan et al, 1983). To obtain an RNA preparation containing GA-induced sequences and to minimize the possibility of mRNA degradation, poly(A)$^+$RNA was isolated from aleurone layers treated with 1 μM GA for 6 hours. RNA isolated at this point has been shown to contain several GA-inducible mRNAs besides α-amylase mRNA as indicated by *in vitro* translation (Muthukrishnan et al, 1979). The isolated poly(A)$^+$RNA was used for the construction of a cDNA library. The procedure used for the construction of the cDNA library is described in the Methods

section. Briefly, oligo(dT) was used as the primer for first-strand cDNA synthesis by reverse transcriptase, RNase H and DNA polymerase I was used in second strand synthesis (Gubler and Hoffman,1983). Neither the vector-primer system nor the hairpin loop cleavage by S1 nuclease were used. cDNA made in this way was used to add C tails using terminal deoxynucleotidyl transferase and cloned into the PstI site of pBR 322 (G-tailed) without further purification or sizing.

A group of 3900 $Tet^{r}Amp^{s}$ clones was obtained from cDNA prepared from $poly(A)^{+}RNA$ sequences present in GA-treated aleurone layers. The library was screened by the colony hybridization procedure of Maniatis et al.(1982). This was accomplished by transferring bacteria from a master plate to a nitrocellulose filter. The colonies on the filter were then lysed and the liberated DNA was fixed on the filter by baking. α-amylase clones were identified by their hybridization to 5'-end labeled insert DNA from a previously characterized α-amylase cDNA designated as clone 103. Clone 103 had been characterized previously by Muthukrishnan et al (1983) and Huang et al (1984).

The clone 103 insert is 595 bp long and is the longest α-amylase cDNA in our collection. It contains only a part of the 3'-terminal region of a high pI α-amylase gene. Among the 3,000 clones that were examined, 21 produced a positive autoradiographic signal in a 16h exposure. Plasmid DNA was prepared from these clones by the method of Birinboim and Doly (1979). The insert sizes of these clones were determined by digestion of plasmid DNA with PstI followed by electrophoresis in

1% agarose gels using HinfI-digested pBR322 as a size marker. The insert sizes of these α-amylase plasmid clones were 200-550 bp.

In a previous study to identify GA-induced sequences, Jeng-Kuen Huang (1985) had carried out a differential cDNA screening procedure. Two cDNAs were prepared from mRNA's isolated from aleurone layers treated for 6 hours either with GA (GA-cDNA) or without GA (control cDNA). Clones that hybridized strongly to ^{32}P-Labelled GA-cDNA but not to ^{32}P-labelled control-cDNA were designated as GA-induced clones. Several of these were identified as α-amylase clones by hybridization with α-amylase cDNA clone 103. GA-induced clones which did not hybridize to α-amylase cDNA probes were classified as GA-induced non-α-amylase clones. Clones identified as GA-induced non-α-amylase clones included clones 85, 98, 121, 132, 137, 159, 162. Among these clones 121, 137, 159 and 162 were shown by DNA sequencing to contain only long poly(G) poly(A) and poly(C) sequence and very little coding sequences (Swegle, unpublished data). Clone 85, 98 and 132 had insert of 175, 160, 150 base pairs respectively.

In order to find longer inserts clones, I screened the cDNA library with 5'-end labeled insert DNAs from GA-induced clones 98, 132 and another clone, 248, whose expression was reduced in GA-treated aleurone cells. 39 clones showed hybridization to these probes (Data not shown). These clones were further grouped as 98-like, 132-like or 248-like as follows. PstI digested plasmid DNAs from these clones were electrophoresed in agarose gels and were blotted onto nitrocellulose. Triplicate Southern blots were individually probed with nick-translated plasmid or insert

DNA from clones 98 or 132 or 248. The result with clone 98 (part of the data show in Fig.1A), indicated that twelve clones (5, 6, 10, 12, 15, 16, 17, 20, 23, 24, 25, 29) appeared to contain inserts related to that of clone 98. The insert sizes varied from 150 bp to 550 bp. Clone 10 is the longest one. Nine clones (1, 7, 8, 9, 18, 19, 21, 39) were identified as related to clone 248 when probed with nick-translated clone 248 insert DNA (Fig.1B). Insert sizes of these clones ranged from 220-700 bp. Clone 8, the longest one had an insert of 700 bp. None of the 39 clones hybridized with nick-translated 132 clone. This result suggests that clone 132 probably represents a very rare RNA species in barley aleurone layers. Clones related to 85 were obtained in a separate screening experiment. When half of the cDNA library was screened with 5'-end labeled insert DNA from clone 85, 3 clones were identified as 85-like (Swegle, unpublished data). They all have 650 size insert. Clone 8, 10 , and 85 which had the longest inserts were used for analysis of RNA as described below. Clone 248 was included as a control for a non-GA-inducible gene.

II. Determination of the size and extent of induction by GA of non-α-amylase clones

The sizes of the RNA species corresponding to the cDNA clones, and the magnitude of the induction by GA were determined by probing RNA gel blots with 32P-labelled plasmid DNA's (Northern blots). The blots contained 10 μg of total RNA from aleurone cells incubated for 12 hours with or without 1 μM GA. These

results (Fig. 2) showed that mRNA corresponding to clones 10 and 85 were more abundant in aleurone cells treated with GA for 12 hours than in the cells incubated without GA. These result indicate clones 10 and 85 are GA-induced clones.

Clone 8 also hybridized to aleurone RNA but in this case, the RNA was more abundant in control aleurone cells than in those incubated with GA for 12 hours. No RNA hybridizing with clone 132 was detected, even when 25 µg of total RNA was used. This result is consistent with the interpretation that clone 132 represents a rare RNA species.

The lengths of the major RNA band detected by the GA-inducible cDNA clones 10 and 85 were found to be 1450, and 1500 nucleotides respectively, and the non GA-inducible clone 8 hybridized to an RNA with a size of 1350 nucleotides (Fig. 2). These values were derived by extrapolate vs. interpolate comparison with the migration of commercially available RNA's of known size. Clones 8, 10, and 85 all hybridized to RNA's of different size, confirming that each represents a different RNA species.

The GA-inducibility of the genes corresponding to these clones was quantitated using slot blots of 5 µg of RNA isolated from barley aleurone cells treated with or without GA for 12h (Fig. 3). The blots were probed with nick-translated cDNA clones and the autoradiogram was scanned using an LKB laser densitometer. mRNA complementary to 103 and E show significant increases in concentration when treated with GA for 12 hour (Fig. 3A, 3B). Concentrations of clone 85 and clone 10 mRNA's increase 1.5-2 fold at 12 hour after treatment with GA (Fig. 3C, 3D). Clone 8

mRNA decreased 3 fold at 12 hour point when incubated with GA (Fig. 3E).

III. Time course of expression of GA-regulated RNA species

To determine whether the accumulation of transcripts for all GA-inducible genes, as well as α-amylase genes follow a similar pattern, the time course of RNA induction following GA treatment was examined. Slot blots containing 5 μg RNA prepared from barley aleurone layers treated with 1 μM GA for increasing periods of time were probed with nick-translated α-amylase and non-α-amylase cDNA clones. The results are shown in Fig.4. The relative intensities of the bands in the autoradiogram were quantitated using an LKB laser densitometer. Because of differences in the specific activity and lengths of inserts in different clones, relative mRNA concentration of different clones could not be determined by a direct comparison of band intensities between sets. However, by assigning a value of 100% to the highest value in each set, and expressing other values relative to the peak value, it is possible to compare the kinetics of accumulation of mRNA for these different clones. Time courses for high pI α-amylase clones 103 and low pI α-amylase clone E (Roger et al. 1983) were included for comparison.

α-amylase mRNA represented by clone 103 becomes detectable at low levels 2 h after addition of 1 μM GA and continues to increase in concentration in aleurone cells up to around 16 h after addition of the hormone (Fig. 4A). Later, there is a decline in

the concentration of α-amylase mRNA. The pattern obtained with clone E as probe is different from that observed with clone 103 (Fig. 4B). α-amylase mRNA detectable by clone E increased gradually in aleurone cells in the early period of GA-treatment reach a maximum value around 18 hours and declined more gradually than RNA detectable by clone 103. These results are consistent with the results of Huang et al (1984) who reported that the regulation of high pI and low pI α-amylase genes are not coordinate.

Clone 85 RNA increased less dramatically and reached its peak at 18 h after GA addition (Fig. 4C). At this point the induction is only 4 fold over the zero-time value. At 24 hours, the level has decreased to 35%.

mRNA complementary to clone 10 (Fig. 4D) was detected as early as 2 h after GA addition, reached a maximum at 14 h after addition of GA and declined thereafter. The pattern of clone 10 mRNA accumulation roughly parallels that of clone 103 mRNA.

mRNA corresponding to clone 8 also increased gradually 2-3 fold before reaching a peak at 10 hours and remained relatively steady showing only a two-fold variation (Fig. 4E). Densitometry analysis showed only a 4-fold increase from 0 time to the point when the maximum level was reached. These results indicated that transcripts corresponding to all these in clones 10, 85 and 8 increase in aleurone during the incubation with GA. But the extent of GA-induction of the individual genes are quite different. The increase in transcripts detectable by clone 8 was somewhat surprising because this clone was classified as not inducible by

GA from the data in Fig.2.

It appears that the level of a specific mRNA could increase in aleurones even in the absence of GA. Therefore, to classify a gene as GA-inducible, a parallel time course of accumulation of mRNA in aleurones incubated without GA, appears to be necessary.

In order to examine the pattern of accumulation of mRNA in aleurones incubated without GA, a time course experiment using aleurone layers incubated without GA was carried out. RNA was extracted after 0, 4, 6, 8, 12, 18, and 24 hours of incubation without GA. Slot blots containing 5 μg of these RNAs were probed with [^{32}P]-labeled cDNA from the five clones (Fig. 6).

Clone 103 RNA concentration in aleurone layer treated without GA showed no significant change with time (Fig. 6A). The concentration of clone E mRNA treated without GA increased slowly and showed a peak at 16 h (Fig. 6B).

Densitometry analysis, showed that clone 85 RNA from aleurones treated without GA had 2 peaks, one at 6 h and the second at 16 h. These peaks represented increase of less than 2-fold compare to the 0 hour value (Fig. 6C).

RNA homologous to clone 10 changed less than three fold in the absence of GA (Fig. 6D).

The clone 8 RNA concentration did not change until 12 hour and then showed a 2 fold increase at 16 hour (Fig. 6E).

Clones 103, E, 10, 85, and 8 RNAs increase to different extents and reach peaks at different times when treated without GA. The RNA concentration increased (2-3 fold) when incubated without GA. However, in the presence of GA, clone 103, E, 10 and 85 mRNAs

were induced 80, 20, 7, and 4 fold, respectively. From these results, it has been conclusively established that clone 103, E, 10, and 85 are GA inducible and clone 8 is not. the results also show that in barley aleurone multiple genes are regulated in different manners by GA.

IV. Effects of ABA and cycloheximide on non-α-amylase clones

The GA-induced synthesis of α-amylase in barley aleurone is inhibited in the presence of ABA (Ho, 1976), and it has been concluded that the regulation of α-amylase genes expression by ABA is primarily at the level of transcription (Muthukrishnan,et al. 1983). To examine the effect of ABA on GA-inducible cDNA clones, slot blots were prepared containing 5 μg of RNA isolated from aleurone layers treated for 12 hours with 1 μM GA in the absence as well as the presence of different concentrations of ABA (10^{-5}, 10^{-6}, 10^{-7}M).

Both clone E and 103 α-amylase mRNA concentration were found to decrease 10 fold to 6 fold when the ABA concentration was increased from 10^{-7} M to 10^{-5} M(Fig. 8A, 8B), confirming the observations of Muthukrishnan et al (1983).

Clone 10 RNA production was not inhibited by ABA when incubated with GA (Fig. 8D). Similarly, different concentration of ABA had little effect on level of mRNA detectable by clone 85 at 12 h after GA treatment (Fig. 8C). The concentration of non-GA-inducible clone 8 RNA also changed little when the ABA concentration was increased. The results obtained with ABA indicate that

regulation of non-α-amylase genes is different from that of α-amylase genes. ABA inhibits GA-induced α-amylase mRNA synthesis but has little or no effect on non-α-amylase mRNA synthesis.

Muthukrishnan et al. (1979, 1983) had reported that an inhibitor of protein synthesis, cycloheximide, blocked the appearance of α-amylase mRNA when added along with GA, indicating that the synthesis of a protein factor(s) is required to bring about GA induction of α-amylase mRNA synthesis. To study whether cycloheximide blocks the appearance of non-α-amylase mRNA in a similar manner, slot blots containing 5 μg of RNA extracted from barley aleurone cells treated with cycloheximide in the presence and absence of GA were hybridized with nick-translated cDNA clone probes.

When probed with clone 103 DNA (Fig. 9A), cycloheximide added at a concentration of 10 μM caused nearly complete inhibition of GA-induced α-amylase mRNA synthesis. In the presence of cycloheximide, induction of clone E and clone 10 mRNA's by GA was affected in the same way as clone 103 mRNA (Fig. 9B, 9D). Cycloheximide decreased clone E and Clone 10 mRNA synthesis when added with GA.

Clone 85 and mRNA concentrations did not decrease when incubated with cycloheximide plus GA (Fig. 9C). The GA-mediated increase in concentration of clone 85 mRNA is not dependent on new protein synthesis. Apparently, unlike α-amylase and clone 10, induction of transcription of clone 85 genes by GA can occur without a new GA-induced protein factor.

mRNA concentration detectable by the non-GA-inducible clone 8

decreased when incubated with cycloheximide without GA at 12 hours (Fig. 9E). However, the concentrations of clone 8 mRNA from aleurone layers treated with GA and treated with GA plus cycloheximide were nearly the same. Thus, cycloheximide appears to have an inhibitory effect on clone 8 mRNA only in incubations without GA. Clearly, the effects of cycloheximide varies for the different clones studies here.

V. Structure genes for clone 8 and clone 10

Southern blot analyses have shown that α-amylases are coded for by multiple genes present on barley chromosomes 1 and 6.(Muthukrishnan, 1983, 1984). High pI α-amylase genes (detectable by clone 103) are on chromosome 6 and low pI α-amylase genes (detectable by clone E) are on chromosome 1.

Genomic DNA from six euplasmic wheat-barley chromosome addition lines and from wheat and barley parents were digested with restriction enzymes and analyzed for the presence of clone 8 and clone 10 genes sequences (Fig. 10, 11). The different addition lines contain 6 of the 7 barley chromosome pairs. Addition lines for chromosome 5 are sterile (Islam et al, 1981). Clone 8 gene sequences of barley were detected only in addition line 7 carrying barley chromosome 7 but not in other addition lines. Since no barley bands corresponding to clone 10 were detected in any of the addition lines, it was concluded that clone 10 gene probably is located in barley chromosome 5.

Fig.1. Identification of cDNA clones containing clone 98 and clone 248 sequences.

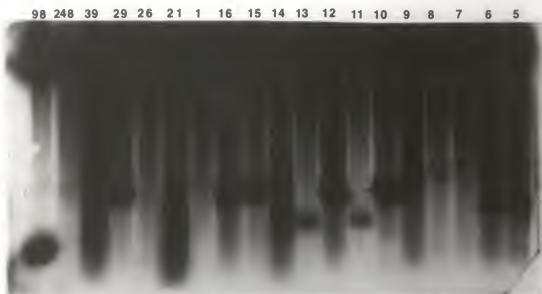
cDNA clones that showed strong hybridization to mixed 32P-labelled 248, 98 and 132 cDNA were digested with PstI followed by electrophoresis in 1.5% agarose gels and blotting onto nitrocellulose membranes. Duplicate Southern blots were probed with;

A. Nick-translated clone 98 cDNA (specific activity 3.6×10^7 cpm/µg).

B. Nick-translated clone 248 insert DNA (specific activity 2.7×10^7 cpm/µg).

The nitrocellulose membranes were exposed to X-ray film for 16 hours.

A. 98



B. 248

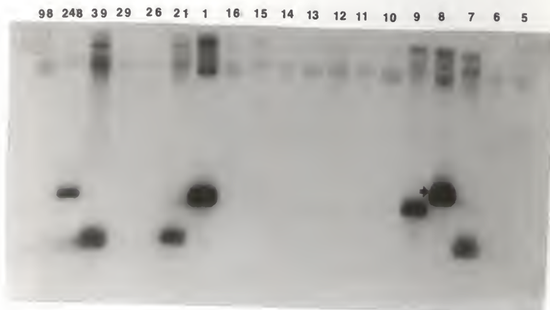


Fig.2: Size determinations of the RNA species represent by non-α-amylase clones.

10 µg of total RNA from aleurone cells incubated with or without 1µM GA were denatured with formaldehyde followed by electrophoresis in 1% agarose formaldehyde gels and blotting onto nitrocellulose membranes. Northern blots were probed with nick-translated plasmid DNA (specific activity cpm/µg);

A. Clone 10 ($3.2x10^7$).

B. Clone 85 ($4.4x10^7$).

C. Clone 8 ($3.5x10^7$).

The size of denatured RNA bands (commercially available from BRL) indicated in Fig.

The nitrocellulose membranes were exposed to X-ray film for 24 hours.

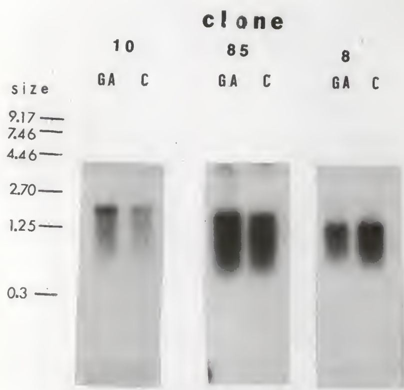

clone
10
85
8
size
GA C
GA C
GA C
9.17
7.46
4.46
2.70
1.25
0.3

Fig.3 α-amylase and non-α-amylase mRNA levels after treatment with or without GA.

Total RNA was isolated from aleurone layers treated with or without 1uM GA for 12 hours. Slot blots containing 5 ug aliquots of denatured RNA were probed with nick-translated cDNA plasmids (specific activity in cpm/ug).

A. Clone 103 ($5.6x10^7$).

B. Clone E ($1.2x10^8$).

C. Clone 85 ($6,8x10^7$).

D. Clone 10 ($9.8x10^7$).

E. Clone 8 ($1.2x10^8$).

The nitrocellulose membranes were exposed to X-ray film for 18 hours.

clone
GA
C
103
E
85
10
8

Fig. 4: α-amylase and non-α-amylase mRNA levels as a function of time.

Total RNA was isolated from aleurone layers treated with 1μM GA for the indicated periods of time. 5μg aliquots of RNA were denatured in 2.2 M formaldehyde and applied to a nitrocellulose membrane as described in Methods.

The slot blots were hybridized with nick-translated cDNA plasmids (specific activity in cpm/μg):

A. Clone 103 (7.8×10^7).

B. Clone E (1.6×10^8).

C. Clone 85 (5.6×10^7).

D. Clone 10 (9.8×10^7).

E. Clone 8 (1.1×10^8).

The nitrocellulose membranes were exposed to X-ray film for 16 hours.

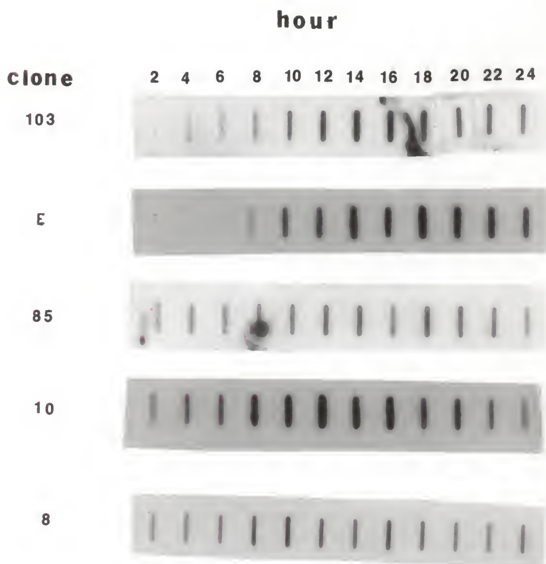

hour
clone
2 4 6 8 10 12 14 16 18 20 22 24
103
E
85
10
8

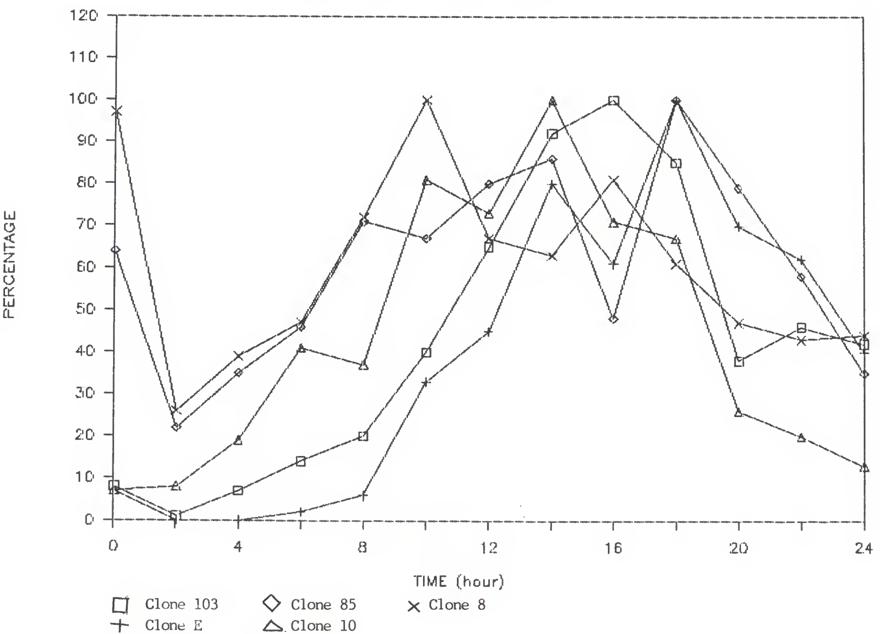
Table I. Densitometry readings for Fig. 3.

Clone	with GA	without GA
103	1706	85
E	875	0
85	895	501
10	1589	964
8	724	1972

Table II. Densitometry readings for Fig. 4.

Clone \ Hour	2	4	6	8	10	12	14	16	18	20	22	24
103	10	63	129	179	364	595	840	912	779	346	424	386
E	0	2	34	94	500	684	1235	942	1535	1069	948	618
85	106	170	219	340	323	387	413	228	480	379	278	170
10	163	376	795	711	1572	1421	1934	1368	1305	509	384	245
8	133	195	235	360	502	338	315	408	306	235	217	220

Fig.5 Computer graph shows densitometry readings of α-amylase and non-α-amylase GA-treated mRNA levels as a function of time. The highest reading for each individual probe was taken to be 100%.



GA RNA TIME COURSE
PERCENTAGE
120
110
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
TIME (hour)
Clone 103
Clone E
Clone 85
Clone 10
Clone 8

Fig. 6: α-amylase and non-α-amylase mRNA levels as a function of time.

Total RNA was isolated from aleurone layers treated without GA for the indicated period of time. 5µg aliquots of denatured RNA were applied to a nitrocellulose membranes as described in Methods.

The slot blots were hybridized with nick-translated cDNA plasmids (specific activity in cpm/µg):

A. Clone 103 (7.8×10^7).

B. Clone E (9.1×10^7).

C. Clone 85 (5.6×10^7).

D. Clone 10 (9.8×10^7).

E. Clone 8 (1.1×10^8).

The nitrocellulose membranes were exposed to X-ray film for 18 hours.

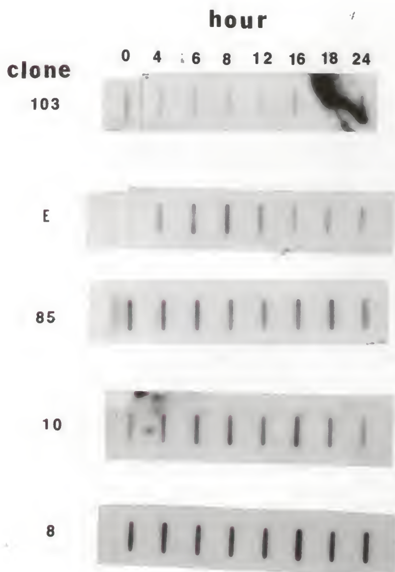

hour
clone
0
4
6
8
12
16
18
24
103
E
85
10
8

Table III. Densitometry readings of Fig. 6.

Hour	0	4	6	8	12	16	18	24
Clone								
103	75	65	73	74	73	76	-	-
E	0	110	115	110	177	293	239	115
85	308	318	359	285	196	310	361	231
10	146	357	350	360	247	405	269	154
8	486	614	509	582	618	1021	654	729

Fig.7. Computer graph shows densitometry readings of α-amylase and non-α-amylase mRNA levels as a function of time treated without GA.

The highest reading for each individual probe was taken as 100%.

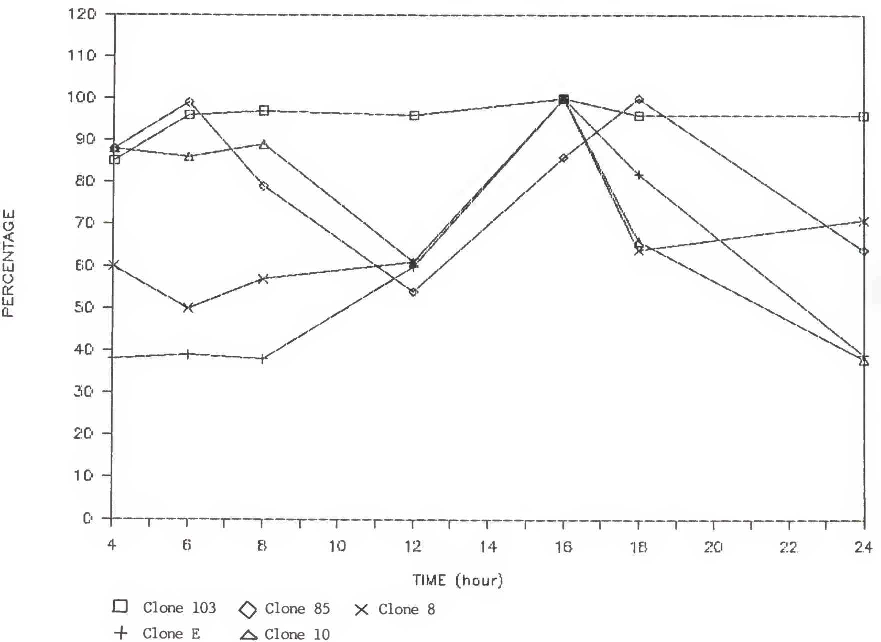

C RNA TIME COURSE
PERCENTAGE
120
110
100
90
80
70
60
50
40
30
20
10
0
4
6
8
10
12
14
16
18
20
22
24
TIME (hour)
Clone 103
Clone 85
Clone 8
Clone E
Clone 10

Fig. 8: Effect of different concentrations of ABA on α-amylase and non-α-amylase mRNA levels.

Total RNA was isolated from aleurone layers treated for 12 hours with 1uM GA in the absence as well as the presence of 10^{-5}, 10^{-6},
10^{-7} M ABA. 5ug aliquots of denatured RNA were applied to a nitrocellulose membrane as described in Methods.

The slot blots were hybridized with nick-translated cDNA plasmids (specific activity cpm/ug):

A. Clone 103 (7.8×10^{7}).

B. Clone E (9.1×10^{7}).

C. Clone 85 (5.6×10^{7}).

D. Clone 10 (9.8×10^{7}).

E. Clone 8 (1.1×10^{8}).

The nitrocellulose membranes were exposed to X-ray film for 18 hours.

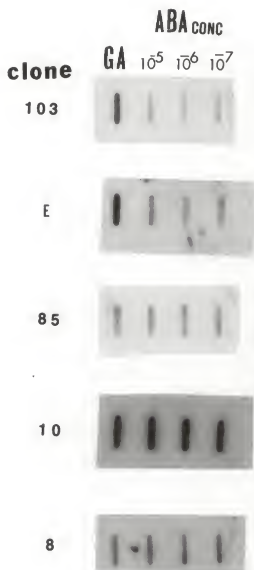

ABA CONC
GA
10^{-5}
10^{-6}
10^{-7}
clone
103
E
85
10
8

Fig. 9: Effect of cycloheximide on α-amylase and non-α-amylase mRNA levels.

Total RNA was isolated from aleurone layers treated with 10µM cycloheximide in the presence and absence of 1 µM GA for 12 hours. 5µg aliquots of RNA were treated with 2.2 M formaldehyde and applied to a nitrocellulose membranes as described in Methods.

The slot blots were hybridized with nick-translated cDNA plasmids (specific activity cpm/µg):

A. Clone 103 (7.8×10^7).

B. Clone E (9.1×10^7).

C. Clone 85 (5.6×10^7).

D. Clone 10 (9.8×10^7).

E. Clone 8 (1.1×10^8).

The nitrocellulose membranes were exposed to X-ray film for 18 hours.

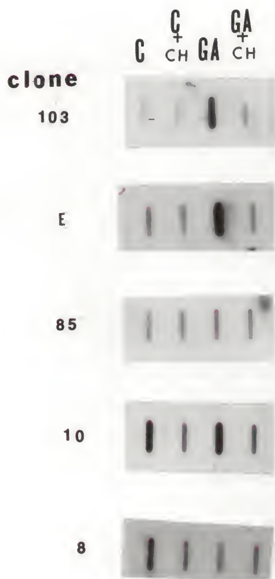

C
C
+
CH
GA
GA
+
CH
clone
103
E
85
10
8

Table IV. Densitometry readings of Fig. 8.

Clone \ treatment.	GA	$GA+10^{-5}ABA$	$GA+10^{-6}ABA$	$GA+10^{-7}ABA$
103	573	81	99	104
E	1100	180	185	260
10	1021	1259	1239	972
85	236	200	171	165
8	354	293	323	352

Table V. Densitometry readings of Fig. 9.

Clone \ treatment.	w/o GA	w/o GA+CH	GA	GA+CH
103	80	70	968	149
E	263	211	1931	192
10	1213	329	1347	392
85	186	180	269	209
8	937	327	283	222

Fig. 10. Structure genes for clone 8.

20 μg of DNA from each addition line and both parents were digested with 60 units of HindIII for 16 hours at 37°C. The digested DNAs were electrophoresed in 0.7% agarose gel, blotted onto nitrocellulose membrane, and probe with nick-translated plasmid cDNA clone 8 (specific activity $3.0x10^{8}$ cpm/μg). The migration position of marker DNA bands and their sizes in kilobases are indicated by arrows on the left. The barley-specific DNA bands in digests of addition line 7 DNA are indicated by small arrow. The nitrocellulose membrane was exposed to X-ray film for 48 hours.

Lane 1. Batzes barley DNA.

Lane 2. Chinese spring DNA.

Lane 3-8. DNA from addition line carrying barley chromosomes 1, 2, 3, 4, 6, 7 respectively.

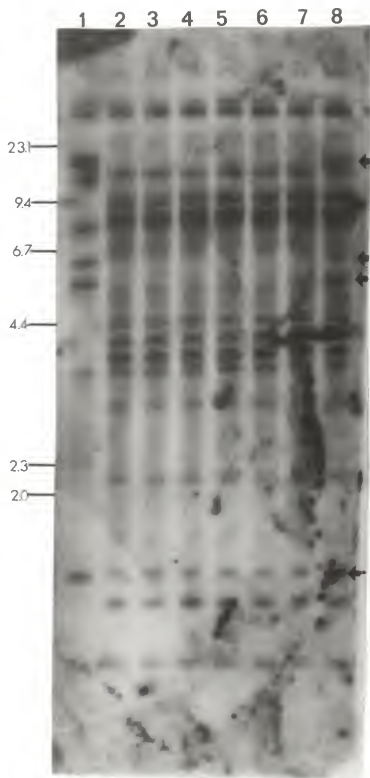

1
2
3
4
5
6
7
8
23.1
9.4
6.7
4.4
2.3
2.0

Fig. 11. Structure genes for clone 10.

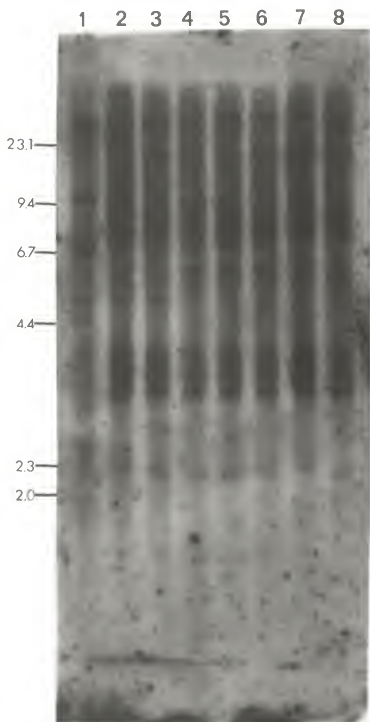
The experimental details are the same as in the legend to Fig. 10, except that nick-translated clone 10 DNA (specific activity $2.0x10^8$ cpm/μg) was used as the hybridization probe. Since no bands could be detected, cDNA clone 10 structural genes may be in the missing addition line 5. The nitrocellulose membrane was exposed to X-ray film for 48 hours.

DISCUSSION

The influence of GA on the expression of genes for several hydrolytic enzymes including α-amylase has been studied as a model system for hormonal control of gene expression in plants. The development of recombinant DNA techniques makes it possible to analyze the action of GA in finer detail. The availability of cDNA copies of mRNA's provides a powerful and sensitive tool for analyzing the expression of genes.

The main focus of this study was to isolate cDNA probes for GA-inducible genes other than α-amylase genes and to use these probes to quantitate the expression of the corresponding genes. A comparison of 1) the kinetics of accumulation of individual transcripts after addition of GA, 2) response to the negative growth regulator ABA, 3) influence of the inhibitor of protein synthesis, cycloheximide, and 4) the chromosomal location of the individual GA-inducible genes could provide some insights as to whether these genes share a common pattern of regulation. I have tried to provide some information on these aspects of gene regulation by GA.

More than 3000 Tet^r Amp^s clones were obtained from a cDNA library prepared from poly(A)+RNA isolated from GA-treated barley aleurone layer cells. Twenty one of these clones were α-amylase clones as shown by their hybridization to α-amylase cDNA clones, E and 103. Several clones were identified as non-amylase GA-inducible by their hybridization to previously characterized non-


1 2 3 4 5 6 7 8
23.1
9.4
6.7
4.4
2.3
2.0

α-amylase clones 98, 248, 132, 85. These non-α-amylase cDNAs which are known to be regulated by GA probably represent genes for other hydrolytic enzymes.

There are two groups of α-amylase isoenzymes in barley aleurone layer cells, and they are classified as low and high pI α-amylases. GA induces both groups of α-amylase mRNA to different degrees. The results reported here indicate that low pI α-amylase mRNAs (clone E detectable) increase only 1.5 fold at 16 hours when incubated without GA, but when incubated with GA the increase is about 14-fold at 18 hours. High pI α-amylase mRNA (clone 103-detectable) are present at very low levels in aleurone prior to incubation with GA, but increase 40 fold at 16 hours of GA treatment. In several such experiments, the increase observed ranged between 5- to 20-fold for clone E mRNA, and 30- to 100 fold for clone 103 mRNA (Muthukrishnan et al, 1983a, Rogers, 1985. Deikman et al, 1983). The peaks reported here are somewhat later than the value of 10-14 hours reported by Muthukrishnan et al (1983a) and Deikman et al (1986). These could due to a different harvest of seeds or to experimental variation.

In order to determine whether our non-α-amylase genes are regulated in parallel to α-amylase, detection and quantitation of non-α-amylase mRNA sequence was done by probing RNA blots with nick-translated non-α-amylase cDNA clones.

The appearance of mRNA complementary to these cDNA clones as a function of time indicated that each clone showed different peak of RNA accumulation. Clone 10 mRNA concentration increases less than 2 fold at 16 hours when incubated without GA, but at 10

hours the levels is 6-times as high as the zero time value.

Clone 85 mRNA concentration increases 1.5 fold in aleurone at 16 hours when incubated without GA, but increases 4 fold at 18 hours of GA treatment.

Clone 8 mRNA is present at high levels in aleurones incubated without GA, and does not increase when treated with GA.

Thus RNA species corresponding to all these clones except clone 8 show increasing levels in aleurone cells when incubated with GA, but the induction of these mRNAs differ from the pattern seen with α-amylase mRNA with respect to the time of peak accumulation and extent of stimulation over the zero time value.

In the presence of ABA, there is a 10 fold decrease in the GA-induced accumulation of high pI α-amylase mRNA sequence at 12 hours, and about 5 fold decrease in low pI α-amylase mRNAs. This is in agreement with the studies of Muthukrishnan et al (1983), and Nolan et al (1987). Presumably, ABA suppresses transcription of both groups of α-amylase genes, but the levels of low pI α-amylase mRNA were decreased to a lower extent by ABA than were high pI mRNA.

The GA accumulation of α-amylase mRNA is blocked by cycloheximide which suggests the requirement of a newly synthesize protein factor for efficient expression of α-amylase gene as reported by Muthukrishnan (1979, 1983). The accumulation of clone 10 mRNA is not affected by ABA but is blocked by cycloheximide. Both ABA and cycloheximide did not block the appearance of clone 85 mRNA both in the presence or absence of GA. This is the first example of a GA-inducible clone whose expres-

sion is induced by GA in the absence of protein synthesis. Thus, this may be one of the early genes to be turned on by GA.

ABA has no effect on clone 8 mRNA, but cycloheximide decreases clone 8 mRNA when added alone. The decrease of mRNA concentration of clone 8 after GA addition may be due to turnover of this RNA following shut off of transcription after GA addition.

It appears that non-α-amylase genes represent by clone 10, 85, and 8 react differently from α-amylase genes in responds to GA, ABA and cycloheximide.

Mundy and Fincher (1986) reported that the hormone regulation of levels of translatable mRNA encoding the (1-3,1-4)-β-glucanase of barley aleurone appears to parallel the regulation of α-amylase genes: levels of both sets of genes are increased 10-fold by GA, and this induction is blocked by addition of ABA. The expression of the thiol protease mRNA is induced 7-fold by GA and peaks after 18 hours of hormone addition. In the presence of ABA, there is in a substantial decrease in the amount of hybridizable RNA (Rogers et al. 1985).

According to DNA sequencing data (Swegle, unpublished data) clone 10 is not similar to the thiol protease cDNA derived from GA-treated aleurone cell mRNA (Roger et. al. 1985).

The carboxypeptidase activity of barley aleurone layers responds differently to GA. In the presence of GA carboxypeptidases activity is enhanced 2 to 3 fold and this activity is not decreased by ABA (Hammerton and Ho, 1986).

Mundy (1984) reported the α-amylase/subtillsin inhibitor as an ABA-induced, GA-repressible protein in barley aleurone layers and

Mundy and Rogers (1986) also reported that mRNA for a probable amylase/protease inhibitor (PAPI) is not affected by either GA or ABA in barley aleurone. From the data presented in this thesis, it can be concluded that the expression of the genes corresponding to clone 10, 85 and 8 differ from those amylase and other well characterized enzymes. Further, there are differences in the pattern of regulation of individual non-α-amylase genes. Thus a common pattern of regulation of all GA-inducible genes appears unlikely.

GA regulated non-α-amylase clones have also been studied in wheat aleurone (Baulcombe and Buffard, 1983). Six non-α-amylase cDNA clone were identified, five were GA-induced, one was GA-inhibited clone. They had concluded that multiple mRNA species are regulated in a manner similar to α-amylase mRNA by GA.

The precise mechanism by which GA regulates gene expression in aleurone layer cells is not clear. The presence of GA receptors has been reported by Carole et al(1977). A GA-inducible protein factor was required for GA-mediated induction of α-amylase (Muthukrishnan et al,1979). There may be different factors controlling expression of genes corresponding to clone E, clone 103 and all these non-α-amylase clones. Alternatively, the binding affinity of these factors for individual GA-inducible genes may vary.

Another possible reason for co-regulation by GA is the clustering of GA-inducible genes on one or two chromosomes. α-amylase genes coding for the low pI and high pI enzymes have been shown to be located on the long arms of barley chromosomes 1 and 6,

respectively (Brown and Jacobsen, 1982; Muthukrishnan et al, 1984; Kam-Morgan, unpublished data). I have attempted to map non-α-amylase genes using wheat-barley chromosome addition lines. Clone 132 could not be mapped because of its short insertion size. Several clone 10 detectable DNA bands were detected in parental Betzes barley DNA indication a large family of genes. However, barley-specific bands were not detected in any of the addition lines. Because only barley chromosome 5 is not represented in the six addition lines, it was concluded that clone 10 genes were probably located in barley chromosome 5. Non-α-amylase clone 8 gene were detected only in addition lines 7 carrying barley chromosome 7, and clone 8 represents a multigene family just as the α-amylase gene family.

Thus it appears that non-α-amylase genes represented by clone 10, 85, and 8 are regulated in different manner to that of α-amylase genes by GA. Their chromosomal locations are also different. Further studies, will be needed to understand the precise mechanism by which GA regulates expression of individual hydrolase genes.

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HORMONAL REGULATION OF NON-α-AMYLASE GENES IN BARLEY ALEURONE CELLS

by

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B.A., Tung-Hai University, 1982

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Genetics

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1987

ABSTRACT

Some effects of gibberellic acid (GA), abscisic acid (ABA), and cycloheximide on gene expression in barley aleurone cells have been studied. Three different non-α-amylase cDNA clones (10, 85, and 8) derived from aleurone cell mRNA were used as probes to study the effects of GA, ABA, and cycloheximide on transcription. The results were compared with the patterns of regulation of 2 known α-amylase clones (E and 103).

Clones 8, 10, and 85 hybridized to RNA's of 1500, 1450, and 1350 nucleotides respectively, showing that each represented a different RNA species.

Southern blot analysis has shown that clones 8 and 10 are members of multiple gene families present on barley chromosome 7 and 5 respectively.

In the presence of GA, clone 103, E, 10 and 85 mRNAs were induced 80, 20, 7, and 3-fold respectively, but clone 8 mRNA declined 4-fold. ABA inhibited GA-induced α-amylase mRNA synthesis but had little effect on non-α-amylase mRNA synthesis. Cycloheximide decreased α-amylase and clone 10 mRNA synthesis when added with GA, but clone 85 mRNA concentration did not decrease. Cycloheximide had an inhibitory effect on clone 8 mRNA synthesis only in incubations without GA.

The results show that in barley aleurone cells multiple genes are regulated in different manners by hormones.