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Seed Dormancy in Red Rice (*Oryza sativa*)[1]

IX. Embryo Fructose-2,6-Bisphosphate during Dormancy Breaking and Subsequent Germination

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Fructose-2,6-bisphosphate (Fru-2,6-bisP) was evaluated as a potential marker for the dormancy-breaking phase or the germination phase before pericarp splitting in red rice (*Oryza sativa*). During 4 h of imbibition at 30°C, Fru-2,6-bisP of dehulled dormant and nondormant seeds increased to 0.26 and 0.38 pmol embryo^{-1}, respectively. In nondormant seeds, embryo Fru-2,6-bisP content remained stable until the onset of pericarp splitting (12 h) and increased rapidly thereafter. In dormant seeds, Fru-2,6-bisP declined to 0.09 pmol embryo^{-1} at 24 h. Embryo Fru-2,6-bisP was correlated with O_2 uptake of dormant and nondormant seeds. A 24-h exposure of dehulled, water-imbibed, dormant seeds to treatments yielding >90% germination (sodium nitrite [4 mM], propionic acid [22 mM], methyl propionate [32 mM], propanol [75 mM], and propionaldehyde [40 mM]) led to changes in embryo Fru-2,6-bisP that were unrelated to the final germination percentages. Furthermore, a 2-h pulse of propionaldehyde increased Fru-2,6-bisP 4-fold but did not break dormancy. Whereas nitrite and propionaldehyde increased Fru-2,6-bisP to 0.33 pmol embryo^{-1} after 2 h of contact, propionic acid and methyl propionate did not increase Fru-2,6-bisP above the untreated control. In all cases, further increases in Fru-2,6-bisP occurred after pericarp splitting. However, the plateau Fru-2,6-bisP attained during chemical contact was inversely correlated with elapsed time to 30% germination ($r = -0.978$). Therefore, although Fru-2,6-bisP is not a universal marker for dormancy release, its rapid increase during nitrite and propionaldehyde treatments suggests that events associated with dormancy breaking can occur within 2 h of chemical treatment.

Physiological observations suggest that the loss of seed dormancy is temporally distinct from germination processes. For example, in many light-dependent species, existence of a photoreversible time interval preceding the irreversible commitment ("escape time") to germination (reviewed by Bewley and Black, 1982) implies that special signaling pathways lead to the termination of dormancy. Embryos isolated from dormant cereal grains also tend to exhibit delayed germination kinetics compared with embryos isolated from nondormant ones (Van Beckum et al., 1993). In red rice (*Oryza sativa*) dormancy-breaking events induced by nitrogen dioxide require 5 to 6 h in addition to the "normal" germination metabolism as indicated by growth kinetics (Cohn and Castle, 1984).

One of the challenges that chronically plagues mechanistic studies of dormancy-breaking treatments lies in identifying specifically induced biochemical events versus those associated with germination per se. We have addressed this problem by looking for markers—similar to those associated with initial cell activation in other model systems (Busa, 1986; Epel, 1990; Storey, 1990)—to delineate changes in development during the application of dormancy-breaking chemicals. Although embryo acidification has been proposed as a marker for the onset of the dormancy-breaking process (Footitt and Cohn, 1992a), it would be beneficial to also identify a biochemical marker for the germination phase to temporally position the dormant/nondormant transition. Increased Fru-2,6-bisP is associated with dormancy loss in tubers (Van Schaftingen and Hers, 1983) and roots (Kowalczyk, 1989) as well as the germination phase of *Avena sativa* (Larondelle et al., 1987). In rice seedlings, Fru-2,6-bisP increased when O_2 was limiting (Mertens et al., 1990); such conditions may prevail in the dense tissues of a germinating seed. As an activator of a potentially near-equilibrium reaction between Fru-6-P and Fru-1,6-bisP (Hajirezaei and Stitt, 1991), Fru-2,6-bisP could serve as a very sensitive indicator for changes in metabolism likely to occur during the dormancy-breaking process and germination. Our objective, then, was to evaluate the use of Fru-2,6-bisP as a germination phase marker for the resumption of intermediary metabolism typically associated with growing tissue.

In this report we present unexpected evidence that (a) some dormancy-breaking chemicals rapidly increase embryo Fru-2,6-bisP, whereas other dormancy-breaking chemicals have no effect on Fru-2,6-bisP until germination and (b) the magnitude of this early increase in embryo

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Abbreviations: GDH, glycerophosphate dehydrogenase; PPi-PFK, pyrophosphate-dependent phosphofructokinase.

Fru-2,6-bisP is highly correlated with subsequent germination rate rather than with the dormancy-breaking process. Part of this work was previously presented in abstract form (Footitt and Cohn, 1992b).

MATERIALS AND METHODS

Mature, dormant red rice seeds (*Oryza sativa*, strawhulled, awnless) were obtained from the South Farm Rice Research Station (Crowley, LA) in 1990. Seeds were harvested by hand-shattering of individual plants. Moisture content at the time of harvest was 24.2%. Seeds were dried for 4 d at 21°C to a moisture content of 13.8% and stored in sealed Mason jars at −15°C. Nondormant seeds were obtained by dry afterripening for 76 d (Footitt and Cohn, 1992a). Germination and viability tests were performed for each experiment (Footitt and Cohn, 1992a). Seedlot viability was ≥99%. Freshly prepared solutions of dormancy-breaking chemicals were used for each experiment. Experiments were repeated at least three times. All data are presented as the means ± SE.

Seed Incubation for Determination of Fru-2,6-bisP

One hundred dehulled seeds were incubated on H_2O-moistened filter paper at 30°C in darkness and sampled for analysis and germination percentages during 24 h. In dormant seed samples, germinating escapes (usually ≤5%) were replaced with similarly hydrated, ungerminated seeds (Footitt and Cohn, 1992a).

For studies with dormancy-breaking chemicals, dehulled seeds were incubated as above for 24 h. Germinated seeds were replaced with similarly treated dormant seeds prior to dormancy-breaking treatments. Seeds were transferred to 250-mL wire-clasp storage jars (Heritage Industries, Millville, NJ) for 24 h at 30°C for pulse applications of dormancy-breaking compounds in 25 mM citrate/phosphate buffer at concentrations that broke dormancy in 90% of the population. Sodium nitrite (4 mM) was applied at pH 3.0 (pK 3.3). Propionic acid (22 mM) was applied at its pK of 4.9. Methyl propionate (32 mM), propanol (75 mM), and propionaldehyde (40 mM) were applied at pH 7.0. At the end of the chemical pulse, seeds were transferred to Petri plates containing distilled H_2O for 24 h at 30°C. Samples were taken at intervals throughout the chemical pulse and postpulse phases. Buffered control treatments were executed under each of the three pH conditions. For dormant controls, only ungerminated seeds were harvested for analysis.

Fru-2,6-bisP Extraction

Fru-2,6-bisP extraction and anion exchange were based on the procedure described by Van Schaftingen (1984). For each determination, 100 embryos were excised and immediately frozen and powdered in liquid N_2. The powder was extracted in a chilled tissue homogenizer with 1 mL of ice-cold 0.2 N NaOH. The homogenate was decanted into a cold centrifuge tube, and the remaining material was recovered by rinsing the homogenizer with 1 mL of 0.2 N NaOH. The homogenate was then centrifuged at 20,000*g* for 5 min at 1°C. The supernatant was heated at 80°C for 5 min and then cooled on ice. The same procedure was used for 40 de-embryonated seeds.

The pericarp of red rice contains anthocyanins (Takahashi et al., 1989). These were removed from the extracts by passing each sample through a column of Dowex 1 × 8 (4 × 40 mm, Cl^- form, 200 mesh) at 5°C. Columns were prepared by washing with 4 mL of 1 M NaCl and 10 mL of H_2O and then stored hydrated at 0 to 5°C. The loaded column was washed with 1 mL of H_2O and 3 mL of 0.15 M NaCl. Fru-2,6-bisP was eluted in 3 mL of 0.3 M NaCl. Samples were frozen at −20°C until assayed. In each experiment one time point sample was spiked with Fru-2,6-bisP to determine the percentage recovery. A recovery control (no tissue included) was also prepared by the same extraction protocol to confirm the amount of the Fru-2,6-bisP used in the recovery sample. Fru-2,6-bisP content was corrected for recovery percentages as reported in the figure legends.

Fru-2,6-bisP Assay

Fru-2,6-bisP was assayed according to the method of Van Schaftingen (1984). Lyophilized enzymes were used: aldolase type X (EC 4.1.2.13, rabbit muscle, Sigma), GDH (EC 1.1.1.8, rabbit muscle, United States Biochemical Corp.), PPi-PFK (EC 2.7.1.90, potato tuber, Sigma), triose-P isomerase type X (EC 5.3.1.1, rabbit muscle, Sigma). GDH, PPi-PFK, and triose-P isomerase type X were stored in 20 mM Tris-HCl, pH 8.2, 20 mM KCl, 2 mM DTT, and 60% (w/w) glycerol at −20°C.

Homogenate samples (1 mL) were neutralized with 0.1 mL of 1 M Hepes, 5.5% (w/v) soluble PVP-10, pH 7.8. An internal calibration was performed for each assay. Endogenous Fru-2,6-bisP was destroyed in the calibration solution by acidifying 0.5 mL of neutralized sample with 0.2 mL of 0.4 N HCl for 20 min at room temperature and then neutralizing with 0.2 mL of 0.4 N NaOH. This gives a sample:HCl:NaOH ratio of 5:2:2, which was used in all sample assays.

To each of four quartz cuvettes was added calibration solution, 0.5 mL of reaction buffer (100 mM Tris-acetate, pH 8.0, 4 mM magnesium acetate, 2 mM Fru-6-P, 0.3 mM NADH; Fru-6-P was first treated with acid to destroy contaminating Fru-2,6-P), and 0.1 mL of enzyme solution (25 mM Tris-acetate, pH 8.2, 150 units L^{-1} PPi-PFK, 20 kilounits L^{-1} aldolase, 100 kilounits L^{-1} triose-P isomerase, 20 kilounits L^{-1} GDH, and 0.2% BSA). Fru-2,6-bisP was added at 0, 0.2, 0.5, and 1.0 pmol. The volume in each cuvette was made up to 0.95 mL with distilled H_2O. In a fifth cuvette equivalent volumes of 0.4 M HCl, 0.4 N NaOH (followed by the reaction buffer), and sample were substituted for the calibration solution. The reaction was started by the addition of 0.05 mL of 10 mM tetrasodium PPi. The assay was monitored at 339 nm for 10 min at 25°C using a temperature-controlled cuvette block in a Gilford (Ciba-Corning Diagnostics Corp., Medfield, MA) Response II spectrophotometer. The Fru-2,6-bisP content of the sample was determined by linear regression analysis of the internal calibra-

tion. Only data from assays in which the correlation coefficient was ≥0.995 were used.

Loss of Desiccation Tolerance

Seeds usually retain desiccation tolerance up to the completion of germination (reviewed by Leprince et al., 1994). As a physiological confirmation of the visual assessment of germination, the time course of the transition from dehydration-tolerant to -sensitive state was determined. Five replicates of 20 dehulled nondormant seeds were used for each time. Seeds were incubated at 30°C in 50-mL Erlenmeyer flasks as for standard germination tests (Footitt and Cohn, 1992a). Seeds were transferred at 2-h intervals to 9-cm Petri plates containing three dry sheets of germination paper and dried for 7 d at 30°C to 6.4 ± 0.1% moisture content (fresh weight basis). Seeds were then transferred to Erlenmeyer flasks, hydrated, and incubated for 7 d at 30°C, when germination was assessed. Necrosis and lack of visible radicle growth were the criteria indicating loss of desiccation tolerance.

Respirometry

O_2 uptake was measured with a Gilson respirometer (Umbreit et al., 1972). Three replicates of 20 dehulled seeds were incubated in 15-mL respirometer flasks at 30°C on two sheets of Whatman No. 1 filter paper and 2 mL of distilled H_2O. The center well contained 0.2 mL of 20% KOH and a fluted filter paper wick to increase the surface area for absorption of CO_2. A film of lanolin at the well rim prevented KOH creep. An internal control without seeds indicated atmospheric pressure fluctuations in each experiment. Respiration was measured during 1-h intervals, every 2 h for 24 h. Dry seed gas exchange was measured over 24 h. At the end of the 24-h period, seeds were transferred to 50-mL Erlenmeyer flasks for the standard germination test.

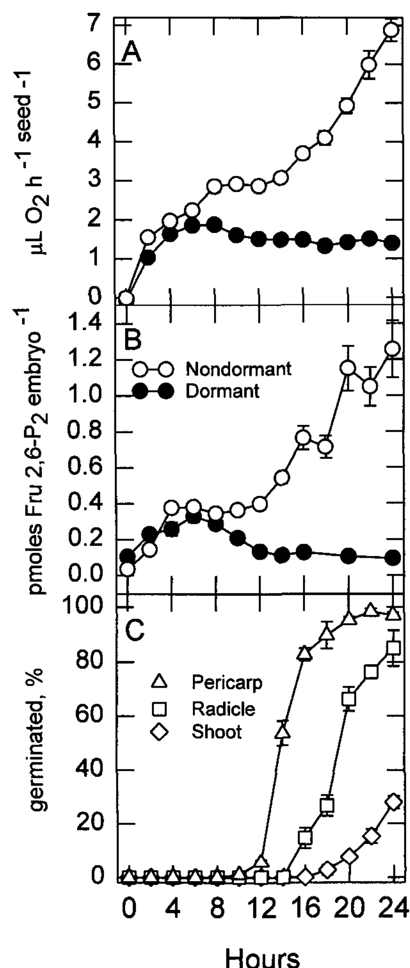

Figure 1. A, Respiration of dehulled, dormant, and nondormant seeds over 24 h at 30°C as O_2 uptake μL^{-1} h^{-1} $seed^{-1}$. B, Fru-2,6-bisP content of embryos in dehulled dormant and nondormant seeds during hydration at 30°C. Data are corrected for full recovery based on within-treatment recoveries. Recoveries: dormant, 77%; nondormant, 86%. C, Utilization of pericarp splitting and radicle and shoot emergence as the criteria for percentage of germination of nondormant seeds in B.

RESULTS

Seed Respiration during Hydration

Respiration rates increased during the imbibition of dormant and nondormant seeds. Following imbibition, the respiration rate of nondormant seeds remained on a plateau from 8 to 12 h. From 12 h onward the respiration rate increased at the point when radicle emergence commenced. In dormant seeds, respiration rate remained stable (Fig. 1A).

Embryo Fru-2,6-bisP during Hydration

In dry seeds, embryo Fru-2,6-bisP was 0.1 pmol $embryo^{-1}$ or less (Fig. 1B). In both dormant and nondormant dehulled seeds, embryo Fru-2,6-bisP increased during imbibition. In nondormant embryos, Fru-2,6-bisP reached a plateau at which it remained from 4 to 12 h, when pericarp splitting commenced (Fig. 1C). Between 10 and 12 h desiccation tolerance declined as germination after dehydration was reduced from 94 ± 5 to 11 ± 5%. Pericarp splitting (germination) reached 83% by 16 h (Fig. 1C). During this period (12–16 h) the embryo Fru-2,6-bisP content doubled (Fig. 1B). Rapid increases in radicle emergence and embryo Fru-2,6-bisP were coincident (18–20 h). In contrast, embryo Fru-2,6-bisP of dormant seeds plateaued between 4 and 8 h and subsequently decreased. Embryo Fru-2,6-bisP and seed respiration rates were highly correlated in dormant and nondormant seeds during the 24-h experiment (r = 0.92, $P < 0.001$). When endosperms were extracted in the absence of adhering embryo tissue (scutellum), Fru-2,6-bisP was detected at approximately 0.25 ± 0.03 pmol $endosperm^{-1}$ during 14 h of hydration independently of dormancy status.

Embryo Fru-2,6-bisP during and after Chemical Contact

During pulse contact with various dormancy-breaking chemicals (Fig. 2A), embryo Fru-2,6-bisP differed depend-

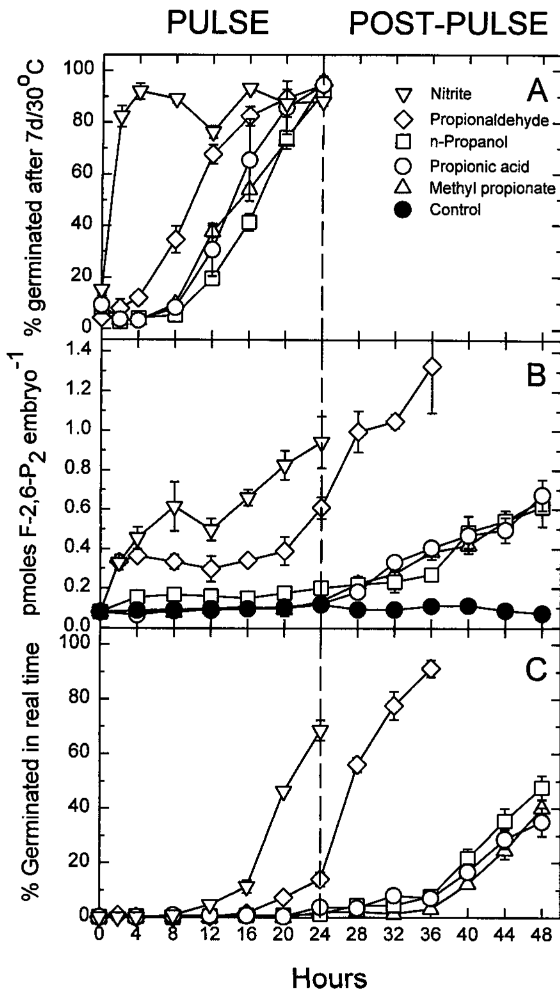

Figure 2. Dormant seeds hydrated for 24 h at 30°C and then exposed to a dormancy-breaking chemical. A, Dormancy breaking after increasing periods of contact. Buffer controls represent 0 h. B, Fru-2,6-bisP content of embryos in dehulled dormant seeds during and after chemical contact. Data are corrected for full recovery, based on within-treatment recoveries. Recoveries: control, 76%; methyl propionate, 91%; nitrite, 97%; *n*-propanol, 97%; propionaldehyde, 90%; propionic acid, 96%. Fru-2,6-bisP values were similar for each pH buffer control and are presented as a composite control. C, Germination of seeds (pericarp splitting) in real time with the continuous presence of the dormancy-breaking chemical during the first 24 h.

ing on the treatment used (Fig. 2B). With nitrite and propionaldehyde, embryo Fru-2,6-bisP rapidly increased after only 2 h of contact and reached plateau levels similar to those of nondormant embryos (Figs. 1B and 2B). Subsequently, embryo Fru-2,6-bisP increased above the plateau level when pericarp splitting commenced, which was during chemical contact for both nitrite (16 h) and propionaldehyde (24 h). Propanol produced a distinct although lower embryo Fru-2,6-bisP plateau. Propionate and methyl propionate did not increase embryo Fru-2,6-bisP above that of the control during chemical contact even though the chemicals broke dormancy. The average plateau embryo Fru-2,6-bisP content during chemical contact was significantly correlated with the total elapsed time to 30% germination (Fig. 3).

After chemical treatment, embryo Fru-2,6-bisP increased coincident with germination of propanol-, propionic acid-, and methyl propionate-treated seeds (Fig. 2, B and C). Following dormancy breaking, germination percentages observed up to 48 h (Fig. 2C) were significantly correlated with embryo Fru-2,6-bisP (Fig. 2B) in chemically treated seeds ($r = 0.905$, $P < 0.001$).

DISCUSSION

In this study, embryo Fru-2,6-bisP was determined and evaluated as a potential marker for the transition from the dormant to nondormant state in red rice. Fru-2,6-bisP determinations were highly reproducible with consistent recoveries in this high phenolic-containing tissue. Fru-2,6-bisP content of red rice was compatible with previous studies of *Oryza* species (Mertens et al., 1990), and PPi-PFK from rice seeds and seedlings can be regulated by Fru-2,6-bisP (Enomoto et al., 1992, 1994).

Embryo Fru-2,6-bisP and Respiration during Germination

The time course of embryo Fru-2,6-bisP and respiration in nondormant seeds followed a triphasic response as shown for embryo moisture content (Footitt and Cohn, 1992a). The main components of increases in embryo Fru-2,6-bisP and O_2 uptake were a result of seedling growth after pericarp splitting. However, O_2 uptake also increased in nondormant seeds prior to pericarp splitting versus dormant controls as early as 8 h of incubation, in accordance with trends and conclusions reported for *Sisymbrium* seeds (Derkx et al., 1993). Loss of desiccation tolerance and the onset of germination occurred at 12 h, immediately prior to the increases in embryo Fru-2,6-bisP and respiration. The use of pericarp splitting identified germination 4 h prior to radicle emergence (the traditional germination marker), showing that the second increase of Fru-2,6-bisP was associated with early seedling growth and not a pre-

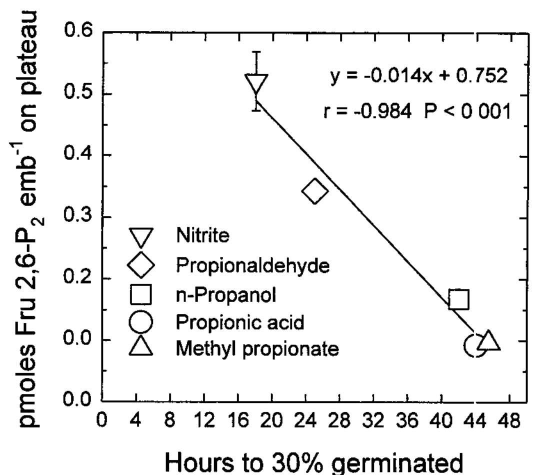

Figure 3. Correlation between mean plateau Fru-2,6-bisP and the time to 30% germination. Data are from Figure 2, B and C.

lude to pericarp splitting and radicle emergence. Therefore, careful consideration of the visual cue used to mark the onset of germination (postgerminative growth) is important in the staging of physiological events.

In dormant red rice seeds, embryo Fru-2,6-bisP increased during imbibition, consistent with results from *Avena* seeds (Larondelle et al., 1987).

Dormancy-Breaking Chemicals and Embryo Fru-2,6-bisP

Although all the chemicals used produced a >90% dormancy-breaking response 7 d after a 24-h seed contact period, their effect on embryo Fru-2,6-bisP prior to pericarp splitting varied greatly. Dormant embryo Fru-2,6-bisP dramatically increased in the presence of nitrite and propionaldehyde after only 2 h of contact. However, only nitrite broke dormancy after 2 h of contact. The propionaldehyde data indicate that a dormancy-breaking chemical can rapidly induce a physiological response without breaking dormancy. Furthermore, methyl propionate and propionate readily broke dormancy, and yet embryo Fru-2,6-bisP remained at control levels during chemical contact. Hence, the timing and attainment of a specific threshold value of embryo Fru-2,6-bisP are not directly related to the onset of dormancy breaking. These data indicate the merits of utilizing several types of dormancy-breaking chemicals to examine the metabolic transitions between dormancy and germination.

Contact with nitrite, propionaldehyde, and propanol increased embryo Fru-2,6-bisP to distinct plateau levels prior to pericarp splitting. The higher the mean plateau embryo Fru-2,6-bisP, the shorter the elapsed time to the onset of germination and the faster the subsequent germination rate. The high plateau values of embryo Fru-2,6-bisP may reflect increased glycolytic activity (Van Schaftingen, 1987; Sung et al., 1988; Stitt, 1990; Hajirezaei and Stitt, 1991; Botha et al., 1992). The different plateau levels may result from the differing effects of the chemicals on glycolysis and respiration (Van Schaftingen et al., 1984; François et al., 1986, 1988; Dumbrava and Pall, 1987; Hue et al., 1988; Pampulha and Loureiro-Dias, 1990; Halinska and Frenkel, 1991; Warth, 1991), despite the fact that 24-h treatments with each chemical yielded 90% germination or better. The implication is that correlations of Fru-2,6-bisP and physiological response reflect some aspect of glycolytic activity. This is, to some extent, borne out by the correlation between embryo Fru-2,6-bisP and respiration in hydrating dormant and nondormant red rice seeds. However, glycolytic flux activation and increased Fru-2,6-bisP may be independent of one another (Hatzfeld and Stitt, 1991). Unfortunately, attempts to measure O_2 uptake via respirometry during dormancy-breaking chemical treatments were unsuccessful because of absorption of chemicals by the KOH trap, an experience also encountered by Perata and Alpi (1991).

After dormancy was broken, the percentages of germination in real time and embryo Fru-2,6-bisP were significantly correlated, as were germination and embryo pH (Footitt and Cohn, 1992a). Such correlations indicate that biochemical changes during germination are independent of the chemicals used to break dormancy. Similar positive correlations were seen during dormancy breaking in *Phycomyces blakesleeanus* spores for germination capacity versus both maximal Fru-2,6-bisP and Glc-6-P (Van Laere et al., 1983).

In other model systems such as *P. blakesleeanus* spores and *A. sativa* seeds, dormancy-breaking chemical treatments resulted in only transient increases in Fru-2,6-bisP (Van Laere et al., 1983; Larondelle et al., 1987). In *Avena*, in contrast to our results, Fru-2,6-bisP was not correlated with germination. These differences may be due to the use of dehulled, hydrated red rice versus intact, dry *Avena* caryopses. During the dormancy-breaking chemical treatment, O_2 uptake by the glumes (Lenoir et al., 1986) may induce anaerobic conditions in the *Avena* embryo, leading to increased Fru-2,6-bisP (Mertens et al., 1990). The glumes also delay the time to germination. Contrary to the effect of ethanol on *Avena* seeds, hydration of dry, dehulled, dormant red rice in the presence of propanol had no effect on embryo Fru-2,6-bisP above that of the control (data not shown). Therefore, physiological responses associated with the transition from the dormant to the germinable state may be masked by events related to tissue hydration if imbibition and dormancy-breaking treatments occur simultaneously. Derkx et al. (1993) reported an analogous situation during the investigation of respiration in *Sisymbrium*.

In summary, it was found that (a) seed respiration increased during hydration and dormant seed respiration remained constant following imbibition; (b) embryo Fru-2,6-bisP increased during hydration, but in dormant seeds this was transient; (c) embryo Fru-2,6-bisP reached a plateau prior to pericarp splitting; (d) embryo Fru-2,6-bisP increased coincidentally with germination; and (e) embryo Fru-2,6-bisP and respiration rate during hydration were highly correlated. When dormant seeds are subjected to dormancy-breaking chemical treatments, (f) dormancy-breaking chemicals could be rapidly perceived by the embryos (nitrite, propionaldehyde, and propanol); (g) a suboptimal, 2-h contact with propionaldehyde induced a physiological response without breaking dormancy; (h) embryo Fru-2,6-bisP content was not related to dormancy breaking; and (i) the magnitude of the embryo Fru-2,6-bisP plateau was highly correlated with the speed of subsequent germination.

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