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Hormonal Regulation of Organic and Phosphoric Acid Release by Barley Aleurone Layers and Scutella[1]

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The release of acid from the aleurone layer and scutellum of barley (*Hordeum vulgare* L. cv Himalaya) was investigated. Aleurone layers isolated from mature barley grains acidify the external medium by releasing organic and phosphoric acids. Gibberellic acid and abscisic acid stimulate acid release 2-fold over control tissue incubated in 10 mM $CaCl_2$. Gibberellic acid causes medium acidification by stimulating the release of phosphoric and citric acids, whereas abscisic acid stimulates the release of malic acid. The accumulation of these acids in the incubation medium buffers the medium against changes in pH, particularly between pH 4 and 5. The amounts of amino acids that accumulate in the medium are low (2–12 nmol/layer) compared to other organic and phosphoric acids (100–500 nmol/layer). The scutellum does not play a major role in medium acidification but participates in the uptake of organic acids. The organic acid composition of the starchy endosperm changes after 3 d of imbibition; malic, succinic, and lactic acids decrease, whereas citric and phosphoric acids remain unchanged or increase. These results indicate that during postgerminative growth, the acidity of the starchy endosperm is maintained by acid production by the aleurone layer.

After germination of a cereal grain, the growing embryo derives nutrients from starch and protein that are stored in the starchy endosperm. These nutrient reserves are hydrolyzed by enzymes that are synthesized and secreted by the aleurone layer and scutellum. In barley (*Hordeum vulgare* L.), the aleurone layer is the principal source of secreted hydrolases, and the capacity of aleurone tissue to produce hydrolytic enzymes is regulated by GA_3 and ABA. GA_3 stimulates enzyme synthesis and secretion, and ABA generally inhibits the formation of these enzymes (Fincher, 1989; Jones and Jacobsen, 1991). The scutellum absorbs the products of hydrolysis and transports them to the embryo. Mobilization of endosperm reserves is thus a process that depends on the coordinated activities of the embryo and endosperm.

We have proposed that pH plays an important role in regulating and coordinating the activities of the endosperm (Drozdowicz and Jones, 1993). The starchy endosperm of cereal grains is maintained at pH 5 throughout most of germination and post-germinative growth (Briggs, 1968; Mikola and Virtanen, 1980), and this acidic environment is essential for the activity of secreted hydrolases. The pH optima for cereal α-amylases (Fischer and Haselbach, 1951), Cys proteases (Koehler and Ho, 1990), and RNases (Wilson, 1975; Green, 1994) are all around 4.5 to 5.0. The low pH within the starchy endosperm also inhibits the association of cereal α-amylases with endogenous inhibitors. The best studied of these inhibitors is the α-amylase/subtilisin inhibitor (Mundy et al., 1983; Weselake et al., 1983). The α-amylase/subtilisin inhibitor forms a complex with α-amylase at neutral or basic pH values and dissociates at acidic pH values (Halayko et al., 1986). Low extracellular pH may also be essential for the response of the aleurone layer to GA_3 and ABA by favoring hormone uptake or the interaction of GA_3 and ABA with receptors (Sinjorgo et al., 1993).

An acidic endosperm pH is also important for the uptake of amino acids and sugars by the scutellar epithelium. At least two peptide carriers (Salmenkallio and Sopanen, 1989; Hardy and Payne, 1991) and four distinct amino acid carriers (Sopanen et al., 1980; Nyman et al., 1983; Sopanen and Vaisanen, 1985; Vaisanen and Sopanen, 1986; Higgins and Payne, 1987) have been identified in the barley scutellum, and all depend on a proton gradient, acid on the endosperm side, to drive amino acid uptake into the scutellum. Less is known about the mechanism of sugar uptake by the scutellum, but by analogy with the uptake of sugars at the plasma membrane of other higher plants, a proton-coupled system is likely to operate (Bush, 1993).

Isolated aleurone layers of developing (Macnicol and Jacobsen, 1992) and mature barley (Mikola and Virtanen, 1980; Drozdowicz and Jones, 1993; Heimovaara-Dijkstra et al., 1994) and wheat grains (Hamabata et al., 1988) are capable of acidifying the medium in which they are incubated. The aleurone layer also plays an important role in acidifying the starchy endosperm in intact grain. In a developing grain, malic and other organic acids that are released from the aleurone layer establish the acidity of the endosperm (Macnicol and Jacobsen, 1992). Heimovaara-Dijkstra et al. (1994) showed that aleurone layers of mature grains also synthesize and release malic acid in response to ABA, but these workers were unable to identify a role for GA_3 in regulating acidification of the external medium.

In this report we examine acid release from the aleurone layers and scutella of mature barley grains and show that both GA_3 and ABA regulate the release of acids from aleurone tissue. Our results establish that GA_3 and ABA regulate the pH of the external medium in distinctly dif-

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Abbreviation: PEPC, PEP carboxylase.

ferent ways. We also show that the acid composition of the starchy endosperm changes during post-germinative growth and present evidence that this change is due to uptake of acids by the scutellum.

MATERIALS AND METHODS

Tissue Preparation and Incubation

Grains of the Himalaya cultivar of barley (*Hordeum vulgare* L.; 1985 and 1991 harvests, Agronomy Club, Washington State University, Pullman, WA) were used throughout these experiments. Whole and half grains were surface sterilized, and aleurone layers were removed from half grains as described by Jones and Jacobsen (1982).

Batches of 10 aleurone layers were rinsed briefly with distilled water and incubated under sterile conditions in 25-mL Erlenmeyer flasks. The incubation medium (2 mL) contained 5 μg mL^{-1} chloramphenicol and 10 mM $CaCl_2$ (control) and had an initial pH of about 5.4. Where indicated, 5 μM GA_3 or 10 μM ABA was added to the medium. Flasks were incubated on a rotary shaker in the dark at 23°C for the specified time.

Whole grains were germinated under sterile conditions at 25°C in the dark on filter paper in 9-cm Petri dishes containing 3 mL of water. After 3 d, the coleoptiles of germinated grains were 1 to 3 cm long. Starchy endosperm was removed from germinated grains after the embryo and distal ends of the grains were excised. A portion of the aleurone/testa/pericarp was carefully shaved off with a scalpel and the starchy endosperm was gently lifted from the remaining aleurone tissue with a spatula. The starchy endosperm was quickly frozen in liquid N_2 and stored at −80°C.

Scutella were isolated from grains germinated for 2 or 3 d by first removing the coleoptile and roots from the embryo and then teasing the scutellum away from the endosperm with a blunt spatula. Batches of 20 scutella were incubated in 1.5 mL of 10 mM $CaCl_2$ containing 5 μg mL^{-1} chloramphenicol at 23°C for 24 h on a rotary shaker in the dark.

Embryos were isolated from grains that had been germinated for 3 d and their roots were removed at their bases with a scalpel. The embryos were incubated in the dark in 5 mL of 10 mM $CaCl_2$ containing 5 μg mL^{-1} chloramphenicol and 5 μM GA_3 with and without de-embryonated half grains that had been sterilized and incubated as described above. Two replicates of 20 half grains with and without embryos were incubated per experiment, and the experiment was repeated three times.

Starchy endosperm was isolated from dry grain by removing the embryo and aleurone layer with a rice milling machine (Satake, Kewaskum, WI) and ground to a powder in a coffee grinder (Regalware Corp. [USA] Inc., Houston, TX).

α-Amylase Activity, pH, Titratable Acidity, Osmolality, and Phosphate

After incubation, the medium in which aleurone layers, half grains, or scutella had been incubated was collected and assayed for α-amylase activity using the starch-iodine method (Jones and Varner, 1967). The pH of the incubation medium was measured using a Radiometer (Logan, UT) PHM84 pH meter. Titratable acidity was determined by diluting a sample of the medium (1.5 mL) to 5 mL with distilled water and titrating with 0.1 N NaOH to pH 7. Osmolality of incubation media was measured with a vapor pressure osmometer (Wescor, Westlake, OH).

Phosphate in the incubation medium was determined using the ammonium-molybdate-semidine procedure (Gabard and Jones, 1986). Semidine reducing agent was prepared by wetting 100 mg of semidine (*N*-phenyl-*o*-phenylenediamine hydrochloride, Kodak) with 3 drops of 100% ethanol and then adding 50 mL of 1% (w/v) sodium bisulfite. The suspension was shaken for 5 min and filtered through Whatman No. 1 filter paper. The reaction mixture contained 2 mL of 10% perchloric acid, 0.2 mL of semidine reducing agent, and 0.2 mL of 6.5% (w/v) ammonium molybdate. A sample of the titrated medium (1 mL) was added to the reaction mixture, shaken, and incubated at room temperature for 20 min before the A_{625} was measured. Phosphate concentrations were determined from a standard curve created from measurements of known concentrations of phosphoric acid.

Organic Acid Analysis

Incubation Medium

Samples of incubation media (250 μL of aleurone layer incubation medium or the combined media from two flasks of scutella) were passed through a 20 × 5 mm DEAE-Sephadex A-25 anion-exchange column as described by Macnicol and Jacobsen (1992). The column was equilibrated with 5 mL of 0.5 M triethylammonium acetate (pH 5.0), and the retained anions were eluted with 4 mL of 1.5 M triethylammonium acetate (pH 5.0). The eluate was dried in a SpeedVac concentrator (Savant, Farmingdale, NY), dissolved in 150 μL of water, and passed through a 0.45-μm filter. Organic acids were separated on a Bio-Rad HPX-87H column using a Hewlett-Packard HP 1090 HPLC system and detected at 210 nm. Organic acids were identified by their characteristic retention times and by spiking samples with authentic organic acid standards.

Endosperm

Starchy endosperm (approximately 0.5 g from about 20 grains) was thawed and ground in a glass Ten Broeck (Fisher, Pittsburgh, PA) homogenizer with 5 mL of 80% methanol. The homogenate was centrifuged at 20,000*g* for 20 min, and the pellet was re-extracted with 2 mL of 80% methanol. An equal volume of water was added to the combined extract. Half of the extract was passed through a DEAE-Sephadex column as described above, dried, and dissolved in 200 to 400 μL of water. Organic acids were separated by HPLC on a Bio-Rad HPX-87H column and detected at 210 nm.

Amino Acid Analysis

Samples of incubation media and endosperm extract were diluted 1:100 and amino acids were determined as

described by Csonka et al. (1994). Free amino acids were derivatized with *o*-phthaldialdehyde, separated by reversed-phase HPLC, and quantitated by fluorescence. HPLC separation was on a Rainin (Woburn, MA) reversed-phase C-18 guard module (AA80-OPA-G3) and matching main column (AA80-OPA-C3). A Beckman 157 fluorescence detector equipped with 305- to 395-nm excitation filters and 430- to 470-nm emission filters was used to quantitate eluted peaks. Peaks were identified by comparing retention times with those of known amino acid standards.

Extraction and Assay of PEPC

Batches of 15 aleurone layers were incubated as described above for 8 and 16 h, and PEPC was extracted immediately after incubation. The tissue was ground in a Ten Broeck homogenizer with 2.5 mL of 100 mM Hepes-KOH, pH 7.5, containing 1 mM $MnCl_2$, 0.5% (w/v) BSA, and 10 mM DTT. The homogenate was centrifuged at 10,000*g* for 10 min. The supernatant was desalted on a Sephadex G-25 column (PD-10, Pharmacia) and eluted with 10 mM Hepes-KOH, pH 7.5, containing 1 mM $MnCl_2$, 0.5% BSA, and 1 mM DTT.

PEPC was assayed by measuring oxaloacetic acid reduction as described by Macnicol and Jacobsen (1992). The assay mixture contained 50 mM Tris-HCl, pH 8.3, 2.5 mM PEP, 5 mM $NaHCO_3$, 5 mM $MgCl_2$, 1 mM Glc-6-P, 4 mM DTT, 0.2 mM NADH, 1 unit of malate dehydrogenase, and enzyme extract. The reaction was conducted at 23°C in a reaction volume of 0.5 mL and was initiated by the addition of PEP. The conversion of NADH to NAD by malate dehydrogenase was followed by monitoring the absorption of NADH at 340 nm.

RESULTS

Acid Release by Isolated Barley Aleurone Layers

Acid release from isolated barley aleurone layers was examined by measuring the pH and titratable acidity of incubation media (Table I). Aleurone layers incubated in unbuffered $CaCl_2$ (control) lowered the pH of the medium from 5.4 to 3.9 and released about 800 nmol of titratable acid per layer during a 24-h incubation. Treatment of layers with either GA_3 or ABA stimulated the accumulation of titratable acid about 2-fold over controls (Table I). GA_3-induced stimulation of acid release did not lower the pH of the incubation medium significantly below that of controls; only ABA treatment was effective in lowering the pH of the incubation medium below controls (Table I). These results show that pH does not provide a reliable measure of medium acidification by aleurone layers, and that total acid release is more accurately determined by titration.

Titration profiles of incubation media showed that medium from GA_3-treated tissue had buffering capacity over a wide pH range compared to controls and buffered particularly well between pH 4 and 5. Medium from ABA-treated tissue had reduced buffering capacity between pH 5 and 9 compared to medium from GA_3-treated tissue (Fig. 1) (Drozdowicz and Jones, 1993).

Although the secretion of α-amylase from GA_3-treated aleurone layers depended on the presence of millimolar concentrations of Ca^{2+}, acid release was independent of Ca^{2+} (Fig. 2). Almost equal amounts of titratable acidity accumulated in the medium of layers incubated in 5 μM GA_3 in the presence or absence of 10 mM $CaCl_2$, but the amount of α-amylase secreted from GA_3-treated layers was less than 30% of that of layers incubated in GA_3 plus $CaCl_2$ (Fig. 2).

The time course of medium acidification shows that during the first 8 h of incubation, aleurone layers that were incubated in $CaCl_2$ released more acid than either ABA- or GA_3-treated tissue (Fig. 3, insets). After about 8 h of incubation, the amount of acid that accumulated in the medium from GA_3- and ABA-treated layers exceeded that of controls. GA_3-treated layers exhibited a sustained increase in acid release for over 24 h, but the amount of acid released from ABA-treated layers did not increase after 16 h of incubation (Fig. 3; data not shown). The time course of the GA_3-stimulated increase in titratable acidity in the incubation medium matches closely the kinetics of phosphate release from GA_3-treated barley aleurone layers (Jones, 1973).

Table I. *Effects of GA and ABA on the pH, titratable acidity, and α-amylase activity of incubation medium of isolated aleurone layers*

Aleurone layers were incubated for 24 h in 10 mM $CaCl_2$ (control), 5 μM GA_3 + $CaCl_2$ (GA), or 10 μM ABA + $CaCl_2$ (ABA). Values are presented as means ± SD (n = 6).

Treatment	pH	Titratable Acidity	α-Amylase
		nmol H^+ layer^{-1}	*units layer^{-1}*
Control	3.92 ± 0.07	797 ± 91.2	0.56 ± 0.21
GA	3.83 ± 0.05	2226 ± 166	1.90 ± 0.55
ABA	3.08 ± 0.06	2346 ± 508	0.34 ± 0.09

Analysis of Acids Released by Aleurone Layers

GA_3 and ABA had markedly different effects on the organic acid composition of the incubation medium. Citric, malic, succinic, and fumaric acids were present in incubation media from all treatments at concentrations ranging from 2 to 500 nmol/layer (Fig. 4; data not shown). Treatment of layers with ABA brought about a 300% increase in malic acid and an 80% decrease in citric acid in incubation media relative to controls. In contrast, GA_3 increased citric acid by about 350% over controls but had no significant effect on malic acid content (Fig. 4). The amount of succinic acid remained constant in all treatments (Fig. 4), and the amount of fumaric acid produced did not exceed 2 nmol/layer under any of the incubation conditions tested (data not shown). GA_3 treatment also stimulated the accumulation of phosphoric acid in the incubation medium by about 150% relative to controls. ABA, on the other hand, decreased phosphoric acid accumulation by 50% compared to controls (Fig. 4).

Amino acids accumulated in the incubation medium surrounding isolated barley aleurone layers, and aspartic and glutamic acids predominated (Fig. 5). GA_3 caused a 100%

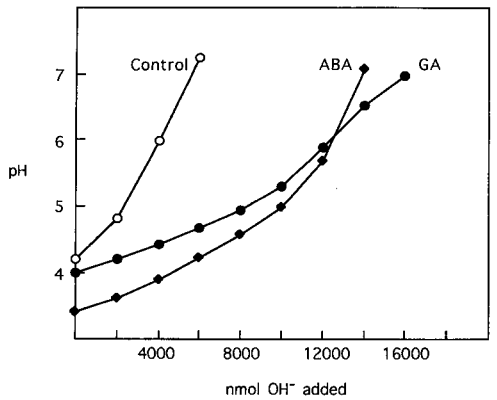

Figure 1. Titration curves of media from isolated aleurone layers incubated for 24 h in 10 mM $CaCl_2$ (control), 10 mM $CaCl_2$ plus 5 μM GA_3 (GA), or 10 mM $CaCl_2$ plus 10 μM ABA (ABA). The means of three replicates are presented. The experiment was repeated three times.

increase in Asp and a 300% increase in Glu accumulation relative to controls. ABA caused a 50% decrease in the accumulation of these amino acids (Fig. 5). Gln was detected in equal amounts in media from control and GA_3-treated layers but was not detected in the incubation medium from ABA-treated layers (Fig. 5). Glu and Asp did not exceed 11 nmol/aleurone layer under any incubation condition (Fig. 5).

Titratable Acids in the Endosperm of Dry and Germinated Grains

The organic and phosphoric acid contents of starchy endosperm from germinated grains that had imbibed for 3 d were compared with those from dry, ungerminated grains (Fig. 6). Starchy endosperm from dry grains contained high amounts of malic acid (almost 800 nmol/endosperm) and lesser amounts of citric, succinic, lactic, and phosphoric acids (Fig. 6). After 3 d of germination, malic

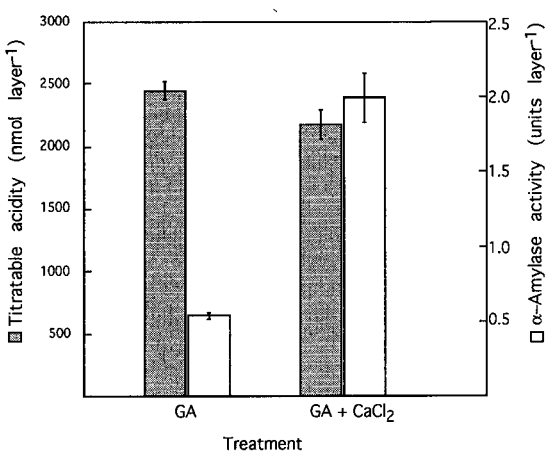

Figure 2. Titratable acidity and α-amylase activity in the incubation media from isolated aleurone layers incubated for 24 h in 5 μM GA_3 with and without 10 mM $CaCl_2$. Means ± SD of three replicates are presented. The experiment was repeated three times.

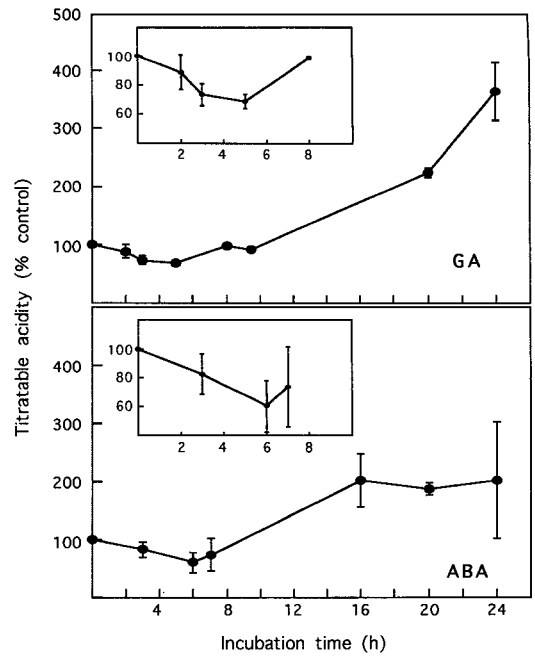

Figure 3. Time course of acidification by aleurone layers incubated in 10 mM $CaCl_2$ and 5 μM GA_3 or 10 μM ABA. Accumulated titratable acidity at each time point is presented as a percent of the acidity accumulated in medium from control tissue incubated in $CaCl_2$ without hormone. Insets show acidification during the first 8 h of incubation on an expanded scale. Means ± SD of three replicates at each time point are presented.

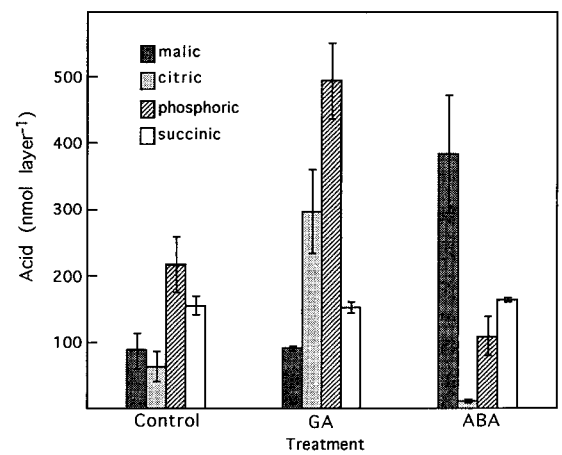

Figure 4. Phosphoric and organic acid composition of incubation media from isolated aleurone layers. Aleurone layers were incubated for 24 h in 10 mM $CaCl_2$ (control), 10 mM $CaCl_2$ plus 5 μM GA_3 (GA), or 10 mM $CaCl_2$ plus 10 μM ABA (ABA). Error bars show the SD values of three replicates from one experiment. The experiment was repeated three times.

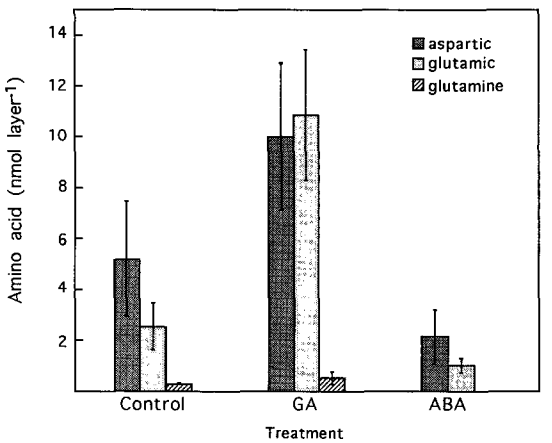

Figure 5. Amino acid composition of incubation media from isolated aleurone layers. Aleurone layers were incubated for 24 h in 10 mM $CaCl_2$ (control), 10 mM $CaCl_2$ plus 5 μM GA_3 (GA), or 10 mM $CaCl_2$ plus 10 μM ABA (ABA). Error bars show SD values of three replicates.

acid decreased by more than 97% to 25 nmol/endosperm and succinic and lactic acid also decreased, but by much less (Fig. 6). The amount of citric acid did not change, and phosphoric acid rose from 50 nmol in dry endosperm to 75 nmol in the endosperm of germinated grain (Fig. 6).

Acid Release from Isolated Scutella

Isolated scutella incubated in unbuffered $CaCl_2$ lowered the pH of the incubation medium to 4.13 and released 30 nmol of titratable acid per scutellum (Table II). Treatment of scutella with GA_3 and ABA had no effect on the amount of titratable acid that accumulated in the incubation medium (Table II), but these hormones had a pronounced qualitative effect on the organic acid composition of the medium (Fig. 7). Incubation media from all treatments contained citric, succinic, and lactic acids. GA_3 treatment caused a 100% increase in citric acid compared to controls,

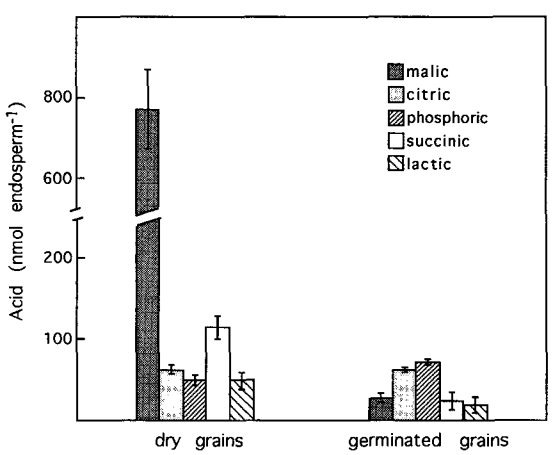

Figure 6. Phosphoric and organic acid composition of starchy endosperm from perled dry grains (dry grains) and from germinated whole grains that had been allowed to imbibe for 3 d (germinated grains). Means ± SD of three replicates are presented.

Table II. *Effects of GA and ABA on the pH, titratable acidity, and α-amylase activity of incubation medium of isolated scutella*

Scutella were incubated for 24 h in 10 mM $CaCl_2$ (control), 5 μM GA_3 + $CaCl_2$ (GA), or 10 μM ABA + $CaCl_2$ (ABA). Values are presented as means ± SD (n = 4).

Treatment	pH	Titratable Acidity	α-Amylase
		nmol H^+ scutellum^{-1}	*units scutellum^{-1}*
Control	4.13 ± 0.06	29.5 ± 3.00	0.50 ± 0.18
GA	4.13 ± 0.04	37.6 ± 4.02	0.69 ± 0.19
ABA	4.31 ± 0.28	22.6 ± 4.09	0.30 ± 0.08

whereas ABA treatment decreased citric acid accumulation by 85% and stimulated lactic acid accumulation 300% over controls (Fig. 7). Succinic acid release from scutellum tissue was not affected by either treatment (Fig. 7).

Acid Release by De-Embryonated Half Grains and Embryos

De-embryonated half grains were incubated alone or in the presence of isolated embryos whose roots had been removed. After incubation, pH, titratable acidity, α-amylase activity, and osmolality of media were measured (Table III). Half grains lowered the pH of the medium to 4.3 and produced 16 units of α-amylase activity in both the presence and absence of embryos (Table III). Much less (33%) titratable acidity accumulated in the medium when half grains were incubated in the presence of embryos, however (Table III). Embryos also reduced the final osmolality of the medium from about 252 to 211 mmol/kg (Table III).

PEPC Activity in Aleurone Layers

Isolated aleurone layers incubated for 8 and 16 h in 10 mM $CaCl_2$ contained 40.6 and 62.6 nmol min^{-1} layer^{-1} PEPC activity, respectively. These activities were comparable to those of developing aleurone layers (Macnicol and Jacobsen, 1992). Treatment of aleurone layers for 8 or 16 h

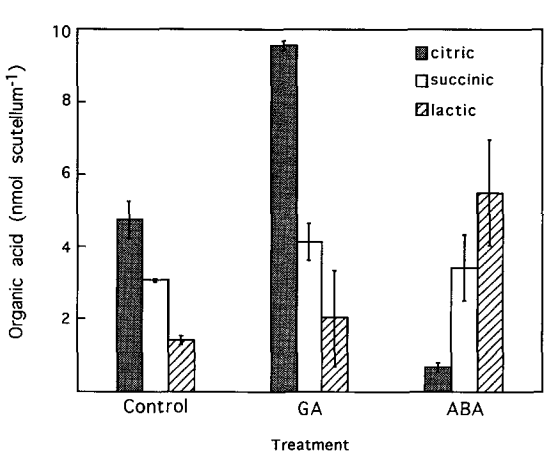

Figure 7. Organic acid composition of incubation media from isolated scutella. Scutella were incubated for 24 h in 10 mM $CaCl_2$ (control), 10 mM $CaCl_2$ plus 5 μM GA_3 (GA), or 10 mM $CaCl_2$ plus 10 μM ABA (ABA). Data from two experiments are shown.

Table III. *pH, titratable acidity, amylase activity, and osmolality of incubation medium from half grains with and without embryos*

Half grains were incubated alone (− embryos) or with an equal number of embryos (+ embryos) for 24 h in 10 mM $CaCl_2$ and 5 μM GA_3. Values are presented as means ± SD (n = 5).

Tissue(s)	pH	Titratable Acidity	α-Amylase	Osmolality
		μmol H^+	*units mL^{-1}*	*mmol kg^{-1}*
− Embryos	4.26 ± 0.08	57.0 ± 4.9	15.7 ± 0.38	252 ± 10.0
+ Embryos	4.36 ± 0.09	19.1 ± 2.9	15.4 ± 3.50	211 ± 9.40

with either 10 μM ABA or 5 μM GA_3 had no effect on PEPC activity.

DISCUSSION

Our results show that aleurone layers isolated from mature barley grains modify the pH of their environment by releasing organic and phosphoric acids. ABA and GA_3 both stimulate medium acidification by isolated aleurone layers, but they do so in different ways. Whereas ABA causes medium acidification by stimulating the synthesis and release of malic acid, confirming the observations of Heimovaara-Dijkstra et al. (1994), GA_3 causes the aleurone layer to release citric and phosphoric acids. The organic acid composition of the starchy endosperm also changes following 3 d of germination and seedling growth, and we present evidence that this change is a result of acid production by the aleurone layer and uptake of these acids by the scutellum.

GA_3 and ABA Regulate Acid Release by Aleurone Layers

Isolated aleurone layers lower the pH of their incubation medium by releasing phosphoric, malic, citric, and succinic acids (Fig. 4). GA_3 and ABA regulate the production of these acids and so alter the buffering properties of incubation media (Figs. 1 and 3). The pKs of the acids that accumulate in the incubation media from GA_3-treated layers (phosphoric acid, pKs of 2.1, 7.2, and 12.3; citric acid, pKs of 3.1, 4.8 and 6.4) confer buffering capacity over a wide range of pH values (Fig. 1) (Drozdowicz and Jones, 1993). Treatment with ABA reduces the amounts of phosphoric and citric acids in the medium but stimulates the production of malic acid. The carboxylic acid groups of malic acid have pKs of 3.4 and 5.1, giving the medium from ABA-treated layers strong buffering capacity between pH 4 and 5 but little buffering strength at higher pH values. These differences in anionic composition and buffering capacity may account for the differences in final pH of the incubation media after the various treatments (Fig. 1; Table I). GA_3 and ABA also regulate the amount of amino acids produced by the aleurone layer, but the amounts of amino acids in the incubation medium are low relative to the other organic acids.

GA- and ABA-induced shifts in aleurone metabolism could account for the lag period in the time course of acidification by aleurone layers (Fig. 3). Treatment of aleurone layers with GA_3 or ABA stimulates acid release after 8 h of incubation, but before this time, hormone-treated layers produce less acid than do controls. Hormone-induced metabolic shifts may result in decreased rates of acidification as new proteins and metabolites are synthesized from the compounds that might otherwise have been released into the medium. These changes would likely occur during the first 8 h of incubation, since many GA_3- and ABA-induced changes in gene transcription and protein synthesis occur within the first 4 to 8 h of incubation (Chandler et al., 1984; Deikman and Jones, 1986; Hammerton and Ho, 1986; Lin and Ho, 1986).

Degradation of phytate (K, Mg salt of inositol hexaphosphate) stored in protein-body vacuoles of the aleurone cell is likely the source of the phosphoric acid that accumulates in the incubation medium. The release of phosphate and cations from GA_3-treated barley aleurone tissue (Jones, 1973; Clutterbuck and Briggs, 1974) and the accumulation of these ions in the starchy endosperm of germinated wheat (Eastwood and Laidman, 1971) is well documented. GA_3 also stimulates the synthesis of acid phosphatase in the aleurone layer of Himalaya barley (Ashford and Jacobsen, 1974; Gabard and Jones, 1986), wheat (Akiyama et al., 1981), and wild oat (Hooley, 1984). In Himalaya barley several isoforms of acid phosphatase have phytase activity, and the activities of some of these phosphatases are increased by GA_3 (Gabard and Jones, 1986). The reduced amount of phosphoric acid in incubation media from ABA-treated aleurone layers is probably a result of suppressed phytate hydrolysis. Light microscopy and EM show that protein body vacuoles and their contents, including phytate, are not mobilized in aleurone layers or protoplasts that have been incubated with ABA (P. Bethke, S. Hilmer, R. Jones, unpublished data).

The organic acids produced by aleurone layers may be the end products of lipid metabolism via the glyoxylate cycle. Aleurone layers from mature barley grains store large amounts of neutral lipid in oleosomes, and glyoxylate-cycle enzymes are present in glyoxysomes in aleurone cells (Jones, 1972). The activities of glyoxylate-cycle enzymes increase after incubation in GA_3 (Jones, 1972; Newman and Briggs, 1976), and Suc synthesis by aleurone layers after GA_3 treatment indicates increased gluconeogenesis (Chrispeels et al., 1973). There is clearly a flux of carbon from lipid through the glyoxylate and citric acid cycles in aleurone cells, and the organic acids in the incubation medium surrounding this tissue could be derived from lipid breakdown.

Lipid metabolism is less active in de-embryonated grains (Newman and Briggs, 1976) and in aleurone layers isolated

from de-embryonated grains (Jones, 1972). Although lipid metabolism has not been studied in ABA-treated aleurone layers, by analogy with de-embryonated grains it is likely that aleurone layers incubated in ABA have reduced activities of enzymes that convert lipids to sugars. However, this lower rate of lipid metabolism may suffice to account for the production of malic acid by ABA-treated aleurone layers.

Aleurone layers may also produce organic acids along the glycolytic pathway. For example, malic acid could be produced in aleurone tissue via PEPC. The presence of PEPC in aleurone layers isolated from mature barley grains is consistent with the observation that this enzyme is active in developing fruits and seeds of many species (reviewed by Lepiniec et al., 1994; Podesta and Plaxton, 1994). The capacity of the aleurone layer of a developing grain to synthesize malate is associated with an increase in PEPC activity in the aleurone/testa/pericarp (Macnicol and Jacobsen, 1992). In aleurone layers of mature grains, PEPC activity is not affected by either GA_3 or ABA and so cannot be correlated with increased malate production by ABA-treated layers (Fig. 4). It is not known if PEPC plays a direct role in grain development or germination.

Little is known about the mechanisms that regulate organic acid synthesis in plant cells. There is general agreement, however, that malic acid plays a role in regulating cytoplasmic pH as well as serving as a key metabolic intermediate (Davies, 1986; Kurdjian and Guern, 1989; Martinoia and Rentsch, 1994). Recently, Heimovaara-Dijkstra et al. (1994) proposed that malate accumulates in aleurone cells in response to ABA-induced cytoplasmic alkalinization. They showed that ABA increased intracellular malic acid in aleurone cells and proposed that the resulting cytosolic acidification may lead to the release of malate. The mechanism of malate release from plant cells is only beginning to be understood. By analogy with malate transport from stomatal guard cells, transport of malate across the plasma membrane might be expected to occur via an anion channel (Martinoia and Rentsch, 1994).

There are other reports indicating that extracellular malic and citric acids play an important physiological role in plants (Martinoia and Rentsch, 1994). For example, Hoffland et al. (1992) showed that citric and malic acids are secreted by rape roots to enhance phosphate mobilization after phosphate starvation. We speculate that the organic and phosphoric acids released by barley aleurone cells also play an important extracellular role. In the case of the cereal endosperm, acids may buffer the pH of the starchy endosperm to favor hydrolase activity, promote dissociation of α-amylase and protease inhibitors, and provide the proton gradient necessary for the uptake of amino acids and sugars by the scutellum and the uptake of ABA and GA_3 by the aleurone layer.

Organic Acids and pH in the Starchy Endosperm

The aleurone layer releases a variety of organic acids, principally malic acid, into the starchy endosperm during grain maturation, which lowers the pH of this compartment to around 4 (Macnicol and Jacobsen, 1992). The starchy endosperm of dry, mature grain is also acidic and remains so during and after germination (Fig. 6) (Briggs, 1968; Mikola and Virtanen, 1980). Our results show that the amount of malic acid is high in dry grains, indicating that it is predominantly malic acid produced by the aleurone layer of the maturing grain that accounts for the low pH of the starchy endosperm of the mature grain (Fig. 6). During post-germinative growth, the amounts of some organic acids drop (malic, lactic, succinic), but the change in malic acid content is especially large. Thus, during the first 3 d after imbibition, malic acid falls from about 800 nmol/endosperm to about 30 nmol/endosperm (Fig. 6). Citric and phosphoric acids, on the other hand, either remain the same (citric) or increase (phosphoric) (Fig. 6). The depletion of malic acid in the endosperm after germination is likely due to uptake by the scutellum (Table III) and reduced malic acid synthesis by the aleurone layer (Fig. 4). The maintenance of an acidic pH in the endosperm after germination must therefore result from the production of citric and phosphoric acids by the aleurone layer (Fig. 4). It is unlikely that the scutellum contributes significantly to the organic acid pool in the endosperm, since an isolated scutellum produces only about one-fiftieth the amount of titratable acidity as an aleurone layer (compare Tables II and III).

The Role of the Scutellum

Our results show that the scutellum does not play a major role in endosperm acidification. Rather, this tissue transports acids and the products of starchy endosperm hydrolysis from endosperm to the embryo (Table III) (Fincher, 1989). When isolated embryos were incubated with de-embryonated half grains, the amount of acidity in the incubation medium decreased (Table III). The importance of protons for the uptake of amino acids and peptides by barley scutellum has been shown by several investigators (Sopanen et al., 1980; Nyman et al., 1983; Sopanen and Vaisanen, 1985; Vaisanen and Sopanen, 1986; Higgins and Payne, 1987; Salmenkallio and Sopanen, 1989; Hardy and Payne, 1991), and we presume, by analogy with proton co-transport mechanisms described for sugar uptake in other plant tissues, that protons are essential for the uptake of sugars by the scutellum (Bush, 1993). The loss of acids from the endosperm during post-germinative growth and after incubation of embryos with half grains could therefore be accounted for by uptake at the scutellar epithelium. The precise mechanism of organic anion uptake, the fate of these metabolites, and their importance in embryo and seedling growth are prospects for future investigations.

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