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Aleurones from a Barley with Low α-Amylase Activity Become Highly Responsive to Gibberellin When Detached from the Starchy Endosperm[1]

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The physiological and molecular bases for contrasting α-amylase phenotypes were examined in germinating seeds of two barley (*Hordeum vulgare* L.) cultivars, Morex and Steptoe. Morex is a high-quality malting barley that develops high α-amylase activity soon after germination. Steptoe is a feed barley that develops only low α-amylase activity levels during this period. The expression of all high- and low-isoelectric point (pI) α-amylase isozymes is reduced in Steptoe. The amount of α-amylase mRNA per gram of seedling tissue is correspondingly lower in Steptoe. Southern blot analysis revealed that the cultivars have the same copy number and organization for most high- and low-pI genes. Steptoe seedlings or embryoless half-seeds produce little α-amylase in response to exogenous applications of gibberellic acid (GA_3) compared with Morex. However, when isolated aleurones of both cultivars are treated with GA_3, they produce similar amounts of high- and low-pI α-amylase RNAs. This suggests that a factor in the starchy endosperm is responsible for lowered α-amylase response in Steptoe. The factor is probably not abscisic acid (ABA), since the two cultivars have similar concentrations of ABA during germination.

Molecular studies of α-amylase gene expression in barley (*Hordeum vulgare*) have focused primarily on the isolated aleurone of the Himalaya cultivar. Little study has been devoted to expression in intact seedlings, especially in commercial cultivars (Chandler and Jacobsen, 1991; Karrer et al., 1991). The Morex (Rasmusson and Wilcoxson, 1979) and Steptoe (Muir and Nilan, 1973) cultivars differ greatly in α-amylase activity during germination. Morex develops high amounts of activity soon after germination, helping to make it one of the top American six-row malting barleys. In contrast, the six-row cultivar Steptoe produces little activity and has exceptionally poor malting quality. These contrasting phenotypes provide an excellent comparative system for analyzing the control of α-amylase genes in intact germinating seedlings.

Extensive research in the past three decades has established a central role for GA as a regulator of α-amylase and many other hydrolytic enzymes, although the exact role played by GA is still clouded by inconsistencies (reviewed by Fincher, 1989). The regulation of α-amylase in cereal grains has been the subject of several reviews (Ho, 1979; Enari and Sopanen, 1986; MacGregor and MacGregor, 1987; Muthukrishnan and Chandra, 1988; Fincher, 1989). Paleg (1960), Yomo (1960), and Sandergren and Beling (1959) initially discovered that GA applications increased α-amylase activity in barley grain. The establishment of an isolated barley aleurone system, in which GA caused a dramatic induction of α-amylase activity (Chrispeels and Varner, 1967), and the finding that GA is produced in the embryo (MacLeod and Palmer, 1967; Radley, 1967) led to the currently held view of cereal seed endosperm mobilization. In brief, GA is synthesized by the germinating embryo, diffuses to the aleurone cells, and induces a battery of hydrolytic enzyme genes, including those for α-amylases. The GA response of isolated barley aleurones has remained an intensively studied phenomenon and forms the basis for speculation on mechanisms controlling α-amylase genes in germinating seedlings. It remains to be seen whether this is a relevant model system or an artifactual system with a unique physiology.

It is well established that GA enhances the transcription of barley α-amylase genes and the net accumulation of α-amylase mRNAs in isolated aleurones (Higgins et al., 1976; Muthukrishnan et al., 1979; Mozer, 1980; Chandler et al., 1984; Huang et al., 1984; Rogers and Milliman, 1984; Deikman and Jones, 1986; Nolan et al., 1987; Khursheed and Rogers, 1988; Nolan and Ho, 1988; Chandler and Jacobsen, 1991), protoplasts (Jacobsen et al., 1985; Chandler and Jacobsen, 1991), and isolated nuclei of protoplasts (Jacobsen and Beach, 1985). Indeed, endogenous GA levels correlate with α-amylase production in a variety of germinating commercial barleys; it has been proposed that a factor determining GA responsiveness may be active in cultivars where poor correlations are found (Kusaba et al., 1991).

The effects of ABA must be considered as a primary factor modifying the GA responsiveness of barley aleurones (Jacobsen, 1983). ABA represses the expression of α-amylase genes and reverses the positive effect of GA on α-amylase mRNA accumulation (Muthukrishnan et al., 1983; Chandler et al., 1984; Jacobsen and Beach, 1985; Nolan et al., 1987; Nolan and Ho, 1988). It is likely that the balance between endoge-

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Abbreviations: DNSA, 3,5-dinitrosalicylic acid; pI, isoelectric point; SSPE, 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA.

nous GA and ABA concentrations strongly controls α-amylase gene expression in intact germinating seedlings, although a cultivar's sensitivity to each hormone would shift the effective (perceived) balance. ABA also induces the accumulation of the α-amylase inhibitor bifunctional α-amylase/subtilisin inhibitor (Mundy, 1984).

In addition to hormonal and other physiological controls, cultivar-specific differences in expression could be caused by gene sequence variations. However, because both the high- and low-pI α-amylases are encoded by multicopy genes, variations must occur in more than one gene to cause significant phenotypic effects. The high-pI α-amylases are represented by at least seven genes or pseudogenes and the low-pI α-amylases by at least three (Knox et al., 1987; Khurseed and Rogers, 1988). Despite this, it is necessary to compare structural genes because cultivar differences may be found in a gene family member that is a dominant contributor to the transcript pool.

In the following study, the Morex and Steptoe cultivars were compared at the molecular and physiological levels. Initially, it appeared that low α-amylase activity in Steptoe could not be caused by GA-related deficiencies. Morex and Steptoe are similar in stature under field conditions. Mutants or variants in the GA system are normally recognized by altered growth habit (Favret et al., 1976; Lanahan and Ho, 1988). However, this study shows that Steptoe represents an unusual case of GA response variance where one organ (the shoot) is entirely normal, whereas another (the endosperm-associated aleurone) appears to be insensitive to or unresponsive to GA.

MATERIALS AND METHODS

Seedling Growth

Seeds of the barley (*Hordeum vulgare* L.) Morex (1989, 1990, and 1991 harvests) and Steptoe (1988 and 1991 harvests) cultivars were provided by the U.S. Department of Agriculture, Agricultural Research Service, Small Grains Germplasm Research Facility (Aberdeen, ID). Seedlings grown under malting conditions were allowed to imbibe ("steep") in 16°C tap water and raised to drain periodically for either 36 h (Morex) or 48 h (Steptoe). Steptoe was steeped longer because of its higher grain weight (47.5 mg) compared with Morex (38.3 mg). They were then germinated aerobically at 16°C with 100% RH in slowly rotating drums for up to 6 d. For ease of discussion, seedling age is given in terms of the day aerobic germination was begun. Thus, "1-d-old" Morex was 36 h old from the initiation of imbibition, and "1-d-old" Steptoe was 48 h old.

For ABA measurements, seeds were sterilized in 1% (v/v) hypochlorite for 10 min, rinsed, allowed to imbibe for 8 h, and then planted on Kimpack[2] germination paper moistened with 20 mM Na succinate/20 mM $CaCl_2$ buffer and 50 μg/mL of gentamycin. Seeds were germinated in the dark at 16°C.

[2] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

Half-Seed and Aleurone Treatments

When Steptoe and Morex seeds were to be used for half-seed and isolated aleurone experiments, the embryo-containing portion of the dry seed was first excised, producing embryoless half-seeds. These were sterilized for 10 min in 1% (v/v) hypochlorite and then allowed to imbibe and rinsed repeatedly for 8 h in deionized H_2O. They were then "planted" on Kimpack germination paper in plastic trays and wetted with 20 mM Na succinate buffer (pH 5.0) containing 20 mM $CaCl_2$ and 50 μg/mL of gentamycin. After 5 d, hulls were removed and aleurones were cleanly separated from the starchy endosperms. Twenty aleurones were placed in 9-cm Petri dishes containing 20 mL of Na succinate/$CaCl_2$ buffer with 150 μg/mL of penicillin and 50 μg/mL of gentamycin, essentially according to Belanger et al. (1986). GA_3 was added to give final concentrations of 0, 10^{-8}, or 10^{-6} M, as indicated. Plates were gently shaken for 24 h at 21°C on a gyratory shaker. After the first 12 h, the GA treatments were repeated. After 24 h, aleurones (or half-seeds) were blotted to remove surface moisture, weighed, and stored at −80°C prior to α-amylase enzyme analysis or RNA extraction. Incubation medium was heat-treated at 70°C for 20 min and stored at −20°C prior to analysis.

α-Amylase Enzyme Activities

Seedling caryopses, half-seeds, and aleurones were prepared for α-amylase enzyme assays and IEF by grinding in buffer containing 200 mM Na acetate (pH 5.5), 10 mM $CaCl_2$, sand, and PVP-360 (Sigma) at a tissue weight-to-volume ratio of 1 g to 10 mL. Homogenates were centrifuged at 12,000 rpm in microfuge tubes for 10 min at 4°C. The supernatants were heated to 70°C for 15 to 20 min to inactivate β-amylase and other carbohydrases (excluding α-amylases) and centrifuged as above. α-Amylase activity was measured using a DNSA assay (Rick and Stegbauer, 1974). Assays were compared to a standard curve based upon maltose. Reactions were incubated at 30°C for 30 min and measured relative to paired samples held at 0°C prior to boiling in the DNSA chromogenic reagent. This allowed a correction for changes in background reaction levels. Units are in terms of μmol of maltose generated from the soluble starch substrate per g fresh weight of seed tissue in 30 min.

For activity gels, heat-treated homogenate was applied to the center of preformed IEF gels of ampholyte pH range 4.0 to 6.5 (Pharmacia). Gels were prerun at 1000 V for 45 min. Samples were electrophoresed at 1200 V and 10°C for 1 h. Amylase activity was detected by impregnating the gels with 1% Lintner starch (Sigma) in 200 mM ethylenediamine (pH 6.0), 10 mM $CaCl_2$ and staining in I_2/KI (Henson and Stone, 1988). After boiling and cooling the starch to 40°C, it would not sufficiently impregnate the 5% polyacrylamide gels. This was overcome by incubating the starch at 40°C with 1 μg/mL of heat-treated α-amylase (Sigma) until its A_{650} was reduced to 0.03. The solution was boiled again, cooled, and incubated with the gels at 40°C while shaking for 3 h. Satisfactory Polaroid photographs were obtained by employing an orange filter.

RNA Extraction

Roots, hulls, and seedling axes (roots and shoots) were removed, and caryopses were frozen in liquid N_2 and stored at −80°C until use. Up to 3 g of caryopses or isolated aleurones were ground to a fine powder in liquid N_2 and then added to 20 mL of 0°C RNA extraction buffer: 250 mM Tris-HCl (pH 9.0 at 22°C), 250 mM NaCl, 50 mM EDTA, 50 mM 2-mercaptoethanol, 1% SDS (BDH Chemicals Ltd.), 1% Na deoxycholate, and 2 mM aurintricarboxylic acid (Sigma Chemical Co.). Aurintricarboxylic acid was employed to inhibit RNase (Stern and Newton, 1984), and the main buffer components selected are known to give high-quality rice kernel RNA (O'Neill et al., 1990).

The powder was homogenized in a Polytron at high speed and centrifuged at 10,000 rpm for 10 min at 4°C in a Beckman JA-20 rotor to pellet starch. The supernatant was quickly transferred to a beaker and stirred briskly on ice with 20 mL of phenol (equilibrated with pH 8.0 Tris and then with extraction buffer). After 10 min, 10 mL of cholorform/isoamyl alcohol (24:1, v/v) was added, and stirring was continued for 5 min. The emulsion was centrifuged as above. The aqueous phase was mixed with a 10% (w/v) solution of calcofluor white (Cellufluor, Polysciences, Inc.) at 615 μg/mL final concentration. The solution was kept on ice for 10 min and centrifuged at 12,000 rpm for 10 min at 4°C, pelleting β-glucans.

The RNA in the supernatant was precipitated at −20°C for 5 h in centrifuge tubes with 0.3 M (final concentration) K acetate, pH 5.5, and 2.5 volumes of ethanol. RNA was pelleted by centrifugation at 12,000 rpm for 10 min at 0°C, washed with 70% ethanol, and lyophilized briefly. The red RNA pellets were readily and completely dissolved in 4 mL of Tris-EDTA buffer (pH 8.0). Two milliliters of 6 M $LiCl_2$ were added, and RNA was reprecipitated for 15 h at 4°C in microcentrifuge tubes. The RNA was pelleted by centrifugation at 12,000 rpm, resuspended and washed in 3 M $LiCl_2$, repelleted, and dissolved in diethylpyrocarbonate-treated H_2O. The RNA was precipitated a final time with K acetate and ethanol, washed with 70% ethanol, and dissolved in H_2O.

Cloned Probes

High-pI α-amylase RNA was detected with the high-pI cDNA clone pM/C (Rogers, 1985) derived from aleurone mRNA of the Himalaya cultivar. Low-pI RNA was detected with the low-pI cDNA clone E (Rogers and Milliman, 1983) from the same source. Both were generously provided by John Rogers (Washington University). The concentrations of cloned insert DNAs used in probe synthesis and as hybridization standards were determined by spectrophotometric measurements and by Hoechst 33258 staining with fluorimetric detection.

Northern Blot Analysis of RNA

Fifteen-microgram samples of RNA were electrophoresed on 1.2% SeaKem (Marine Colloids) agarose-formaldehyde gels and blotted onto Nytran (Schleicher & Schuell) filters (Rave et al., 1979). After baking for 90 min in an 80°C vacuum oven, filters were prehybridized for 8 to 16 h in 50% (v/v) formamide, 2× SSPE (pH 7.4), 250 μg/mL of denatured salmon sperm DNA, 0.1% BSA, 0.1% Ficoll 400 (Pharmacia), 0.1% PVP-40 (Sigma), and 1% SDS (Thomas, 1983). Radiolabeled probe DNA was prepared from gel-purified clone inserts. Clone pM/C and clone E inserts (100 ng) were each labeled simultaneously to high specific activities (1.12×10^9 cpm/μg) using a random hexamer-primed Klenow fragment reaction (Feinberg and Vogelstein, 1983) containing [α-^{32}P]dCTP (3000 Ci/mmol, DuPont). Probe DNA was heated to 100°C for 2 min and added to hybridization bags to give 10^6 cpm/mL. Hybridization solution consisted of four parts of the above prehybridization buffer plus one part 50% (w/v) dextran sulfate (Pharmacia). Hybridization was conducted for 16 h at 62.5°C. Filters were washed four times in 2× SSPE and 0.1% SDS at 22°C for 15 min each and then four times in 0.2× SSPE and 0.1% SDS at 62.5°C for 15 min each.

Filters were exposed to Kodak XAR-5 film between two intensifying screens. Comparative high- and low-pI autoradiographs were made by exposing films for the same duration in the same x-ray cassette. In addition, several exposures were made to ensure that autoradiographs were within the linear response range of the film. Autoradiographs were scanned densitometrically with a soft laser scanner (Biomed).

Southern Blot Analysis of DNA

Genomic DNA was prepared from etiolated seedling shoots by gentle grinding in a Tris-EDTA buffer containing 300 μg/mL of ethidium bromide, followed by isopycnic ultracentrifugation in CsCl (Kislev and Rubenstein, 1980; Sambrook et al., 1989). Ten micrograms of DNA was cut with *Bam*HI, *Eco*RV, *Hin*dIII, or *Xba*I restriction enzyme and electrophoresed through 1.2% agarose gels in Tris-acetate buffer. DNA was Southern blotted onto Nytran nylon membranes (Sambrook et al., 1989), which were processed, probed, and washed as in the northern blot procedure (above).

ABA Measurements

ABA concentrations were determined by the method of Walker-Simmons (1987). Hulls and roots were removed prior to freezing of the seedlings. Methanolic extracts were prepared from mature seeds (0-d-old) and from 1- to 3-d-old seedlings. Seedlings were frozen in liquid N_2, ground to a fine powder in liquid N_2, lyophilized, and extracted in methanol containing butylated hydroxytoluene and citric acid at 100 mg of tissue/mL. Extracts were assayed on ABA monoclonal antibody ELISA plates (Idetek Corp., Sunnyvale, CA). The linear response range was established with 0-d-old samples, and the same volume of homogenate was then used for all samples. ABA levels were determined relative to a standard curve from purified (±)ABA isomers (Sigma).

RESULTS

Developmental Changes in Enzyme Activity

α-Amylase activity increases very slowly in Morex until d 4 (Fig. 1). Activity then increases rapidly in Morex, but the

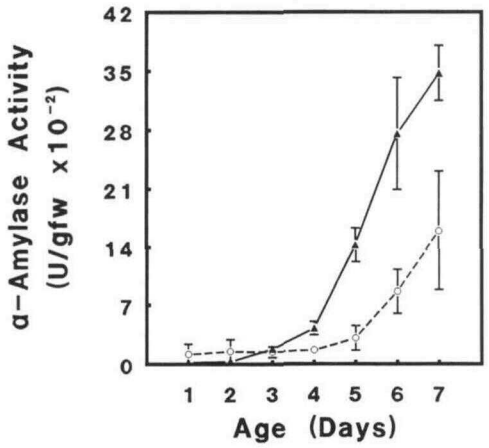

Figure 1. Postgerminative temporal changes in α-amylase enzyme activity in the Morex and Steptoe cultivars. Plant axes and scutella were removed from seedlings at d 1 through 7 of aerobic germination, and caryopsis α-amylase activity was determined by the DNSA procedure. Units are in terms of μmol of maltose produced from potato starch during a 30-min incubation at 30°C. Morex, Solid line. Steptoe, Broken line.

developmental increase in Steptoe does not begin until 1 to 2 d later. From d 4 to 7, the activity in Steptoe is only one-third of that in Morex.

Steptoe's relative deficiency in α-amylase activity results from lowered expression of both the high- and low-pI isozyme groups (Fig. 2A). Also, both cultivars have much more high-pI than low-pI activity. High-pI activity is evident at d 2 in both Morex and Steptoe, and it increases sharply by d 3 in both. The rapid rise seen on activity gels precedes that seen in activity assays (Fig. 1) by at least 1 d. The earlier rise seen on activity gels may result from electrophoretic removal of α-amylase inhibitors. The rise in low-pI activity occurs later than that of high-pI activity. In Steptoe, the low-pI rise lags 2 d behind that in Morex (Fig. 2B).

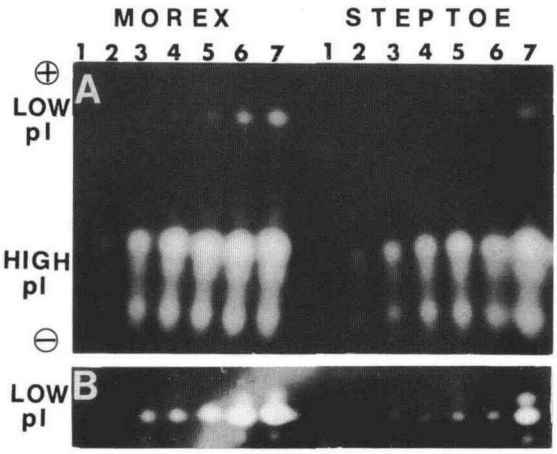

Figure 2. Temporal changes in high- and low-pI α-amylase isozymes. Equal-volume aliquots of heat-treated caryopsis homogenates (representing equal weights of tissue) were applied midway between the anode and cathode on preformed IEF gels. The age of seedling materials (1–7 d) is indicated at the top. After focusing, gels were incubated in potato starch and stained with KI/I_2. A, The separation of isozyme species reveals the abundance of high-pI activity relative to low-pI activity, and the higher level of both isozymes in Morex relative to Steptoe. B, The development of low-pI activity is shown by focusing higher amounts of the same samples used in A.

α-Amylase mRNA Concentrations

The relative amounts of high- and low-pI mRNAs represented on northern blots (Fig. 3) correspond to the isozymal activity levels seen on IEF activity gels (Fig. 2). Morex contains greater amounts of both the high- and low-pI mRNAs. Over the 7-d developmental period, Morex has an average of twice as much high-pI mRNA and three time as much low-pI mRNA relative to Steptoe. In both, the high-pI mRNA rises sharply between d 2 and 3, synchronous with the sharp rise in high-pI isozyme activity. High-pI mRNA reaches a plateau at d 4. The low-pI mRNA, however, continues to increase gradually throughout d 7. In Morex, there is 5.5-fold more high- than low-pI mRNA, whereas in Steptoe there is 9.3-fold more high-pI mRNA over the 7-d period. These differences between Morex and Steptoe and high- and low-pI mRNAs were highly repeatable with different seed lots and with many RNA extractions from stored seeds over a 3-year period.

The main difficulty in determining relative levels of high- versus low-pI mRNA lies in ensuring that both blots were

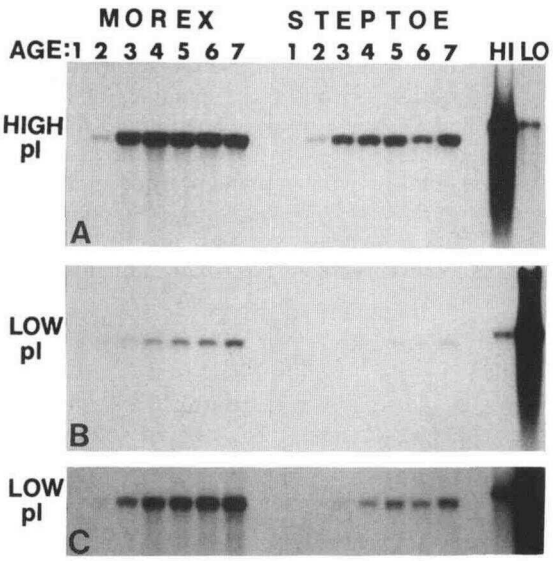

Figure 3. Northern blot analysis of temporal changes in high- and low-pI RNA levels in Morex and Steptoe. Fifteen-microgram samples of total RNA were electrophoresed on formaldehyde-agarose gels, northern blotted, and probed (see "Materials and Methods"). In the right lanes, 5-ng samples of denatured high-pI (HI) and low-pI (LO) insert DNA from cDNA clones pM/C and E (respectively) were electrophoresed to serve as hybridization controls. A, Autoradiogram of RNA blot hybridized with high-pI probe. B, RNA blot hybridized with low-pI probe. C, 16.5-h exposure of B to reveal weak low-pI signal in Steptoe. Autoradiograms of A and B were exposed for 6 h.

hybridized with probes of the same specific activity, that the hybridization solution contained the same concentration of probe activity, and that hybridization conditions and exposure times were the same for both. These factors were rigorously monitored. Probes were synthesized simultaneously, filters were probed simultaneously (in separate bags) in the same hybridization chamber, and filters were exposed together for the same duration in the same x-ray film cassette. The concentrations of high- and low-pI cloned insert DNAs used for probe synthesis were determined to be equal, by both UV spectrophotometric readings and Hoechst 33258 staining. The inclusion of 5 ng of both DNAs as hybridization standards on each northern blot (Fig. 3, A and B), and the comparable degree of hybridization seen for each, provide evidence that the signals for both α-amylase isozymal mRNAs portray their true abundance levels. The amount of cross-hybridization that occurs between low- and high-pI sequences is typically 1 to 5%, which would not significantly influence the interpretation of high- and low-pI mRNA levels.

Another factor that influences the amount of α-amylase transcripts in Morex, relative to Steptoe, is the amount of RNA present per g fresh weight of tissue. Both cultivars experience the same developmental increase in total RNA levels reaching a plateau or peak between d 3 and 4 (data not presented). Before d 4, RNA concentrations are 26% higher in Steptoe, but from d 4 to 7 there is 20% more RNA/g fresh weight in Morex. Therefore, the total α-amylase transcript availability, which is the product of α-amylase transcripts per μg of total RNA (northern blot signal) and RNA concentrations per g fresh weight, is greater in Morex during the high amylolytic period of d 4 to 7. The average transcript availability in Morex, relative to Steptoe, is 2.4-fold greater for high-pI mRNA and 3.6-fold greater for low-pI mRNA over the 7-d postimbibition period.

α-Amylase Gene Analysis

Because the two cultivars differ in germplasm source and in α-amylase gene expression levels, it was possible that the cause of their phenotypic differences could be indicated by Southern blot analysis. After probing restriction enzyme-digested Steptoe and Morex genomic DNA with the high-pI clone, it was found that the high-pI gene copy number and organization of most genes are apparently identical in the two cultivars (Fig. 4A). Six high-mol wt restriction fragments were found to be identical in the two cultivars, and their signal strengths were the same between cultivars.

Similar results were found after hybridizations with the low-pI cDNA probe. Up to four high-mol wt fragments were found, and most were of the same size and signal strength in Morex and Steptoe (Fig. 4B). A polymorphism of weak intensity occurred in *Xba*I digests. Considering the multicopy nature of the high- and low-pI genes in both cultivars, it became apparent that the lowered activity levels of both isozymes in Steptoe is due to the synthesis or response to diffusible regulatory factors, rather than to an altered complement of coding genes.

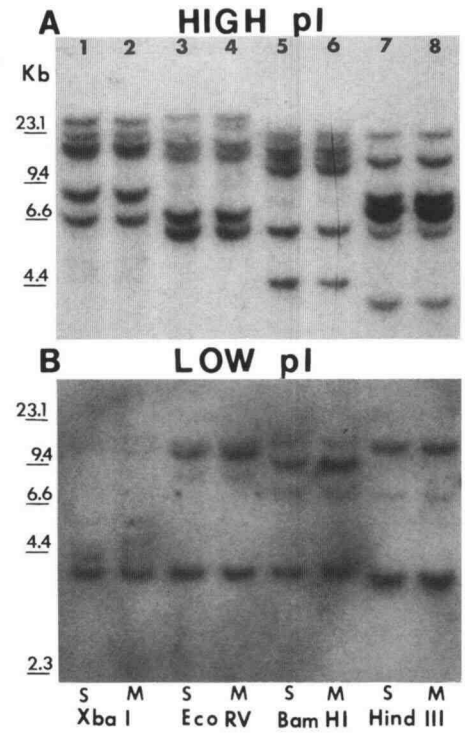

Figure 4. Southern blot analysis of high- and low-pI α-amylase nuclear genes in Morex (M) and Steptoe (S). Ten micrograms of DNA were cut with either *Xba*I (lanes 1 and 2), *Eco*RV (lanes 3 and 4), *Bam*HI (lanes 5 and 6), or *Hin*dIII (lanes 7 and 8). The positions of λ-*Hin*dIII markers are shown at the left. A, Autoradiogram of Southern blot hybridized with high-pI cDNA probe. B, Blot hybridized with low-pI probe.

ABA Concentrations

A likely candidate for a diffusible regulatory molecule that could inhibit the expression of α-amylase (and other GA-inducible genes) is ABA. ABA was measured in the whole caryopsis over the first 3 d of germinative development so that developmental differences would not be overlooked. It was predicted that mature Steptoe seed would have more ABA than Morex, but the opposite was true (Fig. 5); Morex's ABA level was twice that of Steptoe. ABA levels fell in both cultivars during germination, and by d 3, both had equal levels. This is the time just prior to the rise in high-pI mRNA levels in both cultivars (Fig. 3A), so it is unlikely that ABA causes Steptoe's relatively weak α-amylase expression.

GA Response

Because Steptoe's growth in the field is similar to that of Morex, it was not suspected of having deficiencies in GA production or response. However, when seeds were allowed to germinate and grow for 5 d on paper containing different concentrations of GA_3, Morex responded with increases in α-amylase activity, whereas Steptoe had no consistent positive response (Fig. 6). Temporal changes in α-amylase activity in response to GA concentrations of 0, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M were also examined daily from 2 to 7 d from the

Figure 5. Changes in ABA concentrations during germination in Morex and Steptoe seedlings. Methanolic extracts of mature seeds (0-d-old) and 1- to 3-d-old seedlings were applied to ABA monoclonal antibody ELISA plates. Seedlings were germinated on paper rather than under malting conditions. Also, shoots and scutella were not removed prior to freezing as they were in caryopsis samples. Each point is the average of three separate extracts, and 28 μL of extract, representing 2.8 mg dry weight (DWT), was applied to each well.

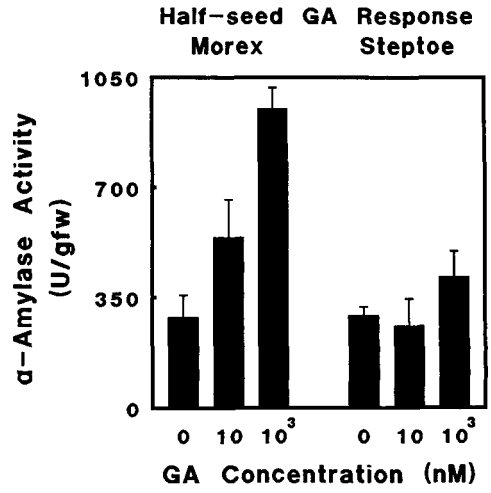

Figure 7. Response of embryoless half-seeds to exogenously applied GA_3. Half-seeds were incubated on germination paper soaked with 0, 10, or 10^3 nM GA_3 for 5 d. α-Amylase activity was determined as in Figure 6. Values are the results of two complete experimental repetitions with two assays conducted for each sample. Each repetition was conducted with seeds from different harvest years.

beginning of imbibition. On each day, α-amylase activity was slightly lower in Steptoe under all GA concentrations relative to untreated controls, whereas Morex responded above control levels at all GA concentrations (data not presented). Maximal response in Morex occurred at 10^{-9} M GA.

To remove the embryo as a possible source of inhibition of Steptoe's GA response, embryoless half-seeds were prepared and treated as above with 0, 10^{-8}, and 10^{-6} M GA. Morex again responded positively to GA, with higher GA concentrations producing increased α-amylase activity (Fig. 7). A slight increase in Steptoe's α-amylase activity occurred at 10^{-6} M but not at 10^{-8} M. This suggests that either the Steptoe aleurones are unresponsive to GA, or the starchy endosperm of Steptoe contains an inhibitor or inactivator of GA.

To explore these possibilities, the GA responsiveness of Steptoe and Morex isolated aleurones was tested at the RNA and enzyme activity levels. Aleurones produced little or no α-amylase mRNA when incubated for 24 h in buffered medium without GA, but both Steptoe and Morex produced high amounts of high- and low-pI mRNA with 10^{-6} M GA (Fig. 8). In contrast to the large high-pI: low-pI mRNA ratios in intact seedlings (Fig. 3), isolated aleurones had similar amounts of both mRNAs. The mRNA data correlated with α-amylase isozymal activity levels (Fig. 9). Both cultivars produce similar amounts of high- and low-pI enzyme activities resulting from GA treatment. In addition, a low level of low-pI activity was produced without GA treatment, although no high-pI isozyme was produced.

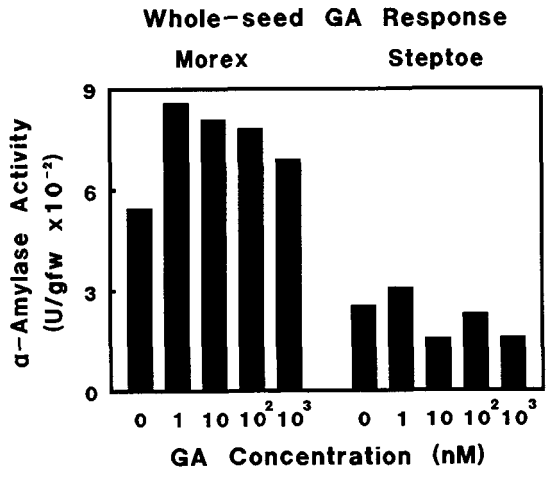

Figure 6. Response of intact germinating seedlings to exogenously applied GA_3. Seedlings were incubated for 5 d on germination paper wetted with various concentrations of GA_3. Hulls and shoot-root axes were removed before preparing homogenates. α-Amylase activity was determined for heat-treated samples of homogenates using the DNSA procedure. Units are the same as in Figure 1.

DISCUSSION

These studies suggest that the low level of α-amylase activity found in the Steptoe cultivar could be caused by a starchy endosperm-associated inhibitor of α-amylase gene expression. Although endosperm-specific inhibitors of α-amylase enzyme activity are known, only ABA is known to inhibit α-amylase expression at the pretranslational level. ABA inhibits a wide range of genes that are positively regulated by GA_3, especially α-amylase and other hydrolytic enzyme genes of germinating cereal seeds (Zeevart and Creelman, 1988). Steptoe is known to exhibit strong postharvest dormancy, or "incomplete after-ripening," and seeds of this cultivar may not germinate for 2 months after maturity (Ullrich et al., 1993). The Steptoe seeds had been stored longer than 1 year under dry conditions prior to their use in these studies. This allowed sufficient time for any dormancy

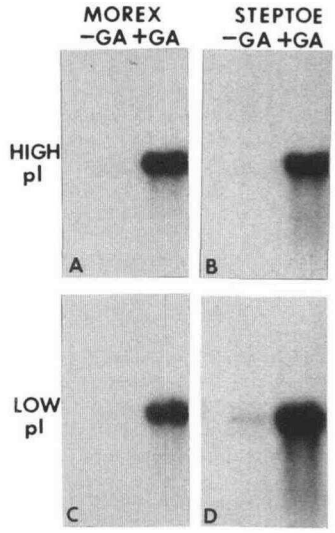

Figure 8. GA responsiveness of isolated Morex and Steptoe aleurones at the RNA level. Aleurones were removed from untreated 5-d-old embryoless half-seeds and treated for 24 h with 10^{-6} M GA_3. RNA was purified from the aleurones, northern blotted, and hybridized with high-pI probe (A and B) or low-pI probe (C and D). Both Morex (A and C) and Steptoe (B and D) aleurones respond strongly to GA.

effects to have passed. It was suspected that high ABA concentrations may contribute to Steptoe's low α-amylase activity and poor response to GA_3. However, ABA concentrations are initially higher in Morex seeds, and by d 3 they decline to identical levels in Morex and Steptoe.

It is possible that Steptoe may be highly responsive to ABA, relative to Morex. Walker-Simmons (1987) found that a wheat cultivar subject to preharvest sprouting and a normal cultivar did not differ significantly in endogenous ABA concentrations. Instead, the preharvest sprouting cultivar was less responsive to increases in ABA. Increased ABA levels accompany late seed development and discourage premature germination in barley (Robertson et al., 1989) and in a range of monocots and dicots (King, 1982; Zeevart and Creelman, 1988). However, in two barley cultivars preharvest sprouting susceptibility was correlated with endogenous ABA concentrations (Goldbach and Michael, 1976). The question of Steptoe's responsiveness to ABA will be examined in future investigations.

Other possible mechanisms may account for Steptoe's apparent endosperm-associated inhibition. Thus, (a) the exogenous GA may not have gained access to the Steptoe aleurone, even though it did in Morex half-seeds and whole seedlings (Figs. 6 and 7); (b) the endosperm of Steptoe may bind or physically inactivate GA; (c) the endosperm of Steptoe may hydrolyze or chemically modify exogenous GA so that it is rendered ineffective; or (d) the aleurone of Steptoe may be very highly sensitive to the ABA remaining in the endosperm. There is partial merit in the first argument. When the GA treatments were repeated on half-seeds that were immersed in GA solution (exactly as in the aleurone treatments), the response of Steptoe half-seeds was improved beyond that observed when GA was passively absorbed from germination paper. However, the response was raised only to one-third of the Morex response (data not presented), compared with one-fourth on paper (Fig. 7). If the GA inactivation or destruction arguments apply, then it would be difficult to explain how the α-amylase genes are induced in Steptoe. High- and low-pI genes have the same developmental expression profile (at the RNA level) in Morex and Steptoe, presumably the result of GA induction.

The lack of GA responsiveness in the Steptoe aleurone, when it is associated with the starchy endosperm, represents a unique phenomenon. To date, genetic stocks of GA variants have been recognizable by altered growth habit. Thus, they display slender (Lanahan and Ho, 1988), dwarf, or even excess growth (Favret et al., 1976). The shoot of Steptoe has no growth abnormalities and appears similar to Morex and other cultivars under field conditions. It also has no GA-response growth deficiencies. After exogenous GA_3 applications, the shoot elongates slightly more than that of Morex (A. Acevedo, unpublished data). Steptoe represents perhaps the only cultivar yet identified in which one organ (the shoot) responds normally to GA_3, while another organ (the starchy endosperm-associated aleurone) has little or no GA response. This is a deceptive phenotype because the Steptoe aleurone, isolated from its endosperm, is very responsive to GA.

The chemical nature of the endosperm factor that may cause Steptoe's poor GA responsiveness is unknown. Inhibitors of α-amylase gene expression have not been extensively explored beyond ABA, bifunctional α-amylase/subtilisin inhibitor, and synthetic growth regulators. The half-seed experiments (Fig. 7) eliminated the possibility that this factor is synthesized from the embryo after imbibition. If such a factor exists, it must be one that is liberated from the starchy

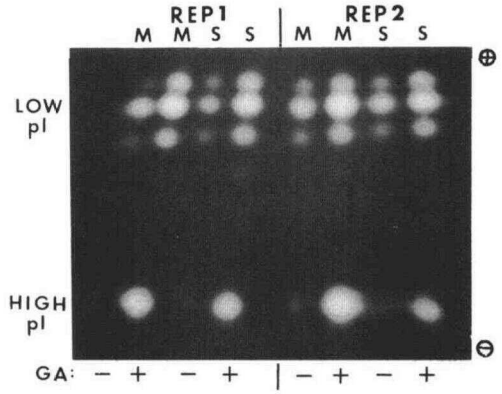

Figure 9. GA responsiveness of isolated Morex and Steptoe aleurones at the enzyme level. Extracts were prepared from aleurones treated for 24 h with GA_3 as in Figure 8. The activities of high- and low-pI α-amylases were displayed on IEF activity gels. Two experimental repetitions (rep1 and rep2) are presented to show the range of response variation. GA-treated (+) and untreated buffer control (−) aleurones are indicated beneath the figure. Samples applied to each lane represent equal fresh weights of aleurone tissue. Both Morex (M) and Steptoe (S) produced similar amounts of high- and low-pI α-amylases. (Left lanes skewed upward and to the right during electrophoresis.)

endosperm and diffuses to the cells of the aleurone. This possibility of chemical signaling from stored starchy endosperm compounds to the aleurone (either directly or as a result of feedback from enzymic activity released from the aleurone) has received little study.

The weaker temporal expression of α-amylase genes in Steptoe may also result from partial inactivation of endogenous GA. If this or other mechanisms effectively exclude active GA molecules from aleurone cells, particularly several days after germination, a factor other than GA must have the capacity to activate α-amylase genes up to the level seen in Steptoe. Whatever this factor is, it must have the capacity to overcome inhibitory effects that appear to arise from the endosperm. These studies suggest that endogenous factors other than ABA may be active as inhibitors of α-amylase gene expression in Steptoe. If such an inhibitor could be identified, it might have application to seed development and germination problems, such as preharvest sprouting in cereals.

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