Effects of fusicoccin and gibberellic acid on the germination of embryos from dormant barley grains: roles of starch degradation and external pH

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Abstract

In isolated embryos from dormant barley grains, synergistic effects of fusicoccin (FC) and gibberellic acid (GA_3) were observed on the induction of α-amylase mRNA expression. However, no α-amylase mRNA expression could be induced by both agents in embryos from non-dormant grains. Both light- and electron-microscopy studies demonstrated that there were large numbers of starch granules present in mature embryos (mainly in scutellum) from dormant barley grains but none or almost none in embryos from non-dormant grains. Furthermore, the content of reducing sugars in embryos from dormant grains was about half of that from non-dormant grains. In contrast to GA_3, FC was able to induce a strong acidification of extracellular pH (pH_e). Clamping the pH_e to prevent FC-induced acidification, by using 50 mM MES buffer (pH 5.6), caused an inhibition of GA_3- or FC-induced α-amylase mRNA expression but did not affect the germination of embryos from dormant grains. In addition, in MES buffer, addition of FC or a combination of FC and GA_3 increased the germination rate of embryos isolated from dormant grains, though large numbers of starch granules were still present in these embryos. Based on these observations, the presence of starch granules and a low reducing sugar level in embryos from dormant grains is not a factor for control of grain dormancy and germination.

Keywords: α-amylase, dormancy, embryo germination, fusicoccin, gibberellic acid, *Hordeum distichum*, pH, starch degradation

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Introduction

In our previous studies, we classified diverse dormancy-breaking compounds into two groups according to their effects on endogenous abscisic acid (ABA) content (Wang *et al*., 1998a). Class I compounds affect the level of endogenous ABA, while class II compounds have no effect on ABA content. Fusicoccin (FC), a toxin produced by the fungus *Fusicoccum amygdali*, is able to break the dormancy of intact barley grains and to stimulate the germination rate of embryos isolated from dormant grains (Lado *et al*., 1974; Galli *et al*., 1975; Wang *et al*., 1998a). FC is a class II compound and induces a wide spectrum of physiological responses in plants including extracellular acidification (Marrè, 1979; Muromtsev, 1996; De Boer, 1997; Wang *et al*., 1998a). Gibberellic acid (GA_3) stimulates seed germination (Bewley and Black, 1982, for references) and is a class I dormancy-breaking compound based on our previous studies (Wang *et al*., 1998a). Acidic external pH (pH_e) is able to enhance GA-induced biological responses such as aleurone α-amylase production (e.g. Karssen, 1976; Sinjorgo *et al*., 1993). Therefore, one goal of this study was to determine if the external acidification induced by FC enhances the function of endogenous GA in isolated barley embryos from dormant grains. Furthermore, we investigated the effects of FC and GA_3 on embryo germination and α-amylase mRNA expression.

In barley grains, dormancy can be induced by variation in growth conditions, although it is also genotype dependent (Schuurink *et al*., 1992). The growth condition-induced dormant barley grains are not able to germinate even under favourable conditions (Schuurink *et al*., 1992; Van Beckum *et al*., 1993). Since variations in growth conditions are able

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to cause dormancy in certain barley genotypes, it is likely that the effects of growth conditions are dependent on developmental stages. In the present work, a comparative study on dormant and non-dormant barley grains revealed a difference in starch granule accumulation in mature dormant and non-dormant embryo scutellar tissue. In addition, we evaluated the possibility that the large number of starch granules in the dormant scutellum had a causal association with the dormant state.

In various plant species, including some cereals, the initial stage of radicle emergence utilizes soluble carbohydrates (Bewley and Black, 1994; Black *et al.*, 1996). In mature wheat embryos, no starch granules are seen (Swift and O'Brien, 1972), while they are observed in developing embryos (Smart and O'Brien, 1983). In the mid–late development stage of wheat, increases in both the amount of starch and α-amylase activity in the embryo have been observed. However, there is only a very low level of α-amylase as compared to the aleurone during germination (Black *et al.*, 1996). It was proposed that this developmentally regulated starch accumulation/degradation and α-amylase activity are related to the desiccation tolerance in embryos during maturation (Leprince *et al.*, 1990), which subsequently may affect the germination capacity of embryos. Therefore, a comparative study of sugar content and α-amylase in the embryos from dormant and non-dormant grains was performed in order to understand the effect of FC and GA on the function of scutellar tissue. Furthermore, the roles of starch degradation in the embryo and the effect of the pH_e on the regulation of germination were investigated.

Materials and Methods

Chemicals

[α-^{32}P]-dCTP (3000 Ci/mmol) was obtained from Amersham (UK). Gene Screen plus was from Dupont (USA). Dextran sulphate was from Pharmacia Biotech (Upsala, Sweden). FC was obtained from Dr A.H. de Boer (Institute for Molecular Biological Sciences, BioCentrum, Vrije Universiteit Amsterdam, The Netherlands). GA_3 (chemical purity 99%) and all other chemicals were from Sigma Chemical Co. (St. Louis, USA).

Barley grains and germination test

Hordeum distichum L. cv. Triumph dormant and non-dormant grains were produced in a phytotron under previously described conditions (Schuurink et al., 1992). Grains were obtained by first growing barley plants at 16 h at 25°C/8 h at 18°C under continuous light for 56 days. Then conditions were changed to produce dormant (8 h light at 15°C and 16 h darkness at 10°C) and non-dormant (24 h light at 21°C and 8 h darkness at 10°C) grains until maturity. Grains were harvested at full ripeness and dried to 7% moisture content in an equilibrium drying cabinet at 12°C and then stored at −20°C to preserve dormancy.

In germination tests of isolated embryos, 10 embryos (in duplicate) were placed in 24-well plates containing 300 µl H_2O or in the presence of GA_3, FC or a combination of both. Plates were incubated at 20°C for 24 h in the dark. Embryos were considered as germinated when the radicles were ≥ 1 mm. In all germination experiments the incubation was prolonged to ensure the observation of growth of young seedlings.

Mean values ± SD of at least three independent experiments are presented unless stated otherwise. Significance differences in mean values were tested with the Student's *t*-test.

Analysis of α-amylase mRNA expression and activity

Ten embryos from dormant or non-dormant barley grains were excised and incubated in 300 µl H_2O, with or without GA_3 and/or FC in 24-well plates in the dark at 20°C for 24 h. As a control, 10 isolated embryos were incubated in 300 µl H_2O with the same amount of carrier (0.07% ethanol) for GA_3 and FC. Total cellular RNA was isolated, purified and analyzed by Northern blotting as described by Wang *et al.* (1992). Hybridization was performed in 1% SDS, 1 M NaCl, 10% dextran sulphate and 0.1 mg ml^{-1} sonicated salmon sperm DNA with random-primed labelled cDNA hybridization probes. The cDNA probe, which recognizes both high pI and low pI α-amylase, is a 0.8 kb SacI fragment encoding the C-terminal half of α-amylase cDNA clone PM/C (accession number K02637, Rogers, 1985). The α-amylase activity measurement was essentially the same as previously described by Sinjorgo *et al.* (1993), except that isolated embryos (20/assay) were used.

Measurements of pH and reducing sugar assay

The pH was measured with a Beckman (Fullerton, USA) Φ 100 ISFET pH meter, especially designed for small volume measurements. The pH meter was calibrated prior to every measurement cycle. Each pH_e measurement was carried out quickly (less than 2 min/determination) and without vigorous stirring. No significant changes in pH_e of incubation solutions during the 2 min measurement were observed with or without embryos present. The reducing sugar assay was carried out as described by Somogyi (1952). The Petri dishes containing samples were sealed with parafilm to prevent evaporation.

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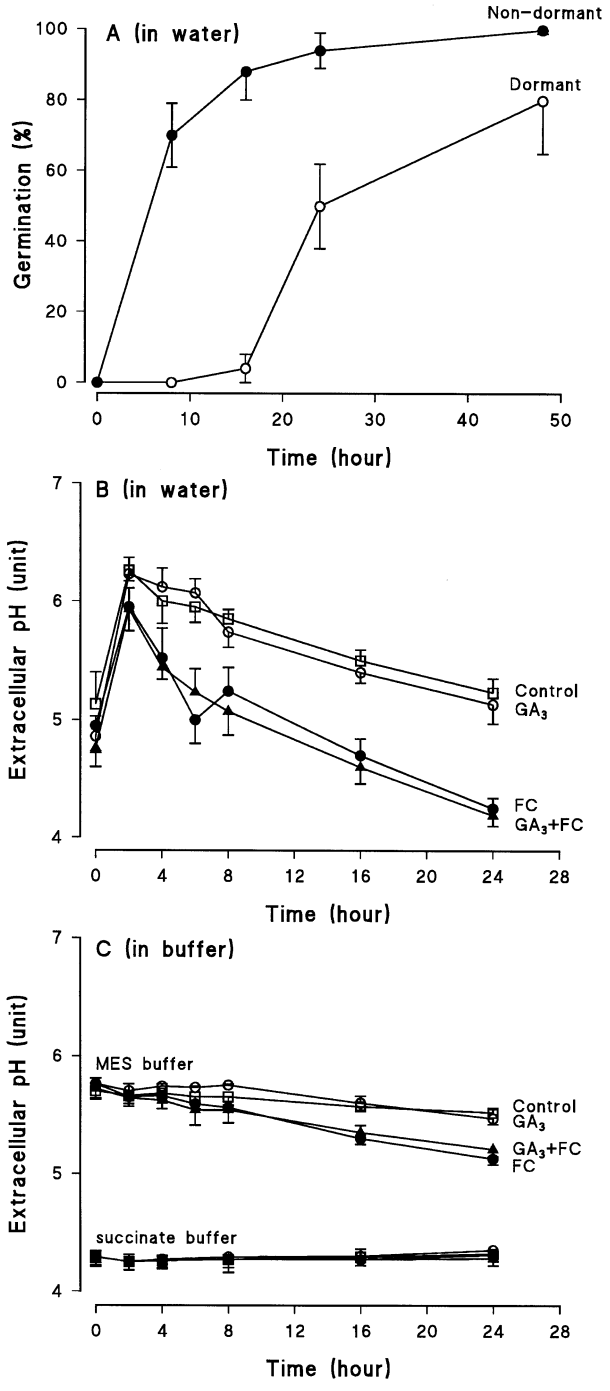

Figure 1. Germination of isolated embryos from both dormant and non-dormant barley grains (A) and effect of FC and GA_3 on external pH for embryos isolated from dormant grains (B, C). Ten embryos isolated from dormant barley grains (in duplicate) were incubated in 300 µl water (B), 50 mM MES buffer (C, pH 5.6) or 50 mM succinate (C, pH 4.2) containing GA_3 (10^{-6} M), FC (10^{-5} M), GA_3 (10^{-8} M) + FC (10^{-5} M) or the carrier (0.07% ethanol) of GA_3 or FC (control) at 20°C in darkness. At different time points, germination was determined and the external pH was measured. Means ± SD of six independent experiments are presented.

Light and electron microscopy

Isolated barley embryos were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate (pH 7.2) for 8 h at room temperature (fixation protocol: In situ Cell Death Detection kit, Boehringer Mannheim product No. 1684795). Subsequently, the embryos were rinsed (2 h) and post-fixed with 1% osmium tetroxide in the same buffer (2 h). After washing in 0.1 M sodium cacodylate and dehydration in 70, 80, 90 and 96% (v/v) ethanol for 30 min each, the embryos were dehydrated further in 100% ethanol for 2 × 30 min. After impregnation with propylene oxide for 2 × 15 min, the samples were passed through a propylene oxide/Epon mixture. The tissue was left in pure Epon overnight before embedding in flat silicon rubber moulds. The Epon was polymerized at 60°C for 48 h.

Ultra-thin cross-sections of the embryos were made using a Reichert-Jung Ultracut-E microtome equipped with a diamond knife. The sections were stained with 2% uranyl acetate in 50% ethanol and 0.4% lead citrate solutions for 10 min each. The sections were examined using both a light microscope (Leitz Laborlux) and a Jeol 100 CX electron microscope operating at 60 kV.

Results

Effects of GA_3 and FC on pH_e in embryos isolated from dormant grains

Intact dormant barley grains were not able to germinate at 20°C in the dark. However, embryos isolated from these grains were able to germinate although with a delayed germination time as compared to embryos from non-dormant grains (Fig. 1A). After 16 and 24 h of incubation, there was no difference in germination kinetics when embryos were incubated in water or in 50 mM MES. However, when embryos were incubated in 50 mM succinate, delayed germination rates were observed (data not shown).

During incubation in water an initial increase in pH_e was observed at 2 h of incubation (Fig. 1B). This was followed by a decrease of pH, and after 24 h of incubation, the pH_e almost equalled the starting value (pH 5.3). In the presence of 10^{-6} M GA_3, the variation in pH_e was about the same as for the control. Addition of 10^{-5} M FC caused a stronger decrease in pH_e (4.2) as compared to controls. This also holds for the combination of FC with 10^{-8} M GA_3 (Fig. 1B). When only 10^{-8} M GA_3 was used, the same pH_e changes as in the control were observed (data not shown). In 50 mM MES buffer (pH 5.6) or 50 mM succinate buffer (pH 4.3), pH_e remained almost constant during 24 h of incubation (Fig. 1C).

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Since FC caused a decrease in pH_e (Fig. 1B), the question was raised if changes in pH_e affected the germination behaviour of isolated embryos. In water, there was less than 10% germination after 16 h of incubation (Fig. 2A). Under the same conditions, 10^{-8} M and 10^{-6} M GA_3 were able to induce about 40% and 70% germination, respectively; 10^{-5} M FC was able to induce about 50% germination and the combination of 10^{-8} M GA_3 and 10^{-5} M FC induced about 100% germination (closed bars, Fig. 2A). Artificial clamping of pH_e at 5.6 with 50 mM MES buffer had no significant effect on the action of GA_3 and FC on germination of isolated embryos (open bars, Fig. 2A). Clamping pH_e at 4.3 with 50 mM succinate buffer showed the same tendency in germination but at a significantly reduced germination percentage in most conditions as compared to in water (hatched bars, Fig. 2A). As a control, non-dormant embryos were used under the same conditions and showed that buffers had no negative effects on germination (Fig. 2B).

Effects of GA_3 and FC on α-amylase mRNA expression

After 24 h of incubation, in embryos isolated from non-dormant grains, GA_3 (even at a concentration of 10^{-4} M) was not able to induce α-amylase gene expression (Fig. 3A). In embryos isolated from dormant grains, 10^{-6} M GA_3 was able to elicit maximum induction of α-amylase mRNA expression (Fig. 3A). In the presence of 10^{-5} M FC, the maximum induction in α-amylase mRNA expression in embryos from dormant grains was already reached at 10^{-8} M GA_3 (Fig. 3A). With addition of FC, 10^{-4} M GA_3 showed a reduced expression of α-amylase mRNA. Nevertheless, FC was able to make isolated embryos about 100 times more sensitive to GA_3 with regard to GA-induced α-amylase mRNA expression. Addition of FC alone also induced some α-amylase mRNA expression in embryos from dormant grains (Fig. 3B). The optimal stimulative effect of FC was at 10^{-5} M (Fig. 3B). However, combinations of FC and GA_3 were not able to induce the expression of α-amylase in embryos isolated from non-dormant grains. A time course of GA_3-induced (10^{-6} M) α-amylase mRNA expression was made for embryos isolated from dormant grains. At this GA_3 concentration, α-amylase mRNA expression was first observed after 16 h (75% germination, Fig. 2A) and reached a maximal expression level after 24 h incubation. Addition of 10^{-5} M FC had no effect on the time course of α-amylase mRNA expression (data not shown). The α-amylase enzyme activities were substantially greater in dormant (7.00 ± 0.11 U/embryo) than in non-dormant (0.22 ± 0.02 U/embryo) embryos treated for 24 h with 10^{-6} M GA_3.

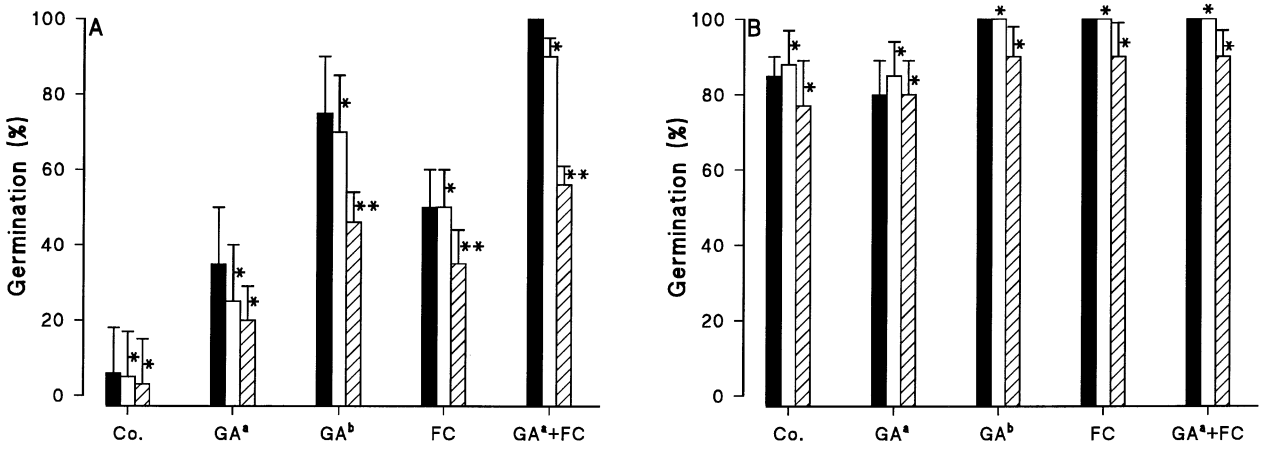

Figure 2. Effects of GA_3, FC and media pH on the germination of embryos from dormant (A) and non-dormant (B) grains. Ten embryos (in duplicate) were incubated in water (closed bars), 50 mM MES buffer, pH 5.6 (open bars) or 50 mM succinate buffer, pH 4.2 (hatched bars) containing Co = control: water or buffer; GA^a: GA_3 (10^{-8} M); GA^b: GA_3 (10^{-6} M); FC: FC (10^{-5} M) or GA^a +FC: GA_3 (10^{-8} M) + FC (10^{-5} M). After 16 h of incubation at 20°C in darkness, germination of different samples was determined and samples were collected for Northern analysis. The means ± SD from three independent experiments are presented. *Mean values not significantly different ($P > 0.95$) from value obtained under corresponding conditions in water. **Mean values significantly different ($P < 0.05$) from value obtained under corresponding conditions in water.

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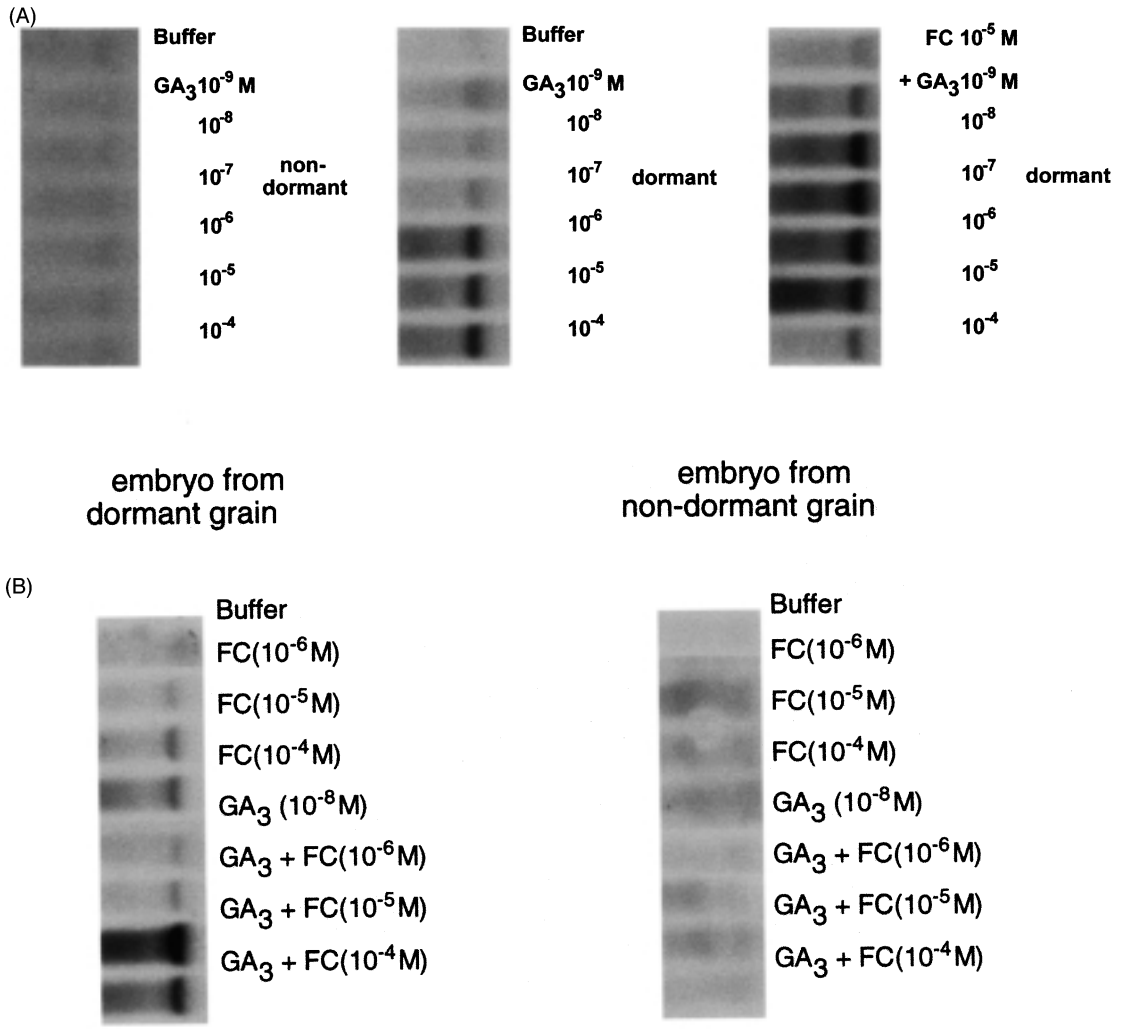

Figure 3. Effects of GA_3 and FC on α-amylase mRNA expression in isolated embryos from dormant and non-dormant grains. (A) A series of GA_3 concentrations ranging from 10^{-9} to 10^{-4} M was used for incubation of embryos in the presence or absence of FC (10^{-5} M). (B) Effects of FC on α-amylase mRNA expression in embryos isolated from dormant and non-dormant grains. In the presence or absence of GA_3 (10^{-8} M), a series of FC concentrations ranging from 10^{-6} to 10^{-4} M was used. In each experiment, 10 dormant or non-dormant embryos were incubated in 300 µl buffer in a 24-well plate at 20°C in darkness for 24 h. Subsequently, the samples were collected for Northern analysis. In this figure, one representative experiment is presented from at least three independent experiments.

Delayed starch catabolism in embryos from dormant grains

Experimental results described above point to the possibility that the effect of FC on germination is via an induction of α-amylase mRNA. Since the function of α-amylase is to degrade the starch, it is possible that degradation of starch is essential to trigger germination. It is likely that the substrates of α-amylase in embryos are the starch granules accumulated during development. Since α-amylase mRNA could only be induced in embryos from dormant grains, we were interested in whether there were any differences in starch accumulation in embryos from dormant and non-dormant barley grains. Embryos were isolated from intact dormant and non-dormant grains which had been imbibed for 4 h and prepared for both light- and transmission-electron microscopy. Light-microscopy studies of embryos from dormant grains detected relatively large numbers of starch granules in the scutellum (Fig. 4B), and a lower number of starch granules was also found in the germ-aleurone (data not shown). However, very little or almost no starch granules were found in any of

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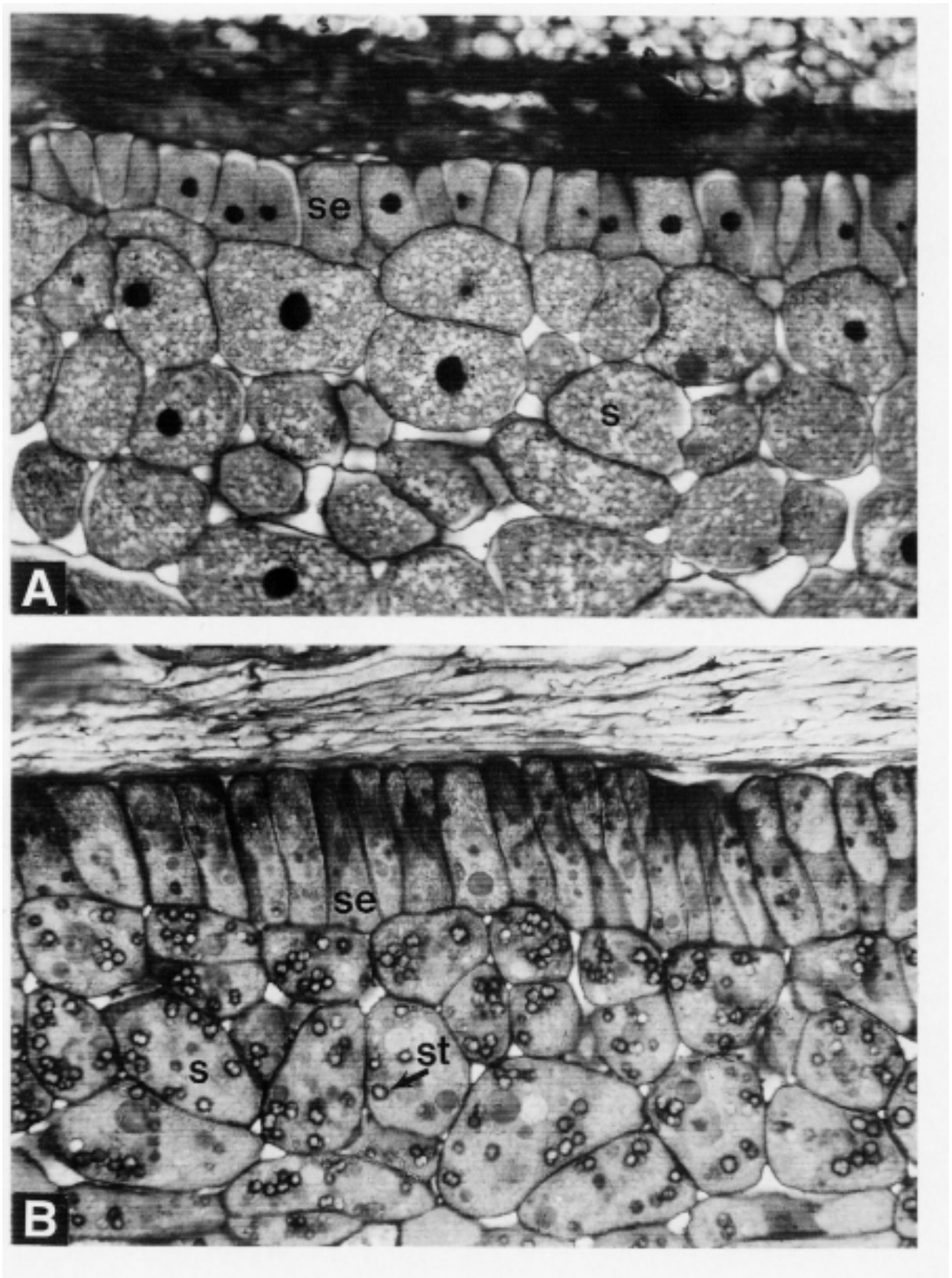

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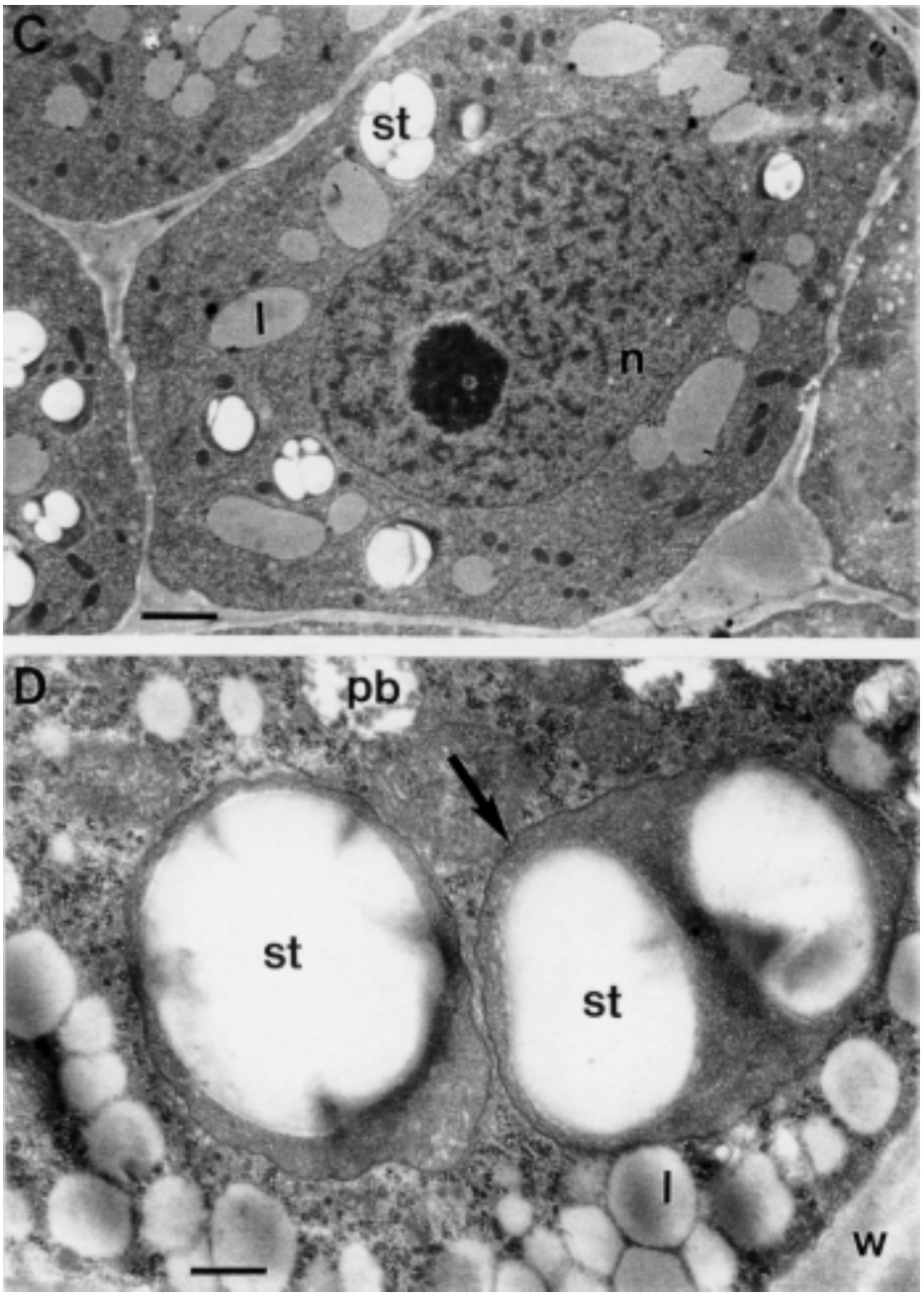

Figure 4. Light- and electron-microscopy studies on embryos from both dormant and non-dormant barley grains. Non-dormant (A) and dormant (B, C and D) grains were imbibed for 4 h at 20°C in darkness, and the embryos were isolated to be used in light- and electron-microscopy studies as described in Materials and Methods. The bar in (C) is 2.0 µm and in (D) is 0.4 µm. s = scutellum; se = scutellar epithelium; st = starch granule; l = lipid body; n = nucleus; pb = protein body; w = cell wall. The arrow indicates the membrane of the amyloplast. At least five embryos from each sample in two independent experiments were observed and typical examples are presented.

these tissues from non-dormant grains (Fig. 4A). No starch was found in the radicle, the coleoptile and the plumule in either dormant or non-dormant embryos (data not shown). The large number of starch granules found in dormant embryos was correlated with a difference in dry weight between dormant (2.10 ± 0.07 mg/embryo, n = 60 embryos) and non-dormant embryos (1.51 ± 0.07 mg/embryo, n = 60 embryos).

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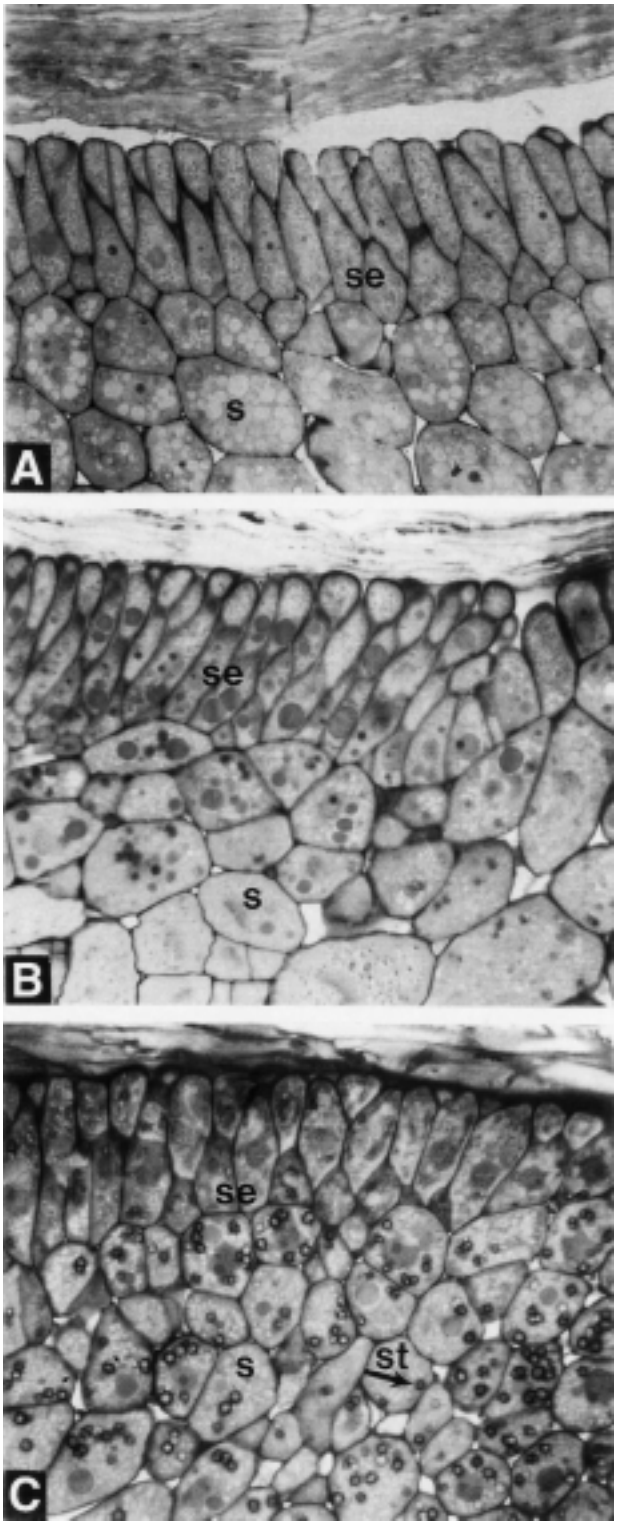

Figure 5. Light-microscopy of embryos from dormant grains treated under various conditions. Isolated dormant embryos were incubated in GA_3 (10^{-8} M) + FC (10^{-5} M) (A), FC (10^{-5}) (B), and GA_3 (10^{-8} M) + FC (10^{-5} M) in 50 mM MES buffer (pH 5.6) (C) for 24 h at 20°C in the dark. The samples were used for light-microscopy studies as described in Materials and Methods. s = scutellum; se = scutellar epithelium; st = starch. At least five embryos from each sample in two independent experiments were observed and typical examples are presented.

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Table 1. The levels of reducing sugars in embryos isolated from both dormant and non-dormant grains. Embryos were incubated in H_2O (10 embryos/300 µl). At different time points embryos were collected, ground in liquid N_2 and taken up in 750 µl distilled H_2O. Reducing sugars were measured according to Somogyi (1952). The means ± SD of three independent experiments are presented

Incubation time (h)	Embryos from non-dormant grains (µmol/10 embryos)	Embryos from dormant grains (µmol/10 embryos)
0	2.68 ± 0.05	1.10 ± 0.07
4	2.75 ± 0.04	1.23 ± 0.05
24	2.52 ± 0.06	2.00 ± 0.04

Since the starch granules observed by light microscopy in embryos from dormant grains could in theory be contamination from the starchy endosperm, it was necessary to verify the location of starch granules within the cell. Scutellum cells from embryos of dormant grains were further studied by electron microscopy. Figure 4C demonstrates that there was a significant number of starch granule groups within these cells. The observed starch granules in scutellum cells of embryos from dormant grains were localized in amyloplasts (Fig. 4D).

It is possible that the initial stage of radicle emergence utilizes soluble carbohydrates and their breakdown products, such as sugars. Thus, the degradation products of starch in embryos may function as primary energy sources and/or in osmotic regulation for water uptake upon the initiation of germination. Therefore, the amount of reducing sugars in embryos from both dormant and non-dormant grains was measured. In embryos from non-dormant grains, the reducing sugar content is about twice as high as in embryos from dormant grains (Table 1). This difference was reduced once the embryos from dormant grains started to germinate (Table 1, 24 h of incubation).

The breakdown of starch granules in dormant embryos is not a prerequisite for germination but necessary for further growth

We hypothesized that the effects of GA and FC on stimulating germination might be at least partly due to activation of α-amylase in embryos. In order to test this hypothesis, experiments were conducted in which α-amylase gene expression in the embryos was manipulated, and under these conditions both germination behaviour and changes in embryo starch granules were studied.

As mentioned above, GA_3 (10^{-6} M)-, FC (10^{-5} M)- and GA_3 + FC (10^{-8} M + 10^{-5} M)-induced germination of embryos from dormant grains was not significantly different in the presence or absence of 50 mM MES buffer, pH 5.6 (Fig. 2). However, GA_3 (10^{-6} M)-, FC (10^{-5} M)- and GA_3 + FC (10^{-8} M + 10^{-5} M)-induced α-amylase mRNA expression in embryos from dormant grains was strongly inhibited by addition of 50 mM MES buffer (Table 2). These data suggested that clamping the pH_e at a relatively high level, although it has no effect on germination, significantly inhibited α-amylase mRNA expression in embryos from dormant grains. As described above, light- and

Table 2. Effects of GA_3, FC and pH_e on α-amylase mRNA expression in embryos isolated from dormant grains. Ten embryos (in duplicate) were incubated in H_2O or 50 mM MES buffer (pH 5.6), containing GA_3, FC, or a combination of GA_3 and FC. After 24 h of incubation at 20°C in darkness, samples were collected for Northern analysis. The amount of ^{32}P-labelled probe hybridizing to the α-amylase mRNA was semi-quantitatively determined by measuring the absorbance on autoradiographs with an UL-Troscan KL densitometer (LKB). The α-amylase mRNA expression for GA_3 + FC was set as 100%. The means ± SD from three independent experiments are presented

Samples	In water (% expression ± SD)	In 50 mM MES buffer (% expression ±SD)
Control	11 ± 7	13 ± 5
GA_3 (10^{-8} M)	14 ± 8	17 ± 7
GA_3 (10^{-6} M)	107 ± 10	22 ± 9
FC (10^{-5} M)	50 ± 15	20 ± 11
GA_3 (10^{-8} M) + FC (10^{-5} M)	100 ± 0	25 ± 13

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electron-microscopy studies demonstrated the presence of a large number of starch granules in embryos from dormant grains but almost none in embryos from non-dormant grains (Fig. 4). The question was raised if addition of FC and GA_3, which stimulated germination of isolated embryos from dormant grains, can cause a decrease in starch granules in these embryos. Figure 5A shows that a 24 h incubation of embryos from dormant grains with FC (10^{-5} M) and GA_3 (10^{-8} M) (at which germination was 100%), caused an almost complete disappearance of starch granules (Fig. 5A). Addition of FC (10^{-5} M) alone gave about 85% germination after 24 h of incubation, and a significant reduction of starch granules in embryos was also observed (Fig. 5B) as compared to 4 h imbibed embryos from dormant grains (Fig. 3B). When embryos were incubated in 50 mM MES buffer (pH 5.6), addition of FC (10^{-5} M) and GA_3 (10^{-8} M) (eliciting 100% germination) did not result in reduction of starch granules in embryos (Fig. 5C), in agreement with the α-amylase mRNA expression data.

Isolated embryos from dormant grains exhibit a reduced germination rate compared to embryos from non-dormant grains (Van Beckum *et al.*, 1993). In addition, although MES (pH 5.6) treatment has no effect on the initiation of germination of embryos from dormant grains (Fig. 2), it significantly reduced the growth rate of young seedlings as compared to germination in water (Table 3).

Discussion

Starch granules in dormant embryos do not inhibit germination

Isolated embryos from dormant grains showed delayed germination (Fig. 1A) and contain a large number of starch granules (Fig. 4). Since both GA_3 and FC were able to break the dormancy of intact grains (Wang *et al.*, 1998a), stimulate the germination rate of embryos isolated from dormant grains (Fig. 2) and induce α-amylase mRNA expression (Fig. 3), it is possible that removal of starch granules by production of α-amylase might be either a first step towards the breaking of dormancy or an accompanying phenomenon of germination. The presence of a large number of starch granules could inhibit the germination of the embryo since the soluble carbohydrates and their breakdown products (e.g. sugars) may be an energy source for initiation of germination and/or an osmoticum for regulation of water uptake. However, clamping the pH_e at pH 5.6 caused the complete inhibition of GA/FC-induced α-amylase mRNA expression, but had no significant effect on GA/FC-induced acceleration of germination (Fig. 2 and Table 2). The consequence of MES treatment was that large numbers of starch granules remained in GA/FC-induced germinating embryos from dormant grains (Fig. 5C). These results show that degradation of starch granules in embryos from dormant grains is not a prerequisite or trigger for the initiation of germination.

Difference in developmental stages

During development of cereal grains (e.g. wheat), starch granules are initially deposited in the embryo (Black *et al.*, 1996). In mature embryos from non-dormant barley grains, almost no starch granules (Fig. 4), a high level of sugars (Table 1) and lack of inducible α-amylase mRNA expression (Fig. 3) suggested that the α-amylase in these embryos has already been expressed during the maturation of the grain and that the starch granules formed during development have been degraded into soluble carbohydrates or sugars. The large number of starch

Table 3. Shoot growth of embryos from dormant grains under various germination conditions. Ten embryos isolated from dormant barley grains (in duplicate) were incubated in 300 µl H_2O with or without GA_3, FC or a combination of GA_3 and FC for 48 h in darkness. The size of the shoot of each individual seedling was measured. The means ± SD of five independent experiments are presented. *Mean values not significantly different ($P > 0.95$) from value obtained under corresponding conditions in water. **Mean values significantly different ($P < 0.05$) from value obtained under corresponding conditions in water

Hormone treatment	H_2O; shoot length (mm)	50 mM MES buffer; shoot length (mm)
Control	1.8 ± 0.7	1.3 ± 0.4*
GA (10^{-8} M)	2.6 ± 0.7	1.8 ± 0.6**
FC (10^{-5} M)	3.2 ± 0.6	2.0 ± 0.1**
GA + FC	3.6 ± 0.4	2.0 ± 0.3**

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granules in embryos from dormant grains (Fig. 4) and the low level of sugar in these embryos (Table 1) may be explained by assuming that during grain maturation, development is stopped or completed before the starch granules are degraded. This hypothesis can be supported by the presence of starch granules in developing wheat embryo/scutellum (Smart and O'Brien, 1983) and an increase in α-amylase activity in the embryo in the middle–late development stage (Black *et al*., 1996).

The α-amylase cDNA probe we used is a conserved cDNA probe which in principle hybridizes with all types of α-amylases. It is possible that the α-amylase mRNAs detected by Northern analysis were not similar to the secretory α-amylases in the aleurone. Plastid localized α-amylase, which is antigenically similar to cereal aleurone α-amylase, has been reported by Maragatha-Vally and Sharma (1995). The α-amylase mRNAs detected in embryos from dormant grains were not high pI α-amylases mRNAs (data not shown) because they could not be detected with a high pI α-amylase specific probe (Rogers, 1985). Garcia-Maya *et al*. (1990) demonstrated that immature wheat embryos produced mainly low pI α-amylase proteins. It is not likely that the embryo α-amylase mRNA originates from the germ-aleurone, because the aleurone of non-dormant grains exhibits GA-induced α-amylase mRNA expression (Sinjorgo *et al*., 1993; van der Meulen *et al*., 2000). However, in non-dormant embryos we did not find α-amylase mRNA expression at the time point measured (Fig. 3). The GA/FC-inducible α-amylase RNA expression in embryos from dormant grains indicates that these grains may stop earlier at the desiccation stage than non-dormant grains (Fig. 3).

During the first 24 h of incubation of isolated embryos from non-dormant grains with GA, levels of α-amylase mRNA and α-amylase activity were very low (Fig. 3). However, it has been reported that α-amylase mRNA is detectable in scutellar epithelium cells after 24 h of incubation of intact non-dormant grains (Wang *et al*., 1998b). It is possible that non-dormant embryos express α-amylase mRNA only in scutellar epithelium cells, but in dormant embryos the entire scutellum is expressing α-amylase. This might be an explanation for the strong difference in α-amylase mRNA levels and activities between dormant and non-dormant embryos.

FC and GA action

GA_3 and FC are able to break dormancy in intact dormant barley grains and to stimulate the germination rate of embryos isolated from dormant grains (Wang *et al*., 1998a and Fig. 1). However, in contrast to GA_3, FC is able to induce a strong acidification of the medium pH in which embryos are incubated (Fig. 1). The artificial clamping of pH_e at 5.6 by MES buffer had no effect on initial germination events. Moreover, an artificial clamping at pH_e 4.3 by succinate buffer caused an inhibition of germination rate in general (Fig. 2). These results suggest that the effects of GA and FC on breaking of dormancy in intact grains are not via the acidification of the medium. The additive effect of FC and GA_3 on germination was not affected by clamping of pH_e. In 50 mM succinate buffer, the reduced germination percentages of embryos from dormant grains most likely reflect the slower germination kinetics of these embryos. However, the activation of H^+-ATPases may still be an essential factor for germination.

The activation of plasma membrane H^+-ATPases may cause the lowering of pH_e brought about by FC (Fig. 1A). Sinjorgo *et al*. (1993) showed that the effect of GA_3 on α-amylase production in barley aleurone is enhanced at low pH_e. In dose response analysis of GA_3-induced α-amylase mRNA expression, FC was able to enhance both sensitivity to GA and the level of α-amylase mRNA expression (Fig. 3). Clamping the pH_e at a relatively high value (5.6) by MES caused an inhibition of GA- and GA/FC-induced α-amylase expression (Table 2).This suggested that the lowering of pH_e caused by FC is the reason for enhancement of GA-induced α-amylase mRNA expression, but not for germination. Therefore, the effect of FC on α-amylase mRNA expression and germination may be via different or at least partially different signal transduction pathways. Our further investigations will focus on this aspect.

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