Journal of Experimental Botany, Vol. 50, No. 332, pp. 311–317, March 1999

Journal of Experimental Botany

Reduced aleurone α-amylase production in aged wheat seeds is accompanied by lower levels of high-pI α-amylase transcripts and reduced response to gibberellic acid

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Received 19 July 1998; Accepted 19 October 1998

Abstract

With the aim of determining the level at which ageing exerts its effect on the expression of α-amylase, GA_3 regulation of α-amylase production was studied in isolated aleurone layers from aged wheat seeds. GA_3-induced α-amylase activity was lower in the tissue from aged seeds than in controls. However, the proportion of ^{35}S-methionine incorporated into α-amylase was higher in the aged than in control tissue. The pattern of α-amylase isoforms was resolved by isoelectric focusing and showed that two isogroups were present with the activity of the high-pI isogroup being higher in the control than in the aged lot. These apparently contradictory results may be explained in terms of differences in isozyme expression. Studies on the expression of α-amylase genes indicated a reduction in the level of high-pI mRNA in aged tissue. Dose–response curves showed lower GA_3-responsiveness of aleurone layers from aged seeds as compared to the controls. From these results, it is proposed that the diminished capacity of α-amylase production in aleurone from aged seeds is apparently due to a decrease in the expression of the high-pI α-amylase genes, and this reduction is associated with a decrease in the response to GA_3.

Key words: Seed ageing, wheat aleurone, gibberellic acid, α-amylase isozymes, gene expression.

Introduction

The aleurone is a peripheral layer of cells surrounding the endosperm of cereal seeds. This tissue synthesizes and secretes several hydrolytic enzymes in response to gibberellic acid (GA_3) of which α-amylase is the most abundant and the best characterized (for review, see Fincher, 1989; Jones and Jacobsen, 1990). In wheat, there are two families of α-amylase isoforms (Macgregor and Macgregor, 1987), encoded by two sets of structural genes (Lazarus *et al.*, 1985). These isoenzymes are secreted into the starchy endosperm of the germinating cereal seed where breakdown of starch occurs, the main reserve in this type of seeds, for use of the growing seedling.

Cereal seed lots of equal high percentage germination may differ in germination rate and in capacity of seedling establishment, which can ultimately influence crop yield at harvest. This behaviour has been attributed to variation in seed vigour (Ellis, 1992). Such symptoms are also characteristic of the effects of storage upon seed ageing (Bernal-Lugo *et al.*, 1994).

During prolonged storage, there is a progressive reduction in the rate of germination and seedling growth, before seed death takes place. It is also known that the embryonic and non-embryonic parts of the seed age at different rates (Aspinall and Paleg, 1971). Previous work performed in this laboratory, using seed lots in which only the aleurone layer had senesced, showed that endosperm of germinating aged seeds produced less α-amylase than the corresponding controls (Bernal-Lugo *et al.*, 1994). This suggests that the decrease in germination efficacy and a delay in seedling emergence as a result of seed ageing may be an expression of the ageing process concerned with the mobilization of reserves in the endosperm. In the present work, earlier studies have been

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extended by investigating the effect of ageing on GA_3-induced α-amylase gene expression in the aleurone layer of aged wheat seeds with the aim of determining at what level ageing exerts its effect on the production of α-amylase.

Materials and methods

Plant material

Seeds of wheat (*Triticum aestivum* L.) cv. Tonichi harvested in 1993 (8% water content dry weight basis) were stored at 4 °C in closed glass jars. Some seeds were 'aged' by storage at 25 ± 2 °C and 53% relative humidity, using a saturated $Mg(NO_3)_2.6H_2O$ solution (Winston and Bates, 1960), for 6 months. After the ageing period, the seeds were dried to 8% water content (dry weight basis) by placing them at 25 °C in a desiccator over saturated potassium acetate solution (23% RH) for 5 d. After this treatment the seeds were stored at 4 °C in closed glass containers.

Germination tests and seedling growth

Seeds were placed in wet paper rolls in the dark at 22 °C. Seeds were scored as having germinated if the axes had broken through the seed coat. Each replicate germination test comprised 50 seeds. Each germination test was done three times.

Batches of 25 seeds were planted in plastic trays of vermiculite in growth rooms with a light intensity of 300 mmol m^{-2} s^{-1} and a photoperiod of 16 h. Temperature was maintained at 22 °C. Coleoptile protrusion of 3 mm at the soil surface was scored as seedling emergence.

Preparation of aleurone layers

Control and aged wheat seeds were cut in half and the ends containing the embryos were discarded. Embryo-less seeds were sterilized for 15 min under vacuum in 1% sodium hypochlorite, washed extensively with sterile distilled water, and thereafter allowed to swell on sterile, moist filter paper for 24 h at 22 ± 2 °C. The aleurone layers were then stripped from the endosperm by squeezing with a sterile spatula, and washed again with sterile distilled water.

Incubation of aleurone layers and α-amylase assay

Twenty isolated aleurones were incubated for 24, 36 or 48 h at 22 ± 2 °C in 2.0 ml of incubation medium (20 mM succinic acid, pH 4.0, 20 mM $CaCl_2$, 5 μg ml^{-1} chloramphenicol, 5 μg ml^{-1} streptomycin) with or without 1 μM GA_3. After incubation, the incubation media were decanted in a Corex tube. Aleurone layers were homogenized with 2 ml of cold 5 mM calcium acetate at pH 6.0. The slurry was transferred to the Corex tube containing the incubation media and heated for 20 min at 70 °C, cooled to 4 °C and centrifuged to 12 000 *g* for 10 min. The recovered supernatant was assayed for a-amylase (EC 3.2.1.1) activity as described by Chrispeels and Varner (1967).

In some experiments (48 h incubation), the incubation media were decanted in a Corex tube and the aleurone layers homogenized as above, but the slurry was transferred to a Corex tube. Both incubation media and aleurone extract were heated and centrifuged as described. Tissue homogenate and incubation media were assayed for α-amylase activity. The α-amylase activity released into the incubation medium in response to added GA_3 was taken as the secreted α-amylase activity and expressed as percentage of the total activity (activity extracted from aleurones and activity in the medium).

Protein synthesis and extraction of labelled proteins

For *in vivo* protein synthesis analyses, 10 incubated aleurone layers were rinsed twice with fresh sterile incubation medium, then pulse-labelled in 1 ml of fresh incubation medium with 20 μCi of ^{35}S-methionine (1000 μCi $mmol^{-1}$) during the last hour of the incubation period. After labelling, the layers were washed twice with fresh incubation medium containing 2 mM non-radioactive methionine. The labelled tissue was homogenized in sample buffer (0.0625 M TRIS, pH 6.8, 10% (v/v) glycerol, 0.125% SDS, and 2% (v/v) β-mercaptoethanol), centrifuged at 10 000 *g* for 3 min and the supernatant was used to determine uptake and incorporation of radioactivity into proteins. The uptake was determined counting 3 μl of the above supernatant in 10 ml of scintillation cocktail in a scintillation counter. Incorporation of ^{35}S-methionine into protein was analysed by hot trichloroacetic acid-insoluble radioactivity of the above supernatant as described by Mans and Novelli (1961). Briefly, 3 μl of the supernatant were spotted on a filter paper disc, air-dried and washed successively in 10% trichloroacetic acid, 5% trichloroacetic acid and ethanol. The filters were air-dried and counted with 10 ml of scintillation cocktail in a scintillation counter.

Protein analysis

Polypeptides from the radiolabelled aleurone layers were analysed by electrophoresis on SDS-polyacrylamide slab gels (Laemmli, 1970). Samples containing equal amounts of incorporated radioactivity (50 000 cpm) were brought to a volume of 75 μl with sample buffer and loaded on to each lane of a gel. The radiolabelled polypeptides were visualized by fluorography at −70 °C according to the procedure of Lasky and Mills (1975). Immunoprecipitation of labelled α-amylase was performed as described by Deikman and Jones (1985).

α-Amylase isoforms activity

The isoforms of α-amylase isolated as described above, were separated on non-denaturing isoelectric slab gels, using a pH gradient from 3 to 10 as described by O'Farrel (1975) with slightly modifications. Isoelectric focusing gels (17 × 18 × 0.5 cm) contained 5% (w/v) acrylamide, 17% (v/v) glycerol, 17% Pharmalyte (pH range 3–10) and Pharmalyte 1.35% (pH range 4–6). Gels were pre-run at 250 V for 1.25 h with 0.5 M phosphoric acid, pH 3.0, as anode buffer and 0.01 M ethylenediamine, pH 10, as cathode buffer. The samples, containing an aliquot of total α-amylase, 10% glycerol, 1% (v/v) Pharmalyte (pH range 3–10), and 2% β-mercaptoethanol, were applied to the gels at the basic end and overlaid with a solution containing 5% glycerol and 1% Pharmalyte (pH range 3–10). Electrofocusing was carried out at 300 V for 30 min, 360 V for 30 min, 420 V for 30 min, 480 V for 30 min, and 540 V for 60 min.

For visualization of the α-amylase bands, the non-denaturing gel was immersed in 0.5% soluble potato starch and incubated at 37 °C for 30 min. The excess starch solution was then washed off, and the gel was flooded with I_2-IK solution for about 3 min. The clear bands in the zymogram represent the α-amylase isoenzymes components (Jacobsen *et al.*, 1970).

Preparation of α-amylase antibodies

Purified α-amylase (Jacobsen and Higgins, 1982), 1 mg in 1.0 ml of phosphate-buffered saline (PBS) was emulsified with an equal volume of complete Freund's adjuvant (Difco) and injected subcutaneously into rabbits. The rabbits were boosted on days 22 and 45 with 0.50 mg of antigen emulsified with

incomplete Freund's adjuvant. Sera collected between day 60 and 70 d were used in this study.

Isolation of RNA and Northern blot analysis

RNA was extracted from aleurone tissue as described by Belanger *et al.* (1986). In Northern blot analysis, 40 μg of total RNA were used in formaldehyde agarose gel electrophoresis as described in Maniatis *et al.* (1982). After electrophoresis, the RNA was blotted overnight on to Hybond-N nylon membrane (Amersham) using 20 × SSC (3 M NaCl, 0.3 M Na-citrate, pH 7.6) as the transfer medium. The membrane was baked under vacuum for 2 h at 80 °C. The baked membrane was prehybridized and hybridized as described by Church and Gilbert (1984), but the temperature used was lowered from 65 °C to 55 °C. The probe was labelled using the Standard Multiprime DNA labelling system (Amersham) with labelled deoxycytosine [α-^{32}P]triphosphate (dCTP). Barley cDNAs for high-pI α-amylase (pM/C) and low-pI α-amylase (clone E) were kindly supplied by Dr J Rogers, Washington University Medical School (Rogers and Milliman, 1984; Rogers, 1985). Following autoradiography, blots were stripped of the probe and rehybridized with ^{32}P-labelled probe for 18S rRNA to standardize for RNA loading.

Autoradiogram quantitation

Autoradiograms were quantitated by scanning with an LKB Bromma 2202 Ultrascan laser densitometer. Each band was corrected for errors in loading the gel by dividing the integration value of the scanned band by the relative amount of rRNA detected by the densitometer. Intensity of the normalized band of controls was arbitrarily set at 1.0.

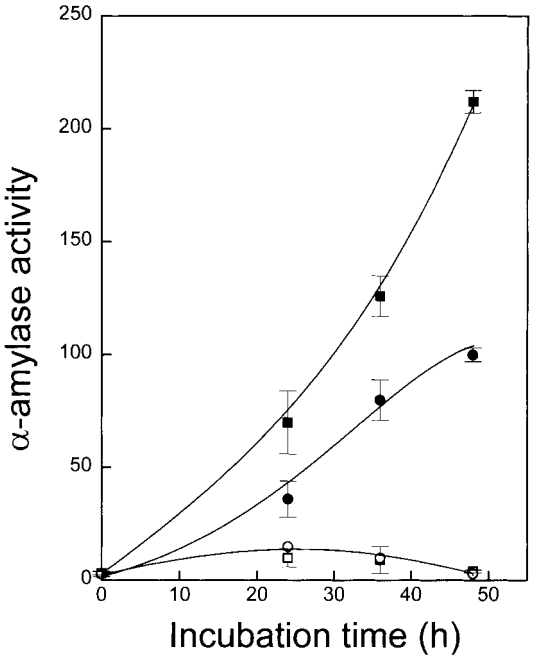

Fig. 1. Effect of GA_3 on α-amylase activities produced by aleurone layers from control and aged seeds. Twenty aleurone layers from control (■, □) or aged seeds (●, ○) were incubated with (■, ●) or without (□, ○) GA_3 during the times indicated. Tissue homogenate and incubation media were mixed and used to measure enzyme activity as described in Materials and methods. Bars represent standard error of the mean.

Results

Effect of seed storage on seed characteristics

Two seed performance parameters, percentage germination and seedling emergence, were used to measure ageing in response to adverse seed storage conditions. The results showed that approximately 90% of the seeds in both wheat lots, control and stored, were capable of germination (Table 1). The time to reach maximum percentage of germination was delayed by ageing from 22 h to 42 h. The period from germination until seedling emergence at the soil surface was delayed from 52 h to 86 h.

Ageing effect on α-amylase production

The functionality of the isolated wheat aleurone layer was measured by its ability to synthesize and secrete α-amylase under the influence of GA_3 (Fig. 1). The rate of increase in total α-amylase activity between 24 h and 36 h was similar for both lots of aleurones. However, during the course of GA_3 treatment, the level of enzyme activity attained in the aged tissue was lower than in the control. Moreover, the activity increased progressively in the control aleurone layers up to 48 h after induction, but not in the aged aleurone. When both seed lots were treated with GA_3, the secreted fraction of the total hydrolase activity at 48 h was similar: in both lots 75% of the α-amylase produced was secreted (results not shown).

The ageing treatment did not affect the basal capacity of protein synthesis of the tissue in the absence of GA_3 (Table 2). Unexpectedly, in the presence of GA_3 this parameter was lower in aged layers than in the control tissue. Fluorographs of SDS-PAGE of protein fractions obtained from aged and control tissue are shown in Fig. 2. In the absence of GA_3, the patterns of labelled protein bands of aged and control tissue were similar.

Table 1. *Effect of storage on seed characteristics*[a]

Seed lot	Viability (%)	Time to maximum percentage of germination (h)	Time to maximum seedling emergence (h)
Control	93 ± 8.4	22 ± 1.1	52 ± 2.34
Aged	89 ± 12.1	42 ± 5.3	86 ± 11.8

[a]Means ± SE are shown.

Table 2 *Effect of GA_3 on protein synthesis in aleurone layers from control and aged seeds*

Ten aleurone layers incubated for 35 h were pulse-labelled with 20 μCi of ^{35}S-methionine during 1 h. The labelled tissue was homogenized and centrifuged. The supernatant was used to determine incorporation of radioactivity in TCA-precipitable proteins.

Tissue	Treatment	Uptake (counts/layer)	Incorporation[a] (% of uptake)
Control	$+GA_3$	251 820 ± 20 238[b]	24 ± 1.4
Control	$-GA_3$	419 605 ± 11 080	29 ± 0.5
Aged	$+GA_3$	221 048 ± 6 008	14 ± 0.2
Aged	$-GA_3$	503 131 ± 24 095	30 ± 1.7

[a](cpm of ^{35}S-methionine in TCA-precipitable material/uptake) × 100.
[b]Results represent the average of three independent experiments ± SE.

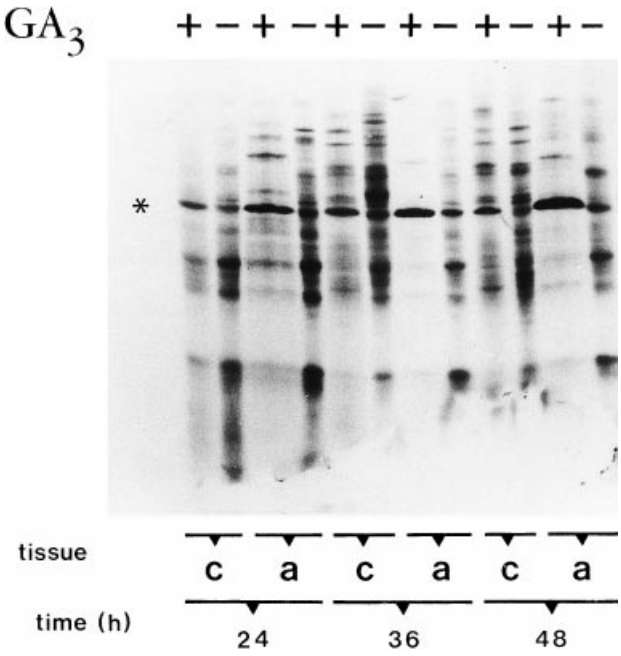

Fig. 2. Fluorography of SDS-polyacrylamide gel electrophoresis. Labelled polypeptides extracted from control (c) and aged aleurone tissue (a) incubated with (+) or without (−) GA_3 (1 μM) for 24 h, 36 h and 48 h. Layers were labelled with [^{35}S]-methionine for the last h of the incubation time. The location of α-amylase is shown by the star. Equal amounts of radioactivity incorporated into protein were loaded in each well. In order to avoid the excess of label at the end of the gel, it was allowed that the bromophenol blue marker dye run off the gel. At least three independent experiments were carried out and all gave similar results. One typical experiment is presented.

The addition of GA_3 caused a decrease, both in intensity and number of labelled protein bands. This effect was much more apparent after 36 h. Moreover, this diminution was more dramatic in aged tissue compared to the control. In both tissues, a polypeptide with the same relative molecular mass as purified α-amylase was synthesized. However, the amount of this polypeptide in aged tissue increased up to 48 h of incubation, when the experiments were terminated, while in control tissue it remained constant for the whole period. To confirm if the label in the 42 kDa band was due mainly to α-amylase, the same amount of ^{35}S-methionine incorporated into protein from both tissues was immunoprecipitated. The results showed that in aged tissue about 3.8 times more label accumulated in the α-amylase band than in the control tissue (Fig. 3).

Expression of α-amylase genes in aged aleurone layers

To study whether the differences in α-amylase activity and synthesis of the polypeptide observed in aged and control tissue were due to differences in the pattern of isoforms produced, α-amylase isoforms were resolved by isoelectric focusing, loading the same amount of α-amylase activity in each sample well of the gel (Fig. 4). In both tissues, the pattern of α-amylase activity showed two isoform groups: a low-pI group (pI = 4.8–6.0), and a high-pI group (pI = 6.0–8.0, Fig. 3). But, at all the periods tested, the detected enzyme activity of the high-pI isogroup was higher in control tissue than in the aged lot (Fig. 4).

The effect of the ageing treatment in the expression of both α-amylase gene groups was measured in order to determine whether the differences in the expression of α-amylase isoenzymes groups in both tissues could be due to differences in the accumulation of specific mRNAs. Total RNA from both control and aged aleurone layers incubated for 24 h with GA_3, was extracted, electrophoresed and probed with specific cDNAs for the low and high-pI isoenzymes (Fig. 5). After 24 h of induction, the level of high-pI α-amylase mRNA was 60% less in aged than in non-aged tissue. However, ageing had no apparent effect on the low-pI α-amylase mRNA. Such findings were in agreement with the levels of isoforms activities depicted in Fig. 4.

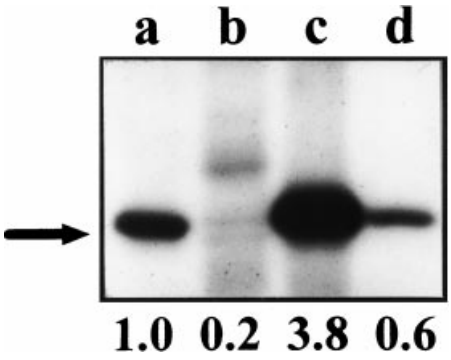

Fig. 3. Fluorography of SDS-polyacrylamide gel electrophoresis of the immunoprecipitated products of [^{35}S]-methionine-labelled polypeptides extracted from control (a, b) and aged (c, d) aleurone tissue treated for 48 h without (b, d) and with GA_3 (1 μM, a, c). The antibody was prepared against purified α-amylase. Equal amounts of TCA-precipitated counts were immunoprecipitated in each case. Numbers below the line give the relative values obtained by density scanning. At least two independent experiments were performed and one of them is presented. The intensity of the band in the GA_3-treated control tissue was arbitrarily set to 1.0.

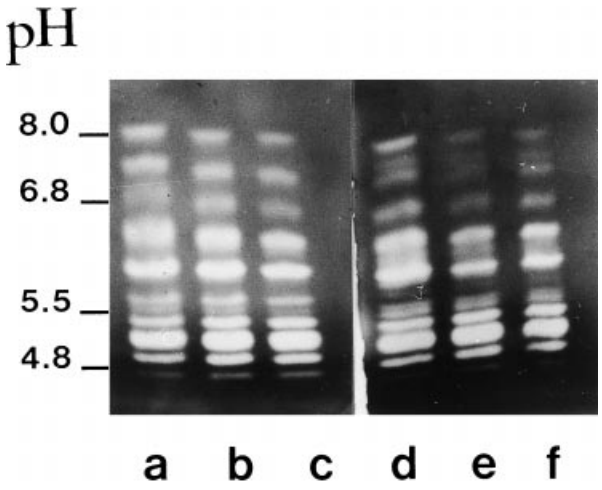

Fig. 4. Isoelectric focusing of α-amylase isozymes from control (a, b, c) and aged (d, e, f) aleurone tissue treated for 24 h (c, f), 36 h (b, e) and 48 h (a, d) with GA_3 (1 μM). Equal units of α-amylase activity were loaded on pH gradient gels, focused and stained for α-amylase activity. Three independent experiments were performed and a representative is presented.

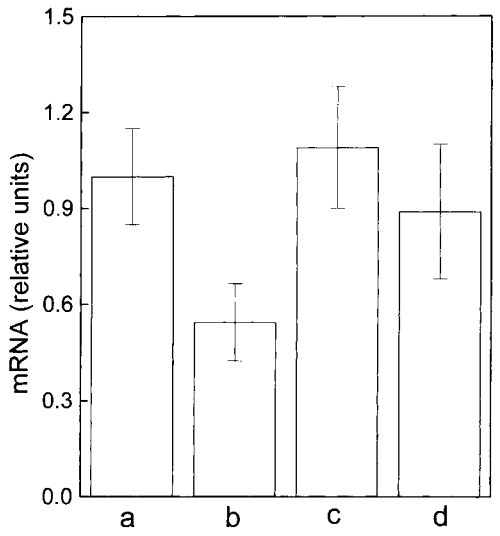

Fig. 5. Hybridization of high-pI (a, b) and low-pI (c, d) cDNA to RNA of control (a, c) and aged (b, d) aleurone tissue treated for 48 h with GA_3 (1 μM). RNA was fractionated by electrophoresis under denaturing conditions, blotted on to nitrocellulose and hybridized with [^{32}P]cDNA inserts. Hybridization was detected by autoradiography. The RNA samples were 20 μg of total RNA. Levels of mRNA accumulation for each treatment were determined by quantifying the intensity of the bands. Individual values for α-amylase transcripts were normalized to 18S rRNA to standardize for RNA loading. The values presented are levels of α-amylase transcripts relative to the normalized levels occurring in control tissue incubated with GA_3, which intensity was arbitrary set at 1.0. Bars represent standard error of the mean of three independent experiments.

Effect of ageing on aleurone GA_3-sensitivity

A GA_3 dose–response curve was constructed for both types of tissue to determine if ageing was modifying the hormonal response of the tissue (Fig. 6). In aged tissue, the ability to respond to GA_3 was decreased as evidenced

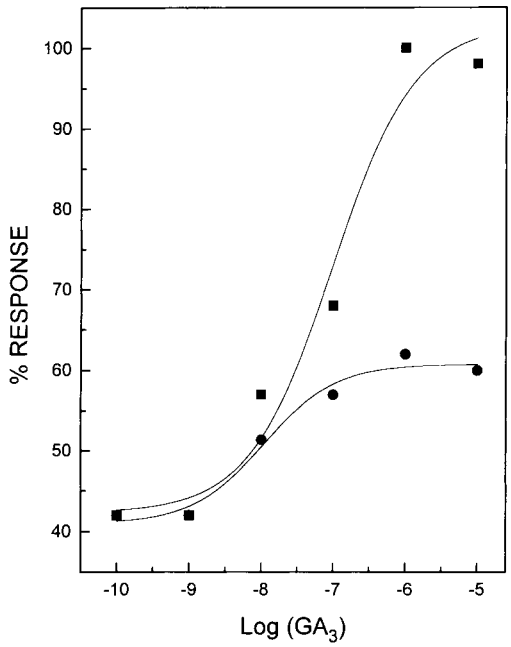

Fig. 6. Dose–response curve for GA_3-induced α-amylase in control (■) and aged (●) tissue. The total α-amylase activity (sum of α-amylase activity in tissue extract and incubation medium) in the control aleurone tissue treated for 48 h with GA_3 at 10^{-6} M was taken as 100%. Two independent experiments were performed in triplicate. The results of both experiments were similar. Results of one typical experiment is presented.

both by a lower slope and plateau as compared to those values from the control tissue.

Discussion

These experiments were undertaken to determine the molecular basis of the decrease in GA_3-induced α-amylase activity produced by aged aleurone layers (Livesley and Bray, 1991; Bernal-Lugo *et al.*, 1994). The differences in α-amylase accumulation in aged and unaged aleurone tissue may have been caused by either a general decrease in protein synthesis or a direct result of ageing on GA_3 responsiveness. The observation that both types of tissue showed the same levels of basal protein synthesis argues against the first possibility.

Although both tissues presented similar basal protein synthesis capacity, the GA_3 induced α-amylase activity in aged tissue was lower than in the control, suggesting that in aged tissue GA_3 responsiveness was impaired. This was confirmed by the lower capacity for protein synthesis of aged tissue after GA_3 treatment. Although, there was a higher proportion of ^{35}S-methionine incorporated into α-amylase polypeptide in the aged GA_3-treated samples. The above results may be a consequence of different ratios of expression of the two α-amylase isogroups (MacGregor and MacGregor, 1987) following ageing. Evidence supporting this proposition is that the two

isogroups of α-amylase have different specific activities against soluble starch: the low-pI isogroup showing lower specific activity than the high-pI isogroup (Marchylo *et al*., 1984). In addition, the amino acid composition of the two groups is different: the abundance of methionine is higher (2.7%) in the low-pI α-amylases than in the high-pI α-amylases (1.7%) (Chandler *et al*., 1984). Therefore, it is proposed that in aleurone tissue from aged seeds, those processes leading to an enhancement in the production of high-pI α-amylase were either absent or reduced, in comparison with their control counterpart and, as a consequence, the members of the low-pI α-amylase isogroup were preferentially synthesized.

The two families of α-amylase isozymes are encoded by two sets of structural genes, whose expression is primarily controlled by GA_3 inducing the accumulation of α-amylase mRNA. The high-pI α-amylase group of isoenzymes is the most highly regulated by GA_3 (Chandler and Jacobsen, 1991). The low levels of high-pI α-amylase mRNA observed in aged tissue may result in a decrease of the amount of high-pI α-amylase isogroup contributing to the total enzymatic activity, as suggested by the isoelectric focusing analysis of α-amylase activity, which in aged tissue showed that the high-pI isogroup had a lower contribution to the total activity. This result also mitigates the likelihood that the low activity of α-amylase observed in aleurones from aged seeds could be due to a higher degradation or inactivation rate of high-pI isoenzymes. The lower levels of high-pI α-amylase mRNA in the aleurone layer from aged seeds may be due to an increased degradation of α-amylase mRNA or to a decrease in the high-pI α-amylase mRNA expression. The first might be ruled out because there is the same level of low-pI α-amylase mRNA accumulation in both tissues. Therefore, one of the effects of seed ageing could be the specific impairment of the expression of GA_3-induced high-pI mRNA.

However, these results could also be explained by an impairment of post-transcriptional, translational and post-translational events, since they may also lead to a decrease in the abundance of the α-amylase high-pI mRNA (Nolan *et al*., 1987).

It is possible that seed ageing reduces the capacity of the aleurone to respond to GA_3 through a differential death or impairment of aleurone cells, or because some component(s) of the GA_3 response pathway is (are) altered, changing for instance, the effectiveness by which a given concentration of GA_3 modifies the levels of high-pI α-amylase mRNA. Both alternatives may be reflected in an altered dose–response curve (Fitzsimmons, 1989; Trewavas, 1991; Hillmer *et al*., 1992; Bradford and Trewavas, 1994). In aleurones from aged seeds, the GA_3 response of the system is restricted as compared to the control, and although the two curves of dose versus response were identical up to a concentration of 10^{-8} M of GA_3, in aged tissue the response was saturated at 10^{-7} M.

The successful response of aleurone cells to GA_3 involves the recognition of the stimulus by outward-facing plasma membrane receptors (Hooley *et al*., 1991; Gilroy and Jones, 1994) and a mechanism for subsequent signal transduction (Bethke and Jones, 1994; Kuo *et al*., 1996; Penson *et al*., 1996). In this scheme, the maximum response of the aleurone may depend on several parameters: (1) the amount and affinity of receptors to GA_3; (2) the overall capacity of the signal transduction system to respond to the number of occupied receptors, and (3) the fraction of aleurone cells that are functional (Hillmer *et al*., 1992). The data and approach in this study can not distinguish which of these factors might be responsible for the low levels of high-pI α-amylase mRNA observed in aleurone layer from aged seeds. However, in the last option ageing may also decrease the low-pI α-amylase mRNA acccumulation, and this was not the case.

In conclusion, these results show that the diminished capacity of α-amylase production in aleurone of aged seeds may be due to a decrease in the expression of the high-pI α-amylase genes, and that this is associated with an alteration in the tissue response to GA_3.

Acknowledgements

We thank AC Leopold and DR Ort for critical review of the manuscript and C Parra and R Gutierrez for technical assistance.

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