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Dormancy of the Barley Grain Is Correlated with Gibberellic Acid Responsiveness of the Isolated Aleurone Layer

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ABSTRACT

The relationship between barley grain dormancy and gibberellic acid (GA_3) responsiveness of aleurone layers has been investigated. Barley (*Hordeum distichum* L. cvs Triumph and Kristina) grains were matured under defined conditions in a phytotron. Grains of Triumph plants grown under long-day/warm conditions had lower dormancy levels than grains of plants grown under short-day/cool conditions. Aleurone layers isolated from grains of long-day Triumph plants secreted more α-amylase and had a higher responsiveness to GA_3 as measured by α-amylase secretion. Storage of the grains increased both the percentage of germination and the responsiveness of the aleurone to GA_3. Use of different sterilization methods to break dormancy confirmed the correlation between germination percentage and aleurone layer GA_3 responsiveness. The response of embryoless Triumph grains to GA_3 was lower than that of the isolated aleurone layers, suggesting a role of the starchy endosperm in regulating the GA_3 response of the aleurone layer. Grains of the cultivar Kristina harvested from short day- and long day-grown plants lacked dormancy, and their isolated aleurone layers had a similar responsiveness to GA_3 as measured by α-amylase secretion. The data indicate that the physiological state of the aleurone layers contributes to the percentage germination of the grains.

During germination, the growing barley embryo synthesizes and secretes gibberellins into the starchy endosperm. Subsequently, the gibberellins diffuse into the aleurone to trigger the synthesis and secretion of hydrolytic enzymes. This classical view, reviewed in detail by Briggs (2), is based on the following observations. Isolated embryos synthesize gibberellins, whereas embryoless grains or isolated aleurone layers generally fail to synthesize hydrolytic enzymes. Addition of gibberellins or gibberellin-synthesizing embryos to half-grains or to aleurone layers induces α-amylase synthesis in a dose-dependent manner (2, 3, 13).

Synthesis of gibberellins by isolated embryos, however, does not necessarily reflect the situation in whole grains. Radley (17) shows that an isolated scutellum synthesizes gibberellins, whereas a scutellum attached to the grain does not; synthesis of gibberellins in isolated embryos can be completely inhibited by low concentrations of sugars. Moreover, the studies on correlations between level of gibberellins and α-amylase activity in the germinating grain yield different results at low and high temperatures. Groat and Briggs (6) show that in decorticated germinating grains as well as in entire husked grains, the level of endogenous gibberellins rises sharply on days 1 and 2 under malting conditions at 14.4°C and is followed by a progressive rise in α-amylase production. At 25°C and under aerobic conditions, gibberellin levels rise after the increase in α-amylase synthesis.

Trewavas (26, 27) proposes that a changing sensitivity of the aleurone cells to gibberellin is as important for the production of α-amylase during germination as the level of gibberellins, because progressively longer imbibition periods of embryoless grains results in a progressive shortening of the lag period of gibberellin-induced α-amylase formation in isolated aleurone layers (29). Furthermore, deembryonation after 1 d of germination leads to subsequent maximum α-amylase formation by the half-grains (6), and the osmotic pressure of the grain content determines the acquisition of gibberellin responsiveness because high osmotic pressure inhibits α-amylase synthesis by the aleurone layer (8).

Although it is known that the response of the barley aleurone layer to added gibberellins varies with the developmental history and genotype of the grain and with the methods used to produce embryoless grains or aleurone layers (2, 12, 25), this phenomenon is poorly understood. Investigations with dormant and afterripened *Avena fatua* grains show that the aleurone cells differ substantially in their capacity to respond to GA_3 (7, 11). The present work was designed to study the GA_3 responsiveness of barley aleurone layers from grains of a given genotype with different levels of dormancy. Grains of *Hordeum distichum* L. cvs Triumph and Kristina were obtained from plants grown in a phytotron, where different growth conditions modulated the dormancy levels in the mature Triumph grains (20). In addition, the Triumph grains were stored for different periods of time to test the effect of dormancy dissipation on GA_3 responsiveness of aleurone layers. Data are presented that suggest that both the aleurone layer and the starchy endosperm play a role in determining the dormancy level of the grain. This is, to our knowledge, the first demonstration that the GA_3 responsiveness of the aleurone layer is correlated with the dormancy level of the mature barley grain.

MATERIALS AND METHODS

Plant Material and Germination Tests

Barley grains (*Hordeum distichum* L. cvs Triumph and Kristina) were obtained from the phytotron experiment per-

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formed in 1990 (20). "Dormant" grains were harvested from SD[2] plants, which developed after flowering under a diurnal regime of 8 h of light at 14°C and 16 h of darkness at 10°C. "Nondormant" grains were from the LD plants, which were grown after flowering for 30 d in continuous light with a thermoperiodicity of 16 h at 15°C and 8 h at 10°C, after which the temperature during the 16-h period was raised to 21°C. Some of the barley grains harvested were stored at 4°C and others at −20°C, and some were dried (after 6 months at −20°C) under mild conditions (15°C, 15% RH) to a dry matter content of 94% and subsequently stored at −80°C to preserve grain dormancy (10). Germination tests were done in Petri dishes (9 cm in diameter) at 10 and 20°C in the dark (in triplicate): fifty grains were germinated on two layers of Whatman paper No. 1 with 2 mL of H_2O added (4). As described previously, the viability of all the grains used in the experiments was 100% (20).

Aleurone Preparations and Incubations

Fifty embryoless grains were allowed to imbibe in Petri dishes (9 cm) between four layers of Whatman paper No. 1 soaked with water (15 mL) containing 75 μg/mL ampicillin (Sigma) and 7.5 μg/mL nystatin (Sigma) for 64 h at 25°C in the dark. Subsequently, starchy endosperm was removed with two spatulas. The aleurone layers were incubated in 20 mM $CaCl_2$ and 1 mM sodium acetate (pH 4.8) in 24-well plates (5 layers/mL) with different concentrations of GA_3 (Sigma) and shaken (135 rpm) in the dark. Samples of the supernatant (15 μL out of 1 mL) were taken during a time course, and α-amylase activity was determined with a colorimetric assay according to the protocol of the manufacturer (Biocon) or with a microassay as described by Sirou et al. (22).

Embryoless grains were incubated in 20 mM $CaCl_2$ and 1 mM sodium acetate (pH 4.8) with different GA_3 concentrations. Crude extracts were prepared by homogenizing 10 half-grains in 4 mL of 50 mM sodium malate, 50 mM NaCl, 2 mM $CaCl_2$, and 3 mM NaN_3 (pH 5.2) at 4°C using an Ultraturrax (5 pulses at 24,000 rpm of 30 s each at 30-s intervals). The resulting homogenate was spun for 10 min at 16,000*g* in an Eppendorf centrifuge at 4°C. The α-amylase activity in the supernatant was determined.

Sterilization of the Grains

Two methods were used to sterilize the grains. In the first method, grains or embryoless grains were incubated in 70% ethanol for 1 min, washed with distilled water, and gently shaken in 50% (v/v) H_2SO_4 for 30 min for dehusking. Afterward, the grains were extensively rinsed with sterile distilled water. In the second method, embryoless grains were incubated in 70% (v/v) ethanol for 1 min, rinsed two times with 0.2% (v/v) NaOCl (sodium hypochlorite), and then incubated in 0.2% (v/v) NaOCl for 30 min (gently shaken at room temperature). The grains were rinsed with sterile (distilled) water, incubated in 10 mM HCl for 10 min, and rinsed extensively with sterile distilled water.

RNA Isolation and Northern Blots

Five to 10 aleurone layers were ground to powder in a mortar and pestle in liquid nitrogen, and total cellular RNA was isolated and purified according to the method of Slater (23). Northern blots were made by separating the RNA on a glyoxal/DMSO 1.4% agarose gel (9) and blotting it onto a nylon membrane, as suggested by the manufacturer (Genescreen Plus, DuPont). Northern blot transfers were hybridized at 65°C in 1% (w/v) SDS, 1 M NaCl, 10% dextran sulphate (Pharmacia), and 0.1 mg/mL sonicated herring sperm DNA (Boehringer) with cDNA probes specific for the high-pI α-amylase mRNA. The probes were labeled with [α-^{32}P]dCTP by the of random-priming method (Pharmacia). Plasmid pJR036 containing the pM/C α-amylase cDNA (kindly provided by Dr. John C. Rogers; 19) was used for isolation of the probe specific for the high pI α-amylase mRNA (*Bam*HI-*Hin*fI fragment). After hybridization, the blots were washed at 65°C twice in 2 × SSC (1 × SSC is 0.15 M NaCl and 15 mM sodium citrate) and 1% SDS, and finally in 0.1 × SSC, 1% SDS. The amount of ^{32}P-labeled cDNA probe hybridized to specific mRNA was determinated semiquantitatively by measuring the absorbance on autoradiographs with the use of an Ultroscan Kl densitometer (LKB). The blots were reprobed with a ribosomal RNA probe as a check for equal loading of the lanes.

RESULTS

Dormancy Levels in Triumph Grains

To compare the percentage germination of grains from plants grown under LD with that of grains from plants grown under SD, germination tests were performed at 10 and 20°C. Table I shows that the LDP grains germinated fully (100%) at 10°C but only partially (8%) at 20°C directly after harvest. The SDP grains did not germinate at either temperature. Because postharvest storage is known to minimize grain dormancy (4, 21), we determined the percentage germination after storage for 1 year at −20°C. After storage, the percentage germination of the LDP grains had increased to 76% at 20°C, whereas the percentage germination of the SDP grains had increased to 41% at 10°C (Table I).

α-Amylase Secretion by Isolated Triumph Aleurone Layers

It was of interest to determine if the differences in percentage germination between the two types of barley grains are reflected in the physiological state of the aleurone layers. Therefore, the secretion of α-amylase by isolated aleurone layers was determined (Fig. 1A). Upon incubation with 10 μM GA_3, the isolated LDP aleurone layers secreted increasing amounts of α-amylase over a period of 4 d. After storage of the grains for 1 year, the lag phase was shorter and maximum α-amylase activity was reached earlier (Fig. 1B). Isolated SDP aleurone layers from freshly harvested grains did not secrete α-amylase within the first 3 d of imbibition in 10 μM GA_3.

[2] Abbreviations: SD, short-day/cool conditions; LD, long-day/warm conditions; SDP, short day/cool conditions plant; LDP, long day/warm conditions plant; $S_{1/2}$, GA_3 concentration at which half the maximum induction of α-amylase secretion occurred.

Table I. *Concentration of GA_3 ($S_{1/2}$) for Half-Maximum Induction of α-Amylase Secretion from Aleurone Layers of Triumph and Percentage Germination of the Grains*

Different storage temperatures and treatments are compared. The data represent the mean (±SE) of two to (mainly) five experiments.

Grains	Treatment	Storage Temperature	$S_{1/2}$ GA_3	Percent Germination	
				10°C	20°C
		°C	$\times 10^{-7}$ M		
SDP	-	-	No α-amylase secreted	0	0
LDP	-	-	2 ± 1	100	8
SDP	dried	−80	No α-amylase secreted	n.d.	
LDP	dried	−80	0.50 ± 0.15	n.d.	
SDP	-	−20	2.1 ± 0.4	41	0
LDP	-	−20	0.16 ± 0.08	100	76
SDP	-	4	1.1 ± 0.4	90	0
LDP	-	4	0.10 ± 0.02	100	100
SDP	NaOCl	−20	1.0 ± 0.2	40	0
SDP	H_2SO_4	−20	0.25 ± 0.1	90	30

After 1 year of grain storage, the SDP layers did secrete α-amylase, but only after a relatively long lag phase (Fig. 1B). Neither type of aleurone layer secreted α-amylase in the absence of GA_3 (data not shown).

Similar differences between the LDP and SDP aleurone layers in rate and amount of α-amylase secreted in response to GA_3 were observed for high-pI α-amylase steady-state mRNA levels (Fig. 1B, inset). The levels of mRNA of this type of α-amylase are known to be highly stimulated by GA_3 (14, 19).

GA_3 Responsiveness of the Isolated Triumph Aleurone Layers

After 1 year of storage (at −20°C) of the Triumph grains, the GA_3 responsiveness of the isolated aleurone layers was tested (Fig. 2). The $S_{1/2}$ was about 2×10^{-8} M for the LDP aleurone layers and 2×10^{-7} M for the SDP aleurone layers. The maximum amount of α-amylase secreted by the SDP aleurone layers in response to GA_3 after 3 d reached only 40% of the level of the LDP aleurone layers.

GA_3 responsiveness of both types of aleurones increased during storage of the grains. At the time of harvest, the LDP aleurone layers had a $S_{1/2}$ of about 2×10^{-7} M, whereas the SDP aleurone layers did not respond to GA_3 concentrations of up to 1 mM after 3 d. Storage at 4°C compared to −20 and −80°C increased GA_3 responsiveness (Table I).

Effects of Grain Sterilization on Germination and GA_3 Responsiveness of the Aleurone Layers

Two widely accepted grain sterilization methods were used to test whether or not these methods could break dormancy of the SDP Triumph grains after 1 year of storage (−20°C). The percentage germination of sulphuric acid-treated SDP grains was higher than that of the nontreated grains. The grains treated with hypochlorite did not germinate better than untreated grains (Table I). Sterilization with hypochlorite did not change the GA_3 responsiveness of the SDP

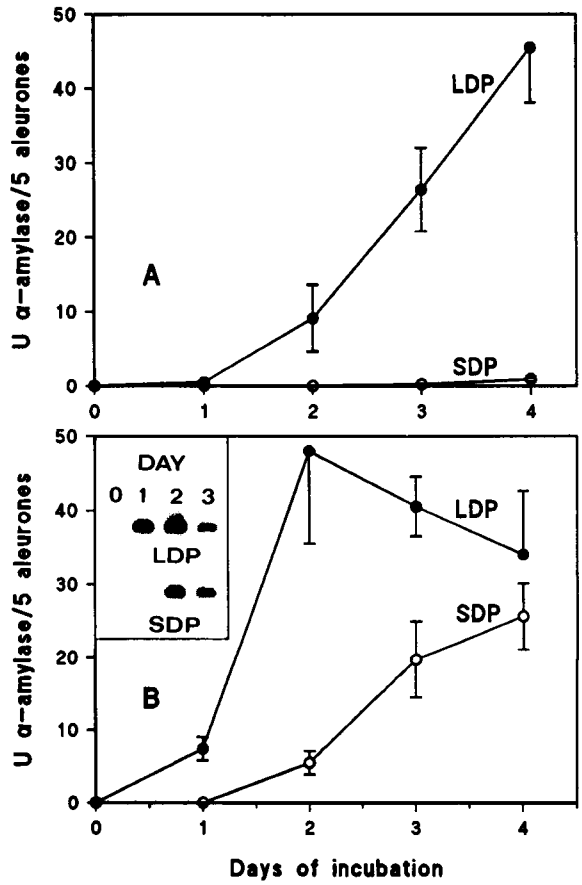

Figure 1. α-Amylase secretion by isolated aleurone layers of the SDP and LDP Triumph grains (unsterilized) incubated in 10 μM GA_3, directly after harvest (A) or after 1 year of grain storage at −20°C (B). The data represent the mean (±SE) of at least five experiments. Inset, Northern blot analysis with high-pI α-amylase probe.

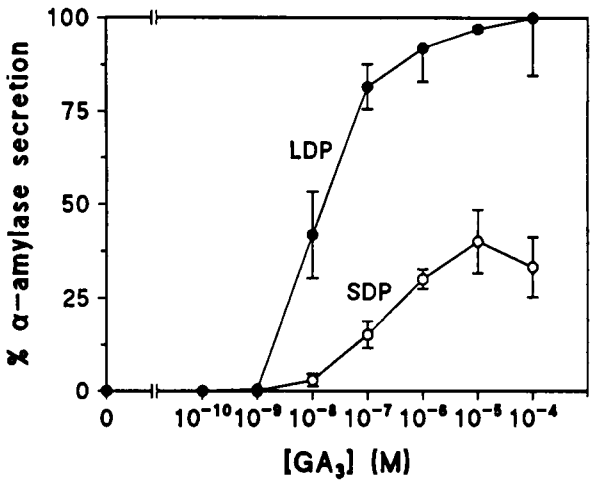

Figure 2. GA_3 concentration-dependent secretion of α-amylase by the aleurone layers of the SDP and LDP Triumph grains (unsterilized; after 1 year of storage at −20°C). The maximum amount of α-amylase secreted by the LDP aleurone layers incubated for 2 d with 100 μM GA_3 was considered 100%. For the SDP aleurone layers, the amount of α-amylase secreted after 3 d is presented. The data represent the mean (±SE) of at least five experiments.

aleurone layers significantly, but sulphuric acid treatment did increase GA_3 responsiveness (Table I).

The rate of α-amylase secretion by aleurone layers of sterilized grains upon incubation with GA_3 was changed as compared with the secretion pattern of unsterilized grains. Both sterilization methods elicited a response to GA_3 by the SDP aleurone layers prepared from grains directly after harvest. Total α-amylase secretion after sulphuric acid treatment was lower than after the hypochlorite treatment, probably because of the stringency of the dehusking treatment (50% sulfuric acid).

α-Amylase Production by Embryoless Triumph Grains

To investigate if embryoless Triumph grains respond to GA_3 in a manner similar to that of isolated aleurone layers, half-grains of dry kernels (stored for 1 year at −20°C) were incubated in 10 μM GA_3. The total production of α-amylase, comprising the activity present in the aleurone layer and in the starchy endosperm, was measured. The data in Figure 3A show that both the LDP and SDP half-grains synthesized α-amylase. The LDP half-grains produced more enzyme, with a shorter lag phase, than the SDP half-grains. The isolated aleurone layers had the same surface area as the aleurone layers of the half-grains. Therefore, the total production of α-amylase by the LDP half-grains, after 3 d, was lower than the amount of α-amylase secreted by the isolated LDP aleurone layers (Fig. 3A versus Fig. 1B). The production of α-amylase by the SDP half-grains after 3 d was about the same as the amount of α-amylase secreted by the isolated SDP aleurone layers (Fig. 3A versus Fig. 1B).

When embryoless grains were prepared from grains that were allowed to imbibe at 20°C for 64 h, instead of half-grains of dry grains, the LDP half-grains produced similar amounts of α-amylase (Fig. 3B). However, the SDP half-grains produced very low amounts of α-amylase upon incubation with 10 μM GA_3. The capacity to produce α-amylase had apparently changed during imbibition by the SDP grains.

Dormancy Levels in Kristina SDP and LDP Grains and GA_3 Responsiveness of the Isolated Aleurone Layers

The grains of the SDPs and LDPs of the barley cultivar Kristina did not show a difference in percentage germination (100% at 10 and 20°C). Nevertheless, the LDP grains germinated faster and produced larger seedlings (after 9 d) than the SDP grains. This could be due to the higher α-amylase production by the LDP aleurone layers. The Kristina grains germinated much faster (100% in 5 d at 20°C) than the Triumph LDP grains.

Isolated aleurone layers of LDP and SDP grains were also tested for their GA_3 responsiveness. The aleurone layers did not display a difference in GA_3 responsiveness as measured by α-amylase secreted ($S_{1/2}$ = 5.6 × 10^{-8} M). However, the lag phase for α-amylase secretion was longer, and the maximum amount of α-amylase secreted was lower for the isolated SDP aleurone layers compared to the LDP aleurone layers (data not shown).

DISCUSSION

Our results led us to conclude that GA_3 responsiveness of the barley aleurone layer might affect dormancy, as it has earlier been postulated to do for the physiological state of the embryo (28) and structures associated with the embryo (palea, lemma, testa, and pericarp; 21). Acquisition by cereal aleurone cells of responsiveness to gibberellins is a significant process in normal grain development and maturation. Our data indicate that GA_3 responsiveness of the isolated aleurone layers, as measured by α-amylase secretion, can be influenced by the environmental conditions during grain development and maturation and is dependent on the genotype. An increased responsiveness of the aleurone layers to GA_3 correlated with a higher percentage germination (Table I). Aleu-

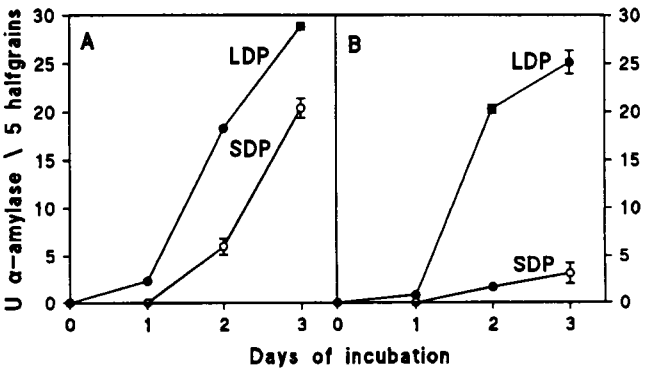

Figure 3. α-Amylase production by embryoless grains of Triumph incubated in 10 μM GA_3 (A) and by embryoless grains from Triumph grains "germinated" for 64 h at 20°C prior to incubation in 10 μM GA_3 (B). The data represent the mean of at least three experiments performed with grains stored for 1 year at −20°C.

rone layers isolated from grains of Triumph plants grown under LDP conditions responded more to GA_3 than grains matured under SDP conditions (Fig. 1); the Triumph LDP grains also had a higher percentage germination (Table I). The grains of the Kristina SDPs and LDPs displayed no difference in percentage germination, and their isolated aleurone layers had a similar responsiveness to GA_3.

Nicholls (12) reported that the amount of α-amylase produced by barley aleurone layers depends on the environmental conditions operating during grain filling and on drying temperature of ears harvested late in grain filling, which is supported by our results. However, the responsiveness of the aleurone layers from different postharvest conditions to GA_3 was found to be similar (12). The absence of differences in GA_3 responsiveness in barley aleurone, as described by Nicholls, may be due to the use of decortication by sulfuric acid.

The data in the study of Hooley (7) indicated that aleurone protoplasts of afterripened *A. fatua* grains produce more α-amylase in response to GA_3 than those of non-afterripened grains. For both type of protoplasts, however, the $S_{1/2}$ values are the same, which contrasts with our results for stored Triumph grains. This might be due to the fact that the protoplasts also produce α-amylase in the absence of gibberellins (7), suggesting a loss of metabolic control during their preparation, as noticed by Briggs (2).

The experimental results with the Triumph half-grains illuminate a gap between the in vivo assay, i.e. the germination test, and the in vitro assay, i.e. the isolated aleurone layer GA_3 response test. Aleurone layers cannot be isolated directly from dry grains, but only from (half) grains that had been allowed to imbibe, in which the starchy endosperm is present. It is striking that the embryoless Triumph SDP grains derived from grains that had imbibed for 64 h produced only very small amounts of α-amylase upon incubation with 10 μM GA_3 (Fig. 3B), in contrast to the LDP half-grains.

During the imbibition period, we observed that the starchy endosperm of the Triumph SDP grains became more friable than the LDP grains. We suggest that there was a different uptake rate and distribution of water in the SDP and LDP grains. The composition of the endosperm starch, which is both genotype and growth condition dependent (24), influences the rate of uptake and distribution of water (15, 16). The friability of the starchy endosperm of the Triumph SDP grains indicates that it is water saturated and that additional GA_3 cannot reach the aleurone layer because the pericarp and the testa-nucellar cuticle are impermeable to GA_3 (1, 5, 15). Alternatively, it cannot be excluded that a high osmotic pressure is built up in the starchy endosperm of the SDP grains, which inhibits α-amylase synthesis by the aleurone layer (8). Briggs (1) concluded that the sugary liquid of the starchy endosperm could also have a depressing effect on gibberellin synthesis by the embryo.

Because α-amylase secretion by the aleurone layer is essentially a postgerminal event (2, 21), the influence of the embryo, including the scutellar epithelium, on the germination process cannot be ignored. An in vitro test showed that all of the isolated embryos of the SDP grains germinated, but germination took 3 d longer (at 25°C) than for embryos isolated from LDP grains (data not shown). Possibly, these SDP embryos produce gibberellins more slowly than LDP embryos or even produce ABA transiently, as has been observed for dormant wheat embryos (18). It would be interesting to investigate if the scutellar epithelia of the isolated LDP and SDP embryos, where initial germination events may occur (2, 21), produce hydrolytic enzymes upon germination. Because low concentrations of glucose (5 mM) sharply reduced α-amylase production by isolated embryos (2), the significance of the role of the starchy endosperm in controlling dormancy must be clarified.

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