Plant Physiol. (1992) 98, 1386–1390
0032-0889/92/98/1386/05/$01.00/0

Received for publication July 23, 1991
Accepted November 6, 1991

In Situ Abscisic Acid Synthesis[1]

A Requirement for Induction of Embryo Dormancy in *Helianthus annuus*

Marie-Thérèse Le Page-Degivry* and Ginette Garello

Laboratoire de Physiologie Végétale, Université de Nice-Sophia Antipolis,06034 Nice Cedex, France

ABSTRACT

When applied to young nondormant embryos of sunflower (*Helianthus annus*) (7–10 day[s] after pollination [DAP]), abscisic acid (ABA) inhibited germination as long as it was present. However, whatever the dose used and the duration of its application, ABA was unable to induce dormancy because after transfer of treated embryos to control (without ABA) medium, germination occurred. Thereafter, exogenous ABA became effective and allowed the dormancy to develop in 13 and 17 DAP embryos, *i.e.* in embryos which after isolation were still able to germinate in high percentage. After embryo dormancy was well established (21 DAP), application of fluridone allowed the germination to occur very quickly on control medium. Isolated dormant axes were also induced to germinate by an application of fluridone. Radioimmunological analysis showed that 24 hours after these treatments, endogenous ABA levels were drastically reduced in the axes. When these fluridone-treated embryos were cultured on ABA medium, germination was again inhibited as long as exogenous ABA was present but germination occurred as soon as embryos were transferred to control medium. Such behavior suggested that *in situ* ABA synthesis is necessary to impose and maintain the embryo dormancy.

The involvement of ABA in the initiation of seed dormancy has been the subject of many recent studies using either mutants deficient in or insensitive to the hormone (4–6) or inhibitors of synthesis, particularly fluridone (3, 11, 12). In *Arabidopsis*, Karssen *et al.* (4) and Koornneef *et al.* (6) showed that the induction of dormancy depended only on embryonic ABA and could neither be influenced by maternal ABA nor by ABA applied to leaves or roots. In *Zea mays*, Hole *et al.* (3) and Smith *et al.* (12) indicated that there is no stringent requirement for ABA produced specifically by the kernel itself; however, dormancy must be induced during a rather narrow time window at a precise stage of embryogenesis.

We have shown (7) that dormancy of sunflower was induced by ABA during embryo development. Application of fluridone to developing achenes, before the increase of endogenous ABA levels, prevented both ABA accumulation and development of embryo dormancy. To determine the conditions in which ABA intervened to induce dormancy in this species, we first studied the changes in responsiveness of embryos isolated at various times after pollination to exogenous ABA. It seemed necessary to distinguish clearly between the physiological consequences of the presence of exogenous ABA in the culture medium and those associated with a genuine induction effect, that is to say those produced by transient application of the hormone and which subsequently persist in its absence. Furthermore, we studied the consequences of an application of fluridone to isolated dormant embryos on their physiological behavior, their endogenous ABA levels and their responsiveness to exogenous ABA.

[1] This work was supported by a Centre Technique Permanent de Sélection des Plantes Cultivées contract.

MATERIALS AND METHODS

Plant Material

Plants of sunflower (*Helianthus annuus* cv Mirasol) were grown in the fields during summer. In September, flowers of male sterile plants were manually pollinated by pollen harvested on small female sterile flowers (seeds generously provided by S.A. Cargill). Flowers were tagged and harvest took place at different times after pollination.

For each age, 25 embryos, *i.e.* a sample considered as sufficient to be representative of the population, were used for *in vitro* culture and extraction. All the experiments were carried out two or three times in two different years (1989–1990).

In Vitro Cultures of Excised Embryos

For each age, embryos were aseptically isolated from the achenes and cultured on water agar with one cotyledon in contact with the medium. The cultures were incubated at 23°C under white fluorescent light (45 Wm^{-2}, 16 h d^{-1}). An embryo was considered to have germinated when the elongation of the radicle was clearly visible (2.0 mm).

Chemicals

(±)-*cis-trans*-ABA from Sigma was aseptically introduced in agar medium at 40°C after filtration on Millipore filter (HA 0.45 μm). Fluridone (1-methyl-3 phenyl-5-[3-(trifluoromethyl)phenyl]-4-(1H)-pyridinone), generously provided by Daw-Elanco, was applied in 10% (v/v) acetone-water, in quantities of 20 μL per embryo or 5 μL per axis, at a concentration of 100 μg/mL.

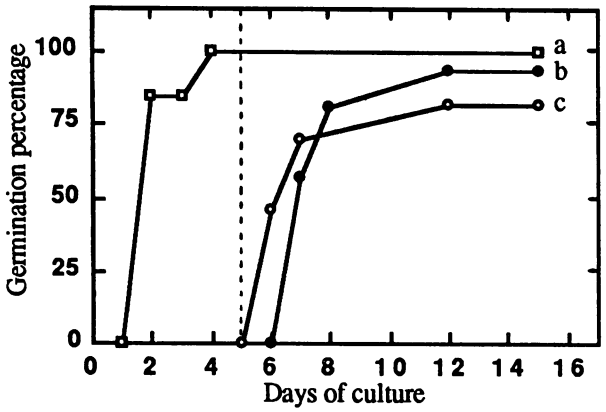

Figure 1. Changes, during culture, of the germination percentage of 10-d-old embryos cultured on water agar directly (a), or after 5 d on ABA 5×10^{-5} M (b) or 5×10^{-6} M (c).

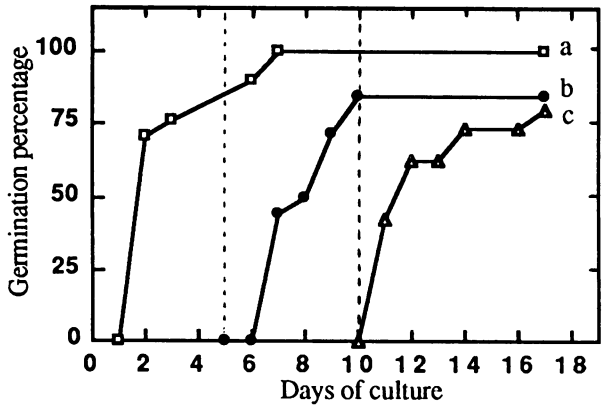

Figure 2. Changes, during culture, of the germination percentage of 10-d-old embryos cultured on water agar directly (a) or after 5 d (b) or 10 d (c) on ABA 5×10^{-5} M.

Extraction Procedure

Thirty isolated embryos were ground in a mortar with chilled 80% acetone containing 2,6-di-*t*-butyl-4-methyl phenol as antioxidant (100 mg·L^{-1}). The homogenate was stirred for 2 h at 4°C and centrifuged for 10 min at 2000*g*. The pellet was reextracted twice with the same volume of cold 80% acetone. The supernatants were collected and evaporated under reduced pressure at 40°C. The remaining aqueous extract was adjusted to pH 3.0 and extracted four times with diethyl ether (v/v).

Endogenous ABA Determination by RIA[2]

ABA was quantified by RIA performed exactly as described earlier (8). Anti-ABA antibodies were obtained by immunization of rabbits with (+)-ABA-human serum albumin conjugate obtained via a mixed anhydride reaction. The tracer was the iodinated (^{125}I) derivative of the conjugate obtained by coupling (+)-ABA to the glycyl-L-tyrosin. Radioimmunological incubations were performed by equilibrium dialysis.

A study of the cross-reactivities of structurally related compounds showed the specific binding of (+)-ABA, when the acid function was free, esterified or linked by amide linkage. The conversion of the carboxyl group of ABA into an amide induced an increase in sensitivity of the free (+)-ABA estimation, allowed ABA values in an extract to be calculated by differential measurement before and after amidation (8). Each value was the average of results obtained from four different dilutions of the extract (two replicates for each).

RESULTS

Responsiveness of Young Nondormant Embryos to Exogenous ABA

When ABA was applied to young nondormant embryos (7–10 DAP), it inhibited germination as long as it was present. However, whatever the dose used (5×10^{-6} M, 5×10^{-5} M) and the duration of its application (5 or 10 d) ABA was unable to induce dormancy since after transfer on control medium, germination occurred (Figs. 1 and 2). An application of fluridone to these embryos did not modify their responsiveness to exogenous ABA or their mode of development (data not shown).

Changes in Embryo Responsiveness to Exogenous ABA during Induction of Embryo Dormancy

As seed development progressed, exogenous ABA became progressively more effective in inducing dormancy (Fig. 3): a 5-d culture on ABA 5×10^{-5} M resulted in an induction of dormancy in more than 50% of the 13 DAP nondormant embryos and in all 17 DAP partially dormant embryos.

Physiological Consequences of an Application of Fluridone to Dormant Embryos

After embryo dormancy was well established (22 DAP) an application of fluridone at the time of isolation allowed germination to occur very quickly on control medium (Fig. 4B).

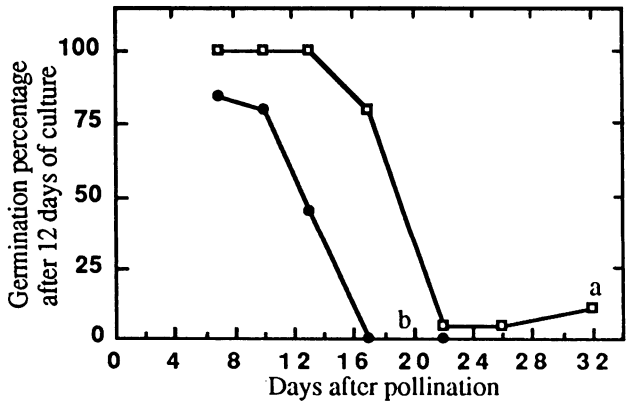

Figure 3. Changes, with time after the pollination, in the germination percentage observed after 12 d of culture, on basal medium directly (a), after 5 d on ABA 5×10^{-5} M (b).

[2] Abbreviations: RIA, radioimmunoassay; DAP, day(s) after pollination; VP, regulatory protein.

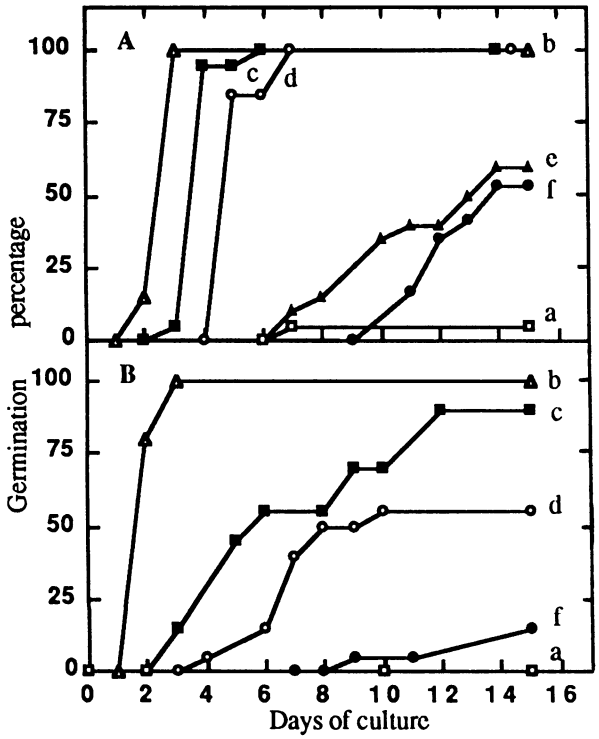

Figure 4. Changes, during culture, of the germination percentage of 22-d-old embryos (A) or 31-d-old embryos (B) cultured on agar medium without any treatment (a), after a treatment with 20 μL of 100 μg/mL fluridone in acetone 10% applied at the time of the isolation (b), after 1 d of culture (c), 3 d (d), 5 d (e), 7 d (f).

This effect was not due to water or even to traces of acetone in which fluridone was dissolved.

An application between the two cotyledons (directly to the axis) resulted in a more rapid response as compared to an application to the upper cotyledon. Thereafter, fluridone (20 μL of 100 μg/mL solution) was always applied with a microsyringe between the two cotyledons. As early as the second day of culture, a high percentage of germination was observed. The growth of the plantlets was quite normal but the cotyledons were white: Chl and carotenoids were only occasionally present in circumscribed areas. However in the majority of cases, the first leaves to appear already contained Chl.

When fluridone was not applied at the time of isolation but after some days of culture, it may in some cases remain totally effective (for example, after 1 or 3 d of culture in 22-d-old embryos, Fig. 4A, lines c and d). However, it was generally less effective: either germination is lower (for example after one day of culture for 31-d-old embryos (Fig. 4B, line c), or the germination percentage reached is lower (Fig. 4A, lines e and f; Fig. 4B, lines d–f). The later the moment of fluridone application, the more Chl was present in the cotyledons of the plantlets.

Physiological Consequences of an Application of Fluridone to Isolated Axes of Dormant Embryos

After embryo dormancy was well established removal of the cotyledons had no significant effect on percent germination (12.5% ± 0.5 for embryos; 11.2% ± 0.4 for axes). This indicates that axes themselves were dormant. When fluridone (5 μL of a solution at 100 μg/mL) was applied to isolated axes at the time of the isolation, germination occurred very quickly (Fig. 5B). Its effectiveness decreased when the application occurred after some days of culture (Fig. 5B, lines c, d, e). The change observed was the same as that observed using the intact embryo (Fig. 4B, lines c, d, and f).

ABA Content in Response to Fluridone Treatment

Because germination induced by fluridone appeared very rapidly, endogenous ABA content was assessed 24 or 30 h after treatment (before any sign of germination was visible). When dormant embryos were cultured *in vitro*, the ABA content decreased markedly during the first day but no significant difference was observed between fluridone treated and nontreated embryos. This decrease must be related to a decrease in ABA content in the cotyledons (Table I); thus for 26-d-old embryos, the ABA content fell from 24 ng per pair of cotyledons to less than 1 ng during the first day of culture. The decrease in ABA in the cotyledons, which represented more than 90% of the fresh weight of the embryos older than 21 DAP, masked the variations that occurred in the axes.

When whole embryos were cultured (Table I), axis ABA content increased in the control, whereas it drastically fell to a nondetectable value in fluridone-treated embryos. When isolated axes were cultured (Table I), a similar change was

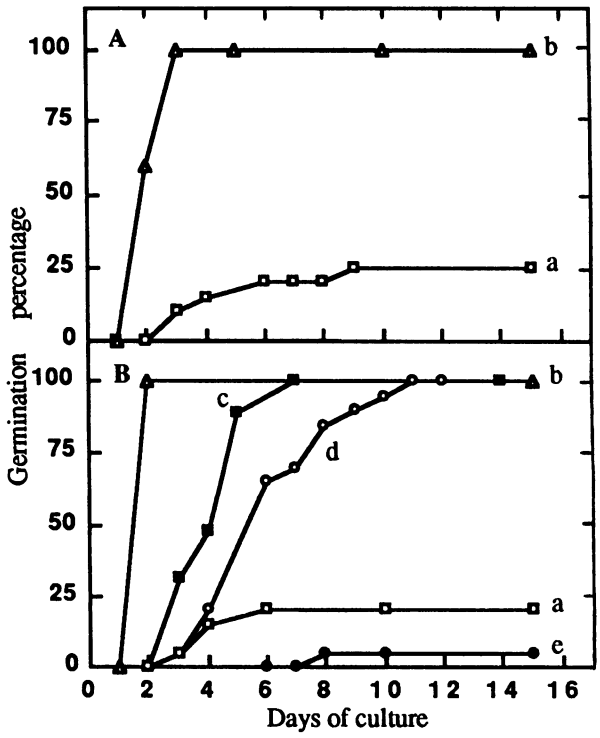

Figure 5. Changes, during culture, of the germination percentage of 28-d-old axes (A) or 31-d-old axes (B) cultured on agar medium without any treatment (a), after a treatment with 5 μL of 100 μg/mL fluridone in acetone 10%, applied at the time of the isolation (b), after 1 d of culture (c), after 2 d (d), after 7 d (e).

Table I. *Changes in Cotyledon and Axis ABA Content of 26 DAP Embryos after 24 h of Culture in the Presence or Absence of Fluridone*

Sample	ABA/Pair of Cotyledons	ABA/Axis	ABA/Axis (g fresh weight)
	pg	*pg*	*ng*
At the time of isolation	24,150 ± 2,400[a]	177 ± 18	38 ± 4
Embryos			
Without fluridone	330 ± 30	290 ± 29	58 ± 6
With fluridone	210 ± 22	ND[b]	ND
Isolated axes			
Without fluridone		423 ± 42	60.9 ± 6
With fluridone		68 ± 7	9.1 ± 0.8

[a] Mean ± SE (n = 6). [b] Not detectable (<1.2 ng/g fresh weight).

observed. In fluridone-treated axes, not only was the synthesis of additional ABA (apparent in controls) inhibited but the ABA already present in the embryo was metabolized.

Responsiveness of Fluridone-Treated Dormant Embryos to Exogenous ABA

When dormant embryos treated by fluridone at the time of isolation were cultured on ABA medium (5×10^{-5} M), germination was inhibited as long as exogenous ABA was present: 5 d (Fig. 6B, line a) or 10 d (Fig. 6C, line a). However, germination occurred as soon as embryos were transferred to control medium (Fig. 6, B and C, line b). If the fluridone application was performed at the time of the transfer to control medium (Fig. 6, B and C, line d) its effectiveness was only partial, but quite similar to that which was observed after 5 d on control medium (Fig. 6A, line c).

DISCUSSION

Isolated immature embryos of *H. annuus* acquire the capacity to germinate very early during seed development (7–10 DAP). Addition of ABA to the culture medium (5 or 50 μM) leads to the inhibition of their germination. This inhibition of premature germination by exogenous ABA has been reported by several authors (reviewed in ref. 5). In nondormant, mature seeds of *Brassica napus* and *Sinapis alba*, Schopfer *et al.* (9) and Schopfer and Plachy (10) suggested that the primary action of ABA involved the control of water uptake. Results obtained by Finkelstein *et al.* (1) during embryogeny in *Brassica* are consistent with this hypothesis. Some authors equate this inhibition of germination to dormancy. However, in *H. annuus*, this inhibition requires the continued presence of the hormone in the culture medium and germination occurs when the embryos are transferred to control medium.

This is therefore a temporary inhibition that is distinct from dormancy which, during development *in situ*, is induced by ABA in less than 1 week. To study the inductive role of ABA *in vitro*, two periods of application were compared: 5 d and 10 d. In young embryos (7–10 DAP) ABA was unable to induce dormancy regardless of duration of application and the dose used (5 or 50 μM).

Exogenous ABA became effective immediately prior to the natural induction of dormancy. For example, 5 d culture on a medium containing 5×10^{-5} M ABA resulted in partial dormancy in 13 DAP embryos while total induction of dormancy occurred in 17 DAP embryos. Hole *et al.* (3) observed also that the addition of exogenous ABA to fluridone treated kernels induced dormancy if it was supplied during a critical time period.

These observations suggest that either a change in sensitivity to ABA occurs during development, or the existence of a second factor is necessary along with ABA to induce dormancy. In accordance with this second possibility, Smith *et al.* (12) proposed a model in which ABA binds to a regulatory protein (VP). Specific induction of dormancy depends upon

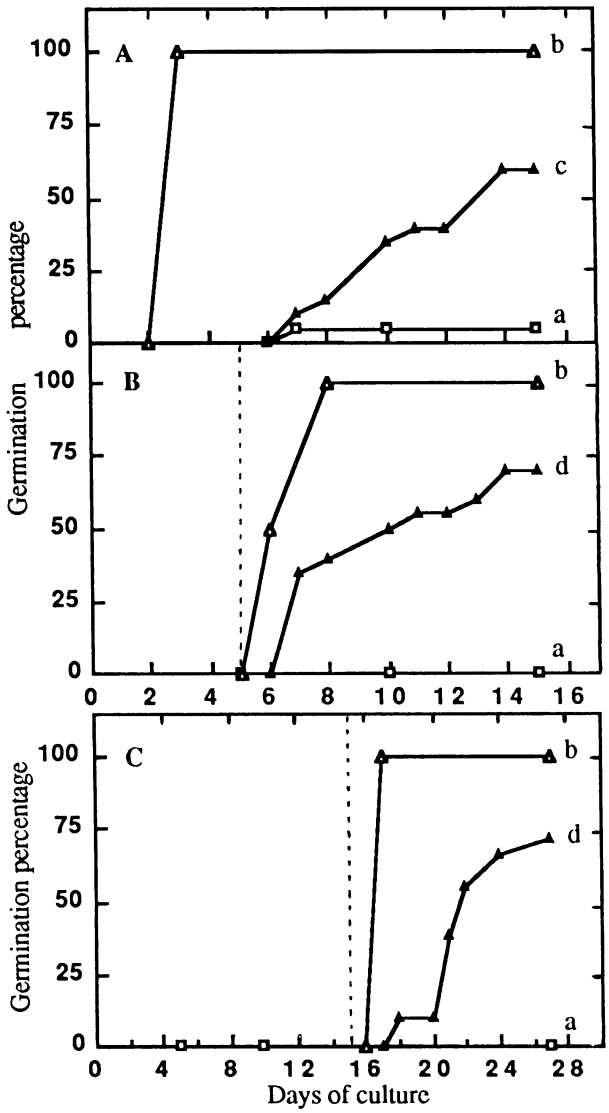

Figure 6. Changes, during culture, of the germination percentage of 22-d-old embryos cultured on agar medium directly (A), after 5 d on ABA 5×10^{-5} M (B), after 15 d on ABA 5×10^{-5} M (C) without treatment (a), after a treatment with 20 μL of 100 μg/mL fluridone in acetone 10% applied at the time of the isolation (b), after 5 d of culture (c), or at the time of transfer to control medium (d).

the state of this ABA/VP aggregate. ABA cannot act as a regulator prior to the time that the VP protein is synthesized. If VP is present, then the regulatory functions depends upon ABA concentration. According to this hypothesis, ABA in *Helianthus* would be unable to induce dormancy in 7 DAP and 10 DAP embryos due to the absence of such a second factor. Beyond 13 DAP, ABA is able to induce dormancy, *i.e.* this second factor must have developed.

It is then possible to use fluridone to modify the endogenous ABA level in older embryos. Removal of the cotyledons has no effect on physiological behavior (*i.e.* the axis itself is dormant). The RIA data indicated that the dormant axis is able to synthesize ABA; after a 1-d culture, the level of ABA increased. An application of fluridone totally inhibited this increase in ABA. It therefore appears that in the axes of *Helianthus* as in *Z. mays* embryos (2) ABA is synthesized via the indirect pathway requiring a xanthophyll precursor. Such fluridone-treated embryos became able to germinate and exogenous ABA was then unable to reinduce their dormancy.

In *Arabidopsis*, dormancy was only induced in seeds if the genome of the embryos contained the dominant ABA allele and thus dormancy was induced only by embryonic ABA (4, 6). For Karssen *et al.* (4), it seemed that in order to induce dormancy, ABA had to be synthesized close to its site of action in the embryo. However, the localization of the different ABA fractions in the seeds could not be studied in detail due to the tiny size of *Arabidopsis* seeds. Our results demonstrate that in *Helianthus*, suppression of ABA synthesis in the axis induces germination and that exogenous ABA cannot reinduce dormancy. It thus appears that a process associated to the synthesis of the inhibitor is required in order to induce dormancy.

ACKNOWLEDGMENT

The technical assistance of H. Le Bris is gratefully acknowledged.

LITERATURE CITED

1. **Finkelstein RR, De Lisle AJ, Simon AE, Crouch ML** (1987) Role of abscisic acid and restricted water uptake during embryogeny in *Brassica. In* Molecular Biology of Plant Growth Control. Alan R Liss, New York, pp 73–84
2. **Gage DA, Fong F, Zeevaart JAD** (1989) Abscisic acid biosynthesis in isolated embryos of *Zea mays* L. Plant Physiol **89:** 1039–1041
3. **Hole DJ, Smith JD, Cobb BG** (1989) Regulation of embryo dormancy by manipulation of abscisic acid in kernels and associated cob tissue of *Zea mays* L. cultured *in vitro.* Plant Physiol **91:** 101–105
4. **Karssen CM, Brinkhorst-van der Swan DLC, Breekland AE, Koornneef M** (1983) Induction of dormancy during seed development by endogenous abscisic acid: studies on abscisic acid deficient genotypes of *Arabidopsis thaliana* (L.) Heynh. Planta **157:** 158–165
5. **King RW** (1982) Abscisic acid in seed development. *In* AA Khan, ed, The Physiology and Biochemistry of Seed Development, Dormancy and Germination. Elsevier Biomedical Press, New York, pp 157–185
6. **Koornneef M, Hanhart CJ, Hilhorst HWM, Karssen CM** (1989) *In vivo* inhibition of seed development and reserve protein accumulation in recombinants of abscisic acid biosynthesis and responsiveness mutants in *Arabidopsis thaliana.* Plant Physiol **90:** 463–469
7. **Le Page-Degivry MT, Barthe P, Garello G** (1990) Involvement of endogenous abscisic acid in onset and release of *Helianthus annuus* embryo dormancy. Plant Physiol **92:** 1164–1168
8. **Le Page-Degivry MT, Duval D, Bulard C, Delaage M** (1984) A radioimmunoassay for abscisic acid. J Immunol Methods **67:** 119–128
9. **Schopfer P, Bajracharya D, Plachy C** (1979) Control of seed germination by abscisic acid. I. Time course of action in *Sinapis alba* L. Plant Physiol **64:** 822–827
10. **Schopfer P, Plachy C** (1984) Control of seed germination by abscisic acid. II. Effect on embryo water uptake in *Brassica napus* L. Plant Physiol **76:** 155–160
11. **Smith JD, Fong F, Magill CW, Cobb BG, Hole DJ** (1986) Dual sources of abscisic acid in maize kernels. Maize Genet Coop News Lett **60:** 38–39
12. **Smith JD, Fong F, Magill CW, Cobb BG, Bai DG** (1989) Hormones, genetic mutants and seed development. *In* RB Taylorson, ed, Recent Advances in the Development and Germination of Seeds. Plenum Press, New York, pp 57–69