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The effects of gibberellic acid and abscisic acid on α-amylase mRNA levels in barley aleurone layers studies using an α-amylase cDNA clone

Peter M. Chandler, John A. Zwar, John V. Jacobsen, Thomas J. V. Higgins & Adam S. Inglis[1]
CSIRO Division of Plant Industry, P.O. Box 1600, Canberra, 2601, Australia
[1] *CSIRO Division of Protein Chemistry, 343 Royal Parade, Parkville, 3052, Victoria, Australia*

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Summary

Two cDNA clones were characterized which correspond to different RNA species whose level is increased by gibberellic acid (GA_3) in barley (*Hordeum vulgare* L.) aleurone layers. On the criteria of amino terminal sequencing, amino acid composition and DNA sequencing it is likely that one of these clones (pHV19) corresponds to the mRNA for α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1), in particular for the B family of α-amylase isozymes (Jacobsen JV, Higgins TJV: Plant Physiol 70:1647–1653, 1982). Sequence analysis of PHV19 revealed a probable 23 amino acid signal peptide. Southern hybridization of this clone to barley DNA digested with restriction endonucleases indicated approximately eight gene-equivalents per haploid genome.

The identity of the other clone (pHV14) is unknown, but from hybridization studies and sequence analysis it is apparently unrelated to the α-amylase clone.

Both clones hybridize to RNAs that are similar in size (~1500b), but which accumulate to different extents following GA_3 treatment: α-amylase mRNA increases approximately 50-fold in abundance over control levels, whereas the RNA hybridizing to pHV14 increases approximately 10-fold. In the presence of abscisic acid (ABA) the response to GA_3 is largely, but not entirely, abolished. These results suggest that GA_3 and ABA regulate synthesis of α-amylase in barley aleurone layers primarily through the accumulation of α-amylase mRNA.

Introduction

A major event during post-germination development of many plant embryos is the mobilization of seed reserve materials. The reserve carbohydrate (starch) and protein are located principally in the non-living endosperm of the seeds of the Gramineae. In developing barley (*Hordeum vulgare* L.) seedlings the reserves are hydrolysed by enzymes secreted from the living cells of the aleurone layer in response to gibberellin produced by the developing embryo (for a recent review see 18). α-Amylase is involved in starch hydrolysis and is the most abundant of these enzymes.

Isolated aleurone layers respond in organ culture to applied gibberellic acid (GA_3) by synthesis and secretion of α-amylase: in the absence of GA_3 little α-amylase is produced (31). Associated with the effect of GA_3 on α-amylase synthesis is the proliferation of rough endoplasmic reticulum, an increase in polysomes (9, 31) and accumulation of poly(A) RNA presumably including α-amylase mRNA (13, 16). Cell-free translation studies of RNA extracted from GA_3-treated layers showed that enhanced α-

Abbreviations

ABA: abscisic acid
CHA: cyclohepta-amylose
CMC: carboxymethyl cellulose
GA_3: gibberellic acid

amylase synthesis resulted from increased levels of translatable mRNA for the enzyme (11, 12, 23, 22).

A second growth regulator, abscisic acid (ABA), antagonises the effect of GA_3 on the induction of α-amylase synthesis (7, 22, 12). The mechanism by which ABA reverses the GA_3-effect is not well understood. Cell-free translation studies have indicated that it prevents the GA_3-induced accumulation of α-amylase mRNA (12). This result is consistent with that obtained in hybridization studies where a radioactive probe derived from size-fractionated RNA which included α-amylase mRNA was used to assess α-amylase mRNA levels (3). However, a different study which was also based on cell-free translation (22), as well as inhibitor studies (14) indicated that there was little effect of ABA on the GA_3-induced accumulation of α-amylase mRNA, and this has been interpreted as evidence for ABA control at the level of translation of α-amylase mRNA.

In an attempt to clarify this situation recombinant DNA plasmids containing sequences complementary to α-amylase mRNA were constructed. These have been used to study the kinetics of α-amylase mRNA accumulation during GA_3 treatment of aleurone layers, and the effect of ABA on GA_3-enhanced α-amylase mRNA levels. Such plasmids have the advantage that they allow the direct measurement of sequences related to α-amylase mRNA whether these RNAs are translatable *in vitro* or not. While this manuscript was in preparation the results of two studies on the effect of GA_3 on barley α-amylase mRNA levels using cloned DNAs were published (24, 25). The results of these studies agree well with similar experiments reported here. In addition we have examined the effect of GA_3 on an unrelated RNA, the effects of ABA, and address the issue of whether total α-amylase mRNA levels can be assessed using a single clone as probe.

Materials and methods

Isolation, incubation and processing of aleurone layers. Aleurone layers from seeds of barley (*Hordeum vulgare* L.) cv Himalaya (1972 and 1979 harvests, Department of Agronomy, Washington State University, Pullman, Washington) were prepared as described (8) and incubated in 10 mM $CaCl_2$ solution with or without 1 μM GA_3 (Sigma Chemical Co.) (12). Abscisic acid (Sigma Chemical Co.) was used at a concentration of 25 μM.

Preparation of α-amylases A and B. 500 aleurone layers (1979 Himalaya) were prepared and incubated (20 layers/flask) in 1 μM GA_3 and 10 mM $CaCl_2$ for 30 h. The medium was pooled and centrifuged. α-Amylase was purified using the CHA* Sepharose affinity chromatography procedure of Silvanovich & Hill (27) as described previously (17) except that the buffer used was 0.02 M succinate pH 5.0 containing 10 mM $CaCl_2$. Eluted α-amylase was concentrated to 6 ml, using an Amicon UF 100 pressure cell, clarified by centrifugation and the CHA removed on a G-25 Sephadex column equilibrated with 0.02 M succinate buffer pH 4.3 containing 10 mM $CaCl_2$. The A and B α-amylase components were resolved by CMC* column chromatography at room temperature. The column was eluted with a linear NaCl gradient 0–1.2 M (150:150 mls) in pH 4.3 succinate buffer. The first (A) and second (B) peaks of α-amylase were each concentrated to 8 ml and the salt removed by dialysis against 6×1 l changes of distilled water. Protein was precipitated with 4 vol acetone, recovered by centrifugation and dried under N_2. Each lot of 500 aleurone layers yielded about 1.8 mg of α-amylase A protein and 3 mg of B.

Determination of amino terminal sequences of α-amylase. The NH_2-terminal sequence of purified α-amylase A was determined using a modified Edman-Begg-type sequenator incorporating a stainless steel spinning cup as previously described (30).

Amino acid composition of α-amylase. Samples of purified α-amylase A and B (0.25 mg each) were analysed on a modified Beckman analyzer after hydrolysis *in vacuo* in 4 M methane sulfonic acid (0.4 ml) containing tryptamine (0.8 mg) for periods of 39 and 72 h at 115 °C (28). The values obtained were corrected for destruction of the labile amino acids and for incomplete hydrolysis of resistant peptide bonds (15).

RNA isolations. Total RNA was isolated from aleurone layers as previously described (11). Poly(A) RNA was obtained by chromatography of total RNA on oligo dT cellulose columns (Type 3, Collaborative Research, Waltham, Massachusetts) as recommended by the manufacturer.

DNA isolations. DNA was isolated from barley seedlings by a modification of an earlier method (1). Sterilized seed was germinated, the radicles and plumules collected and homogenized in SDS/proteinase K, extracted with phenol/chloroform and precipitated with ethanol as previously described (1). The pellet was dissolved in 30 ml of TE (TE = 0.01 M Tris. HCl, 0.001 M EDTA pH 7.5) followed by addition of 28.5 g CsCl and 3.0 ml of ethidium bromide solution (10 mg/ml). When the CsCl had dissolved the solution was centrifuged at 44 000 rpm in a vertical Ti 50 rotor (Beckman Instruments, Palo Alto, California) for 16 h. The band of DNA was visualized under UV-light and collected by side-puncture of the tube. Ethidium bromide was removed by serial extractions with isoamyl alcohol and the solution dialysed to remove CsCl before precipitation with ethanol. DNA was dissolved in 0.1 × TE and concentrated by freeze-drying if necessary.

Construction of recombinant plasmids. Poly(A) RNA (25 μg) from aleurone layers treated with GA_3 for 24 h was copied into double-stranded DNA and cloned into the Pst I site of pBR 322 using conditions which have been described previously (6).

Screening of colonies. Transformants were screened by colony hybridization (10) for GA_3-responsive cDNA inserts using ^{32}P-labelled cDNA probes made from poly(A) RNA of untreated or GA_3-treated (24 h) aleurone layers.

DNA sequencing. DNA was sequenced in both strands using either the G, G + A, T + C, and C reactions of Maxam & Gilbert (20), or the dideoxy method of Sanger *et al.*, (26) following sub-cloning of Hpa II or Taq I fragments of insert DNA into the Acc I site of M13mp8 (21).

Hybridizations to filter-bound RNA. Total RNA was electrophoresed, transferred to diazotised aminothiophenol paper and hybridized as previously described (6).

Preparation of GA_3-specific cDNA probe. Poly(A) RNA (1 μg) isolated from aleurone layers incubated with GA_3 for 24 h was used as a template for cDNA synthesis in a 100 μl reaction under conditions previously described (6) except that the concentration of dATP was reduced to 50 μM and 40 μCi [α-^{32}P] dATP was added to the reaction. RNA template was hydrolysed at 60 °C for 1 h in 0.1 M NaOH, the solution neutralized with Tris-HCl and extracted with buffer-saturated phenol. One microgram of poly(A) RNA from control layers (incubated for 24 h without GA_3) was added to the aqueous phase and nucleic acids precipitated with the addition of two volumes of ethanol. The pellet was washed in cold 70% ethanol, dried briefly and dissolved in 10 μl H_2O. After addition of 100 μl of hybridization buffer the solution was heated to 80 °C for 5 min and incubated at 42 °C for 6 h before the addition of a further 5 ml hybridization buffer. During this hybridization any sequences in the cDNA population representing RNAs which are present in the poly(A) RNA of control layers as well as GA_3-treated layers will hybridize with their complementary RNA, and therefore be unavailable for subsequent hybridization. This solution was then used without further heating for hybridization to an RNA filter. Hybridization buffer was identical to that used previously for hybridization to RNA filters (6).

We estimate the efficiency of cDNA synthesis to be approximately 30% (relative to the poly(A) RNA population). Therefore the conditions of hybridization in the 100 μl solution are such that any sequences which increase in relative abundance more than about 4-fold following GA_3 treatment will not be saturated during hybridization to the control RNA and will subsequently be free to hybridize to complementary sequences on the RNA filter.

Southern hybridizations to barley DNA. Barley DNA (20 μg per sample) was digested with a 10-fold excess of three different restriction enzymes and electrophoresed in 0.8% (w/v) agarose. Gels were processed and DNA transferred to nitrocellulose filters as described (29). Filters were prehybridized and hybridized as for RNA filters (see above) presence except for the presence of 10% dextran sulfate (w/v) in both prehybridization and hybridization solutions, and the use of plasmid inserts labelled to high specific activity (>10^8 cpm/μg DNA) using a nicktranslation system (New England Nuclear, Boston, Massachusetts).

Results

Isolation and characterization of cDNA plasmids

Poly(A) RNA extracted from aleurone layers treated with 1 μM GA_3 for 24 h was cloned into the Pst I site of pBR322 (see Methods). Five hundred colonies containing inserts (on the basis of sensitivity to ampicillin) were screened with two cDNA probes – one was derived from control layers (i.e. no GA_3 during the 24-h treatment), the other from layers which had been treated with 1 μM GA_3 for 24 h.

Plasmids were prepared from 36 clones which hybridized with sequences of greater relative abundance in the cDNA from GA_3-treated layers than from control layers. Two distinct hybridization families were identified among these plasmids, and one representative of each was chosen for detailed study. The two representative clones, designated pHV14 and pHV19, contain inserts of approximately 1 000 and 1 100 nucleotide pairs, respectively. Hybridization studies and DNA sequencing indicate that the inserts are unrelated.

To determine if either of these clones contained sequences derived from α-amylase mRNA, hybrid release translation experiments were undertaken. The insert in pHV19 selected a mRNA which in cell-free translation directed synthesis of a polypeptide that co-migrated with *in vitro* synthesized α-amylase (5). pHV14 hybridized to a 1 500 nucleotide RNA species in Northern hybridizations, and was used to purify such an RNA, but attempts to translate this in the wheat germ system were unsuccessful.

DNA sequencing studies

To confirm the identify of pHV19 as an α-amylase clone DNA sequencing studies were undertaken. In Fig. 1 the sequence of the insert in pHV19 is shown, together with the deduced amino acid sequence of the only open reading frame. It codes for a polypeptide of 368 amino acids and included a stretch of amino acids near the amino terminus (residues 24–35) of which ten are identical to an NH_2-terminal amino acid sequence determined for α-amylase A (Fig. 2), one of the two major families of α-amylase (7). Therefore on the basis of sequencing and hybrid release translation we conclude that pHV19 represents an α-amylase plasmid, although for reasons detailed later it is likely to represent mRNA for the B family of α-amylase.

Twenty-three amino acids before the NH_2-terminal amino acid sequence of pHV19 is a Met residue which is followed by a stretch of predominantly hydrophobic amino acids. This sequence is likely to represent the signal peptide of barley α-amylase suggested in earlier studies (12). The nucleotide sequence surrounding this Met residue is consistent with it being the site of translation initiation as it includes purines at –3 and +4 (19). However, the possibility cannot be excluded that it represents an internal Met residue in an unusually long signal peptide (>28 amino acids).

An unusual featuare of the pHV19 sequence is its relatively high G + C content (65%). Less extensive sequencing has been done on the insert in pHV14, but for 700 nucleotides which have been sequenced it has an even higher G + C content (68%, data not shown).

From the molecular weight of α-amylase ($Mr \cong 44\,000$) the mature enzyme is estimated to contain approximately 400 amino acids. The sequence of pHV19 includes 345 amino acids of the mature protein, and therefore represents about 85% of the sequence. The deduced amino acid composition of this sequence agrees well with that determined for purified α-amylase B (Table 1) and is discussed in detail at a later stage.

α-amylase mRNA studies

Effect of GA_3. pHV14 and pHV19 were used as probes in RNA blot hybridizations to examine the kinetics of accumulation of their respective mRNAs in aleurone layers which had been treated with GA_3 (Fig. 3A). Both plasmids hybridized to RNAs of approximately 1 500 nucleotides which increased in abundance relative to the 25s rRNA during the course of GA_3 treatment – the pHV14 mRNA increased 10-fold over control levels, whereas that for pHV19 increased 50-fold (Fig. 3B). Maximal levels of both RNAs were attained 18–24 h after the addition of GA_3. At later stages (36–48 h after GA_3 addition) a decline in the level of the pHV19 mRNA was observed. From this result it is concluded that GA_3 stimulates accumulation of α-amylase mRNA, confirming earlier observations based on *in vitro* translation studies (11, 12, 23, 22).

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                    Gly Lys Asn Gly Ser Leu Cys Cys Phe Ser     Leu
 C  TAGC CGCGC       G    G   C GG  AGC CTG TG   G  T    CT   G C G
AGAGAGCTGAAGAAC ATG GCG AAC AAA CAC TTG TCC CTC TCC CTC TTC CTC GTC
                Met Ala Asn Lys His Leu Ser Leu Ser Leu Phe Leu Val
                 1

        Leu     Leu     Gly               His
  G   G CTG   T CTC     G G       G       C CAC                       G
CTC CTT GGC CTG TCG GCC AGC TTG GCC TCC GGG     CAA GTC CTC TTT CAG GGC
Leu Leu Gly Leu Ser Ala Ser Leu Ala Ser Gly     Gln Val Leu Phe Gln Gly
                       20

                           Gln Ser                     Met Met
                             G  GC                     A G A
TTC AAC TGG GAG TCG TGG AAG CAC AAT GGC GGG TGG TAC AAC TTC CTG ATG
Phe Asn Trp Glu Ser Trp Lys His Asn Gly Gly Trp Tyr Asn Phe Leu Met
                               40

                                     Val
          C                   T       A G    C               G   A
GGC AAG GTG GAC GAC ATC GCC GCC GCC GGC ATC ACG CAC GTC TGG CTC CCT
Gly Lys Val Asp Asp Ile Ala Ala Ala Gly Ile Thr His Val Trp Leu Pro
                                           60

    Pro     His         Ser Asn Glu
    C         C         T   A C G     T           T   T
CCG GCG TCG CAG TCC GTC GCC GAG CAA GGG TAC ATG CCG GGC CGG CTG TAC
Pro Ala Ser Gln Ser Val Ala Glu Gln Gly Tyr Met Pro Gly Arg Leu Tyr
                                                    80

    Ile                           Ala     Glu
    A C       G                   GC      G
GAC TTG GAC GCC TCC AAG TAC GGC AAC AAG GCG CAG CTC AAG TCC CTC ATC
Asp Leu Asp Ala Ser Lys Tyr Gly Asn Lys Ala Gln Leu Lys Ser Leu Ile

                              Gln
  C                         G C
GGG GCG CTC CAC GGC AAG GGC GTC AAG GCC ATC GCC GAC ATC GTC ATC AAC
Gly Ala Leu His Gly Lys Gly Val Lys Ala Ile Ala Asp Ile Val Ile Asn
100

        Cys     Asp Tyr        Ser
        TGC   C   C T         T A     C
CAC CGC ACG GCG GAG CAC AAG GAC GGC CGG GGC ATC TAC TGC ATC TTC GAG
His Arg Thr Ala Glu His Lys Asp Gly Arg Gly Ile Tyr Cys Ile Phe Glu
            120
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Fig. 1. For caption see page 413.

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            Ser      Gly
            T        G                       C                    T
GGC GTC ACC CCC GAC GCC CGC CTC GAC TGG GGG CCC CAC ATG ATC TGC CGC
Gly Gly Thr Pro Asp Ala Arg Leu Asp Trp Gly Pro His Met Ile Cys Arg
                        140


        Thr Lys     Ser             Ala     Leu
        ACC AAA     T C   T          CA      TC           A
GAC GAC CGG CCC TAC GCT GAC GGC ACC GGC AAC CCG GAC ACC GGC GCC GAC
Asp Asp Arg Pro Tyr Ala Asp Gly Thr Gly Asn Pro Asp Thr Gly Ala Asp
                                    160


    Ala                                     Asp             Arg
    CC       G                              GAC   G         CGC
TTC GGG GCC GCC CCC GAC ATC GAC CAC CTC AAC CTG CGC GTC CAG AAG GAG
Phe Gly Ala Ala Pro Asp Ile Asp His Leu Asn Leu Arg Val Gln Lys Glu
                                            180


    Lys             Leu             Ser     Leu             Ala
    AAG             CT              AG      C                CG
CTC GCC GAG TGG CTC AAC TGG CTC AAG GCC GAC ATC GGC TTC GAC GGC TGG
Leu Ala Glu Trp Leu Asn Trp Leu Lys Ala Asp Ile Gly Phe Asp Gly Trp
                                                    200


    Leu             Arg             Pro Glu Met         Val
    C T           T  G           G C     G A G         G G       C
CGC TTC GAC TTC GCC AAG GGC TAC TCC GCG GAC GTC GCC AAG ATT TAC ATT
Arg Phe Asp Phe Ala Lys Gly Tyr Ser Ala Asp Val Ala Lys Ile Tyr Ile


    Gly Thr Ser         Leu                 Val     Asp Asn Met
    G   A A TCC   G     C                   G G     GAC AAT A G   C
GAC CGC TCG GAG CCC AGC TTC GCC GTG GCC GAG ATA TGG ACG TCG CTC GCG
Asp Arg Ser Glu Pro Ser Phe Ala Val Ala Glu Ile Trp Thr Ser Leu Ala
    220


Thr                             Tyr Asp         Ala             Asn
AC        C                     TA  G           GC              A T
TAC GGC GGG GAC GGC AAG CCC AAC CTC AAC CAG GAC CAG CAC CGG CAG GAG
Tyr Gly Gly Asp Gly Lys Pro Asn Leu Asn Gln Asp Gln His Arg Gln Glu
                240


                                        Ala Ala Ser Ala Gly Met Val
                                G       GCG GCC TC  G A  GC  TG GT
CTG GTG AAC TGG GTG GAC AAG GTT GGC GGC AAA     GGG CCC GCT ACC ACG TTC
Leu Val Asn Trp Val Asp Lys Val Gly Gly Lys     Gly Pro Ala Thr Thr Phe
                            260
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                                        Ala
          G       A   G   A   G       CT
GAC TTC ACC ACC AAG GGC ATC CTC AAC GTG GCC GTG GAG GGC GAG CTG TGG
Asp Phe Thr Thr Lys Gly Ile Leu Asn Val Ala Val Glu Gly Glu Leu Trp
                                    280

        Ile Asp Pro Gln                     Val Met
A       AT   A  C G C G   G       C   C     G    G   A           G
CGG CTG CGC GGC ACA GAC GGT AAG GCG CCA GGC ATG ATC GGG TGG TGG CCA
Arg Leu Arg Gly Thr Asp Gly Lys Ala Pro Gly Met Ile Gly Trp Trp Pro
                                        300

           Ala                                                  Ala
         C  CC       C   C               T   A                 GC
GCC AAG GCG GTG ACC TTT GTG GAC AAC CAC GAC ACC GGC TCC ACG CAG CAC Ala
Lys Ala Val Thr Phe Val Asp Asn His Asp Thr Gly Ser Thr Gln His
                                                        320

                        Lys
          A       C   C     A                 C   C   G
ATG TGG CCC TTC CCT TCT GAC AGG GTC ATG CAG GGA TAT GCC TAC ATC CTC
Met Trp Pro Phe Pro Ser Asp Arg Val Met Gln Gly Tyr Ala Tyr Ile Leu

               Ile                                      Asn
  C       C   C  TC                          C         A         G
ACG CAC CCA GGG ACG CCA TGC ATC TTC TAC GAT CAT TTC TTC GAC TGG GGC
Thr His Pro Gly Thr Pro Cys Ile Phe Tyr Asp His Phe Phe Asp Trp Gly
    340

                    Ala Ala
 Phe      Asp Gln                       Ala Ile      Lys Arg Asn
 T T        C C         CG GCG C        G G A         AG C   AA
 CTG AAG GAG GAG ATC GAT CGC TTG GTG TCA GTC AGG ACC GGC GCC
 Leu Lys Glu Glu Ile Asp Arg Leu Val Ser Val Arg Thr Gly Ala
                 360
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of pHV19. The lower two lines of each row of four indicate the pHV19 sequence. The upper two lines of each row show the sequence of clone E determined by Rogers & Milliman (25), where it differs from that of pHV19. To maximize homology between the two sequences two gaps have been introduced into the pHV19 sequence (residues 25 and 264). For details of the comparison between the clone E and pHV19 sequences see Discussion. Residue 1 is the assumed start of translation. The NH_2 terminus of mature α-amylase A is the His residue at position 25 (see text for details).

(a) His gln val leu phe gln gly phe asn trp glu

(b) Gly gln val leu phe gln gly phe asn trp glu

Fig. 2. Comparison of NH_2-terminal amino acid sequences (a) determined for purified α-amylase A, and (b) a portion of the deduced amino acid sequence of pHV19.

GA_3 also stimulated accumulation of the RNA detected by pHV14, but since the identity of this RNA is unknown the physiological significance of this observation is difficult to assess.

Effect of ABA. The influence of ABA on the GA_3-stimulation of α-amylase mRNA levels was studied

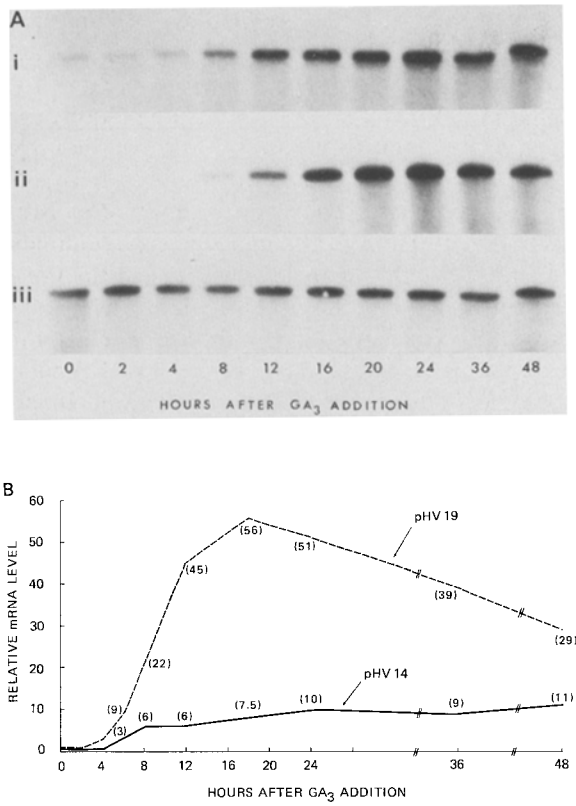

Fig. 3. A: Hybridization of pHV14 (i), pHV19 (ii) and a 25s rRNA probe (iii) to RNA extracted from aleurone layers treated with GA_3 for various times. Total RNA (20 mg) from each stage of treatment was electrophoresed and transferred to filters as described in Methods. Filters were hybridized with ^{32}P-labelled pHV14 or pHV19, washed and exposed to X-ray film. After suitable exposures were obtained, filters were rehybridized with a 25s rRNA probe and processed similarly.

B: Quantitation of densitometric tracings of the fluorographs in A. Areas of each mRNA peak were normalized for the area of the 25s rRNA peak and expressed relative to the zero time level which was taken as 1 unit.

by hybridization using pHV19 against RNA isolated from aleurone layers which had received either no growth regulator, GA_3, ABA or ABA + GA_3 (Fig. 4A). The marked stimulation in α-amylase mRNA levels seen in the GA_3 samples (48-fold) is largely abolished in the sample which received ABA + GA_3 (3-fold). ABA by itself had little effect on α-amylase mRNA levels, reducing it to slightly below the control level. It is therefore likely that the failure of aleurone cells to synthesize α-amylase in the presence of GA_3 + ABA is a consequence of low levels of mRNA.

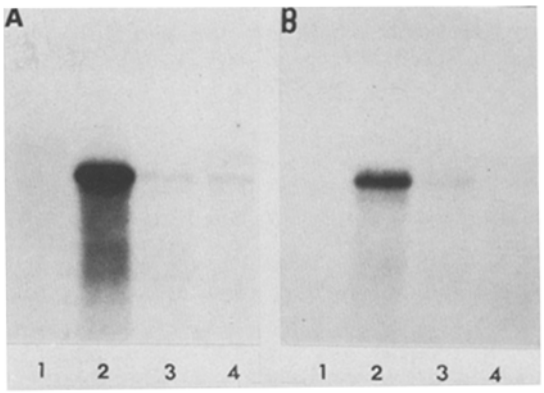

Fig. 4. Hybridization of pHV19 (A) and GA_3-specific cDNA (B) to fractionated RNA from aleurone layers which had received no growth regulator (1), GA_3, (2), GA_3 + ABA (3) or ABA (4) for 24 h. Quantitation of relative mRNA levels was made as in the legend to Fig. 3.

To investigate whether this observed effect of ABA on mRNAs detected by pHV19 was general for GA_3-induced mRNAs or specific for that detected by pHV19, the same four RNA preparations were hybridized with a cDNA probe which should represent all major GA_3-induced RNAs (see Methods for preparation of the probe). A result very similar to that using the cloned sequence was obtained (Fig. 4B). An analogous result was also obtained with pHV14 as probe (P.M.C. unpublished data). It is concluded that GA_3 induces the appearance a major class of mRNAs (including α-amylase) ~1500 nucleotides in length, and that this effect is largely prevented by ABA.

α-amylase genes

Genetic studies have indicated that there are at least two unlinked loci for α-amylase in barley (4). Four distinct isozymes of α-amylase appear following GA_3-treatment of aleurone from Himalaya barley (17). To obtain an estimate of α-amylase gene copy number in Himalaya barley Southern hybridizations were performed on restricted barley DNA using pHV19 as prove. In addition, a reconstruction experiment was performed in which Pst I-digested pHV19 was mixed with Eco R1-digested salmon sperm DNA in amounts corresponding to 1, 3 or 5 copies of the gene for α-amylase per haploid barley genome (Fig. 5). Densitometry of hybridization to the digested samples indicated that

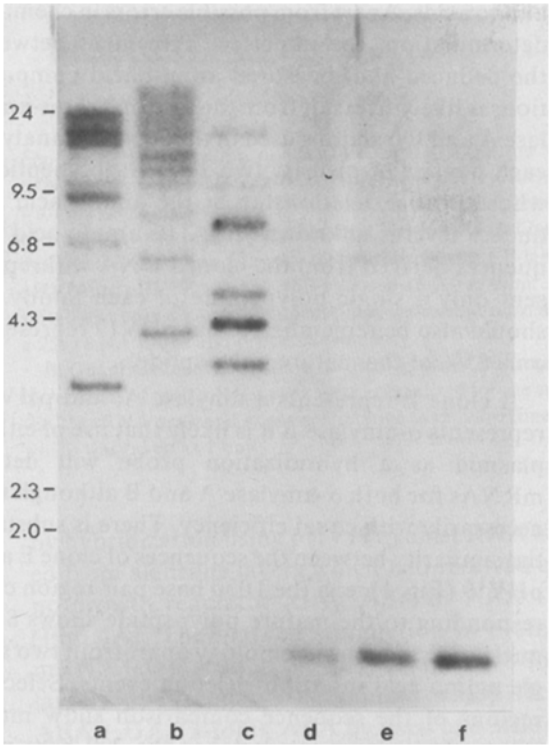

Fig. 5. Southern hybridization of pHV19 to Himalaya barley DNA digested with various restriction enzymes. DNA was digested with Eco R1 (a), Bam H1 (b) or Bgl II (c). The hybridization in lanes (d)-(f) corresponds to 1, 3 and 5 copies, respectively, of pHV19 insert added per haploid equivalent of DNA (assumed to be 5.5 pg, ref. 2). The numbers refer to sizes in kilobases of marker DNAs.

relative to the reconstruction experiment there are approximately eight copies of an α-amylase 'gene' per haploid genome. Since none of these enzymes cuts the insert in pHV19 it is likely that most of the bands in Fig. 5 represent a complete gene, although the possibility that cuts may occur in regions of the gene which are not represented in the cDNA clone cannot be excluded.

Discussion

This study reports the isolation of cDNA clones for two major GA_3-inducible RNAs in barley aleurone layers. One of these corresponds to α-amylase mRNA; the identify of the other is unknown, although if the RNA it detects functions as a mRNA *in vivo* among the possible candidates are mRNAs for the other hydrolases secreted by aleurone cells in response to GA_3 (see 18). Using the α-amylase clone as a probe to measure α-amylase mRNA levels, we found that GA_3 stimulated α-amylase mRNA levels approximately 50-fold over control levels. Similar results using other cDNA plasmids for α-amylase have recently been reported by Muthukrishnan *et al.*, (24) and Rogers & Milliman (25). Furthermore, it was shown that in the presence of excess ABA, the GA_3 stimulation of α-amylase mRNA levels was largely abolished. Thus, the available evidence suggests that the growth regulators gibberellin and ABA exert their well documented effects on α-amylase synthesis primarily by influencing the level of α-amylase mRNA. This conclusion supports previous ones which were based on *in vitro* translation and hybridization studies (11, 12, 23, 22, 3). The possibility of translational control, particularly in the case of ABA (14, 22) is not entirely eliminated in the present study, but on the basis of the results in Fig. 4 and Bernal-Lugo *et al.*, (3) it is unlikely to be the major level of ABA action, at least in terms of α-amylase synthesis. Whether the effects of GA_3 and ABA on α-amylase mRNA levels results from changes in transcription, processing of transcripts, or degradation of the mRNA is the subject of current investigation.

An earlier study defined two major families of α-amylase isozymes in Himalaya barley (17). Each family (A and B) consisted of two polypeptides which were closely related to each other on the basis of partial proteolytic cleavage patterns. Further evidence for heterogeneity in α-amylase isozymes is provided in Fig. 1 which shows a comparison between the nucleotide sequences and deduced amino acid sequences of clone E (25) and pHV19. Apart from two single amino acid insertion/deletions (at positions 25 and 264) there is 23% divergence between the two amino acid sequences in the region corresponding to the mature polypeptide. The amino acid sequence divergence in the putative signal peptide is much higher (58%) although most of this occurs at the proximal rather than distal end. The 201 base substitutions in the region corresponding to the mature polypeptide have resulted in 40 amino acid substitutions.

Given this heterogeneity in isozymes is it possible to relate the major α-amylase families to the individual clones? Two lines of evidence suggest that clone E (25) corresponds to a clone for α-amylase

A, whereas pHV19 is a B-type clone. The NH_2-terminal amino acid sequence determined for purified α-amylase A agrees exactly with that sequence predicted from the DNA sequence of clone E, but differs by a single amino acid from the corresponding sequence predicted by pHV19. We would predict either glycine or glutamine as the NH_2-terminus of α-amylase B, but attempts to determine this experimentally have been unsuccessful, perhaps because it is blocked.

A second line of evidence suggests that pHV19 is an α-amylase B clone, and not simply a variant of the A family. The amino acid compositions of purified A and B α-amylase do not differ markedly (Table 1) except in their contents of alanine, methionine and serine. When the deduced amino acid compositions of the polypeptides encoded by clone E and pHV19 are compared with those of α-amylase A and B, the best matches occur with the combinations α-amylase A/clone E and α-amylase B/pHV19, particularly for the three diagnostic

Table 1. Comparison of amino acid compositions of α-amylases A and B with pHV19 and clone E[a].

	α-amylase		cDNA clones	
	A	B	E	pHV19
Ala	12.0	9.4	11.3	9.0
Asp + Asn	14.4	14.1	13.0	12.8
Glu + Gln	6.6	8.1	6.0	7.0
Phe	3.8	4.5	3.6	4.6
Gly	12.2	12.8	10.8	11.9
His	1.9	2.2	3.1	3.5
Ile[b]	5.7	6.5	5.5	5.5
Lys	3.4	3.7	5.3	5.5
Leu	6.5	7.6	6.0	6.7
Met	2.7	1.5	2.9	1.7
Pro + Cys[c]	4.4	6.0	5.1	5.8
Arg	2.2	2.8	3.6	4.4
Ser[d]	5.3	3.6	5.1	3.5
Thr[d]	4.5	4.6	4.1	4.9
Val[b]	7.5	6.6	6.8	5.5
Trp[e]	2.9	2.6	3.9	4.4
Tyr	4.2	4.0	3.9	3.5

[a] Values are expressed as residues per 100 total residues. Clone E is an α-amylase cDNA plasmid described by Rogers & Milliman (25).

[b] Values from 72 h hydrolysis.

[c] Comparison of A440 nm/A570 nm to proline and cysteine reference standards suggests that cysteine is present in the proline peak.

[d] Corrected by extrapolation to zero time.

[e] Allowing for 10% loss during hydrolysis.

amino acids. Apart from possible errors in chemical determination, the imperfect agreement between the deduced and measured amino acid compositions is likely to result from the fact that the α-amylase A and B fractions used in the chemical analysis each consist of at least two related polypeptides whose precise relationship at the amino acid sequence level is unknown (17). The amino acid sequences derived from the cloned DNA will represent only a single polypeptide of each family. It should also be remembered that pHV19 represents only 85% of the mature polypeptide.

If clone E represents α-amylase A, and pHV19 represents α-amylase B it is likely that use of either plasmid as a hybridization probe will detect mRNAs for both α-amylase A and B although not necessarily with equal efficiency. There is substantial similarity between the sequences of clone E and pHV19 (Fig. 1), e.g. the 1030 base pair region corresponding to the mature polypeptide shows 81% nucleotide sequence homology apart from two single amino acid insertion/deletion events. Selected regions of the sequence comparison show much greater homology, for instance the 200 base pair region between amino acid positions 85 and 152 shows 90% homology. As a consequence cross-hybridization between inserts of clone E and pHV19 is expected, and is in fact observed, under the usual conditions of stringency. The high G + C content of these sequences enhances the stability of the mismatched hybrids formed between the inserts such that in 0.1 × SSC, 0.1% SDS they are not denatured until a temperature of between 60 and 65 °C is reached (P.M.C. unpublished data). On this basis therefore it is likely that use of either plasmid as a probe in Northern hybridizations will measure hybridization to mRNAs for both A and B α-amylases, however it is expected that regions in a mRNA which differ from corresponding regions in the probe by 15% or greater are likely to hybridize with significantly reduced efficiency. It may be necessary to use sub-cloned fragments of 3′ and 5′ untranslated regions before mRNA levels for the individual α-amylase families (A or B) can be assessed, as these regions are frequently less conserved than the translated region.

Several different estimates have been made of the degree of GA_3-induced stimulation in α-amylase mRNA levels. Based upon *in vitro* translation studies Higgins *et al.*, (12) estimated a 10–15-fold in-

crease in α-amylase synthesis (relative to total protein synthesis) following GA_3 treatment. A much smaller increase (2-fold) was observed following GA_3 + ABA treatment. Bernal-Lugo *et al.* (3) used a size-fractionated poly(A) RNA preparation to synthesize radioactive cDNA enriched in α-amylase sequences. With this probe they estimated a 40-fold increase in α-amylase mRNA levels (in the poly(A) RNA population) following GA_3 treatment, and a 3-fold increase following GA_3 + ABA treatment. They pointed out however that the probe contained sequences other than α-amylase mRNA since many RNA species are of similar length to this RNA (e.g. the GA_3-inducible pHV14 RNA). Muthukrishnan *et al.* (24) used an α-amylase clone 103 as a hybridization probe and reported a 'large increase' in amounts of α-amylase mRNA (relative to total RNA) following GA_3 treatment. Rogers & Milliman (25) used clone E as a hybridization probe to estimate a 5-fold increase after GA_3 treatment in levels of α-amylase mRNA in the poly(A) RNA population, but found a 17-fold increase using a cDNA probe made from total GA_3-stimulated poly(A) RNA. This difference was interpreted as evidence for at least two populations of α-amylase mRNA molecules. If, as suggested above, there is a cross-hybridizing population of α-amylase mRNAs, an alternative explanation for their observation may be the contribution of GA_3-induced sequences unrelated to α-amylase mRNA (e.g. pHV14).

We have measured changes in α-amylase mRNA levels relative to the amount of 25s rRNA to minimize variation due to growth regulator effects on the size of the poly(A) RNA population as well as variation in recovery and purity between different preparations of poly(A) RNA. Our estimates of a 50-fold increase following GA_3 treatment and a 3-fold increase following GA_3 + ABA treatment are in general agreement with earlier studies. Differences between individual measurements may result from the different methods employed. In addition we have observed differences in the levels of α-amylase mRNA in untreated (control) RNA preparations from different harvest of grain. This can lead to large numerical differences when increases are expressed relative to a low initial value.

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