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# Regulation of Embryo Dormancy by Manipulation of Abscisic Acid in Kernels and Associated Cob Tissue of *Zea mays* L. Cultured *in Vitro*[1]

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## ABSTRACT

**Sectors of *Zea mays* cobs, with and without kernels were cultured *in vitro* in the presence and absence of fluridone. Cultured kernels, cob tissue, and embryos developed similarly to those grown in the field. Abscisic acid (ABA) levels in the embryos were evaluated by enzyme-linked immunosorbant assay. ABA levels in intact embryos cultured in the presence of fluridone were extremely low and indicate an inhibition of ABA synthesis. ABA levels in isolated cob tissue indicate that ABA can be produced by cob tissue. Sections containing kernels cultured in the presence of fluridone were transferred to medium containing fluridone and ABA. Dormancy was induced in more than 50% of the kernels transferred from 13 to 15 days after pollination, but all of the kernels transferred at 16 days after pollination or later were viviparous. ABA recovered from kernels that were placed in medium containing fluridone and ABA suggest that ABA can be transported through the cob tissue into developing embryos and that ABA is required for induction of dormancy in intact embryos.**

The plant hormone ABA has long been associated with dormancy in plants (27). Many other regulatory roles have been attributed to ABA, including stomatal closure, inhibition of protein synthesis, and adaptation to stress (27). Critical determinations of specific physiological effects of a regulator may first require that endogenous concentrations be brought to very low levels. This can be accomplished for ABA in maize kernels by the use of genetic mutants or chemicals which inhibit ABA biosynthesis.

Continuous development of an embryo from zygote to mature caryopsis, with no intervening period of quiescence, is known as vivipary. A survey of the albino seedling mutants of maize indicated that albino mutants which accumulated intermediate carotenoids were also viviparous (21). The reduced ABA content detected in carotenoid-deficient viviparous mutants indicated that an ABA deficiency was responsible for the viviparous phenotype (3, 18, 22, 25). Fluridone, 1-methyl-3-phenyl-5(3-(trifluoromethyl)phenyl)-4(1H) - pyridinone, interrupts carotenoid synthesis in plant leaves by inhibiting phytoene desaturation (2). If applied prior to the onset of carotenoid biosynthesis in the maize kernel, fluridone also blocks carotenogenesis in the kernel (6, 7) and generates morphological and biochemical phenocopies of the viviparous mutant *vp5* (24), including reduced ABA levels.

Although carotenoid-deficient mutant and fluridone-treated maize embryos have significantly reduced levels of ABA, a substantial amount of ABA has always been detected. However, no ABA was detected in seedlings grown from mutant embryos (16), which suggested that ABA found in these embryos originated in the phenotypically normal, though heterozygous, maternal plants (23).

Maize kernels can be successfully cultured (4, 9, 10, 12, 13), and work has been done to compare kernel development in ear sections cultured *in vitro* with field-grow material. Cobb and Hannah (4) reported that *sh1* and *sh2* developed similarly in cultured and in the field with regard to physical appearance, germination percent, starch, sugar, and selected enzyme levels. Growth and development *in vitro* allows environmental control to be exercised while maintaining similarity to field conditions in other ways.

If sectors of ears are cultured *in vitro* for a few days, the contribution of the maternal plant to ABA in the kernel should be negligible, since endogenous ABA is rapidly catabolized (5, 11). Thus, homozygous mutant and fluridone-treated kernels should have low levels of ABA after several days in culture. In this study, emphasis was placed on determining the effects that manipulation of ABA concentrations in developing kernels had on embryo dormancy. The potential contribution of cob tissue to ABA in the embryo was also investigated.

## MATERIALS AND METHODS

### Genetic Material

The genetic material included TX5855, a standard WT[2] inbred line, and *w3*, *vp7* and *vp5*, viviparous mutant stocks. Mutant stocks that were used had all been backcrossed to TX5855 at least twice and subsequently selfed for two generations prior to the study. Plants were grown at the Texas A & M University Research Farm under normal cultural conditions and irrigated as necessary during the growing season. Ear shoots were bagged prior to silk emergence. When treated with fluridone, ear shucks were pulled back and the ears were

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[2] Abbreviations: WT, wild type; DAP, days after pollination.

sprayed either with a 100 mg $L^{-1}$ concentration of fluridone or distilled water. The shucks were then repositioned around the ear and the ear was rebagged.

### *In Vitro* System

Ears that were to be placed in culture were harvested at 5 DAP. The tissue culture medium used was that described by Gengenbach (9) as modified by Cobb and Hannah (4). The specific procedure for sectioning the ear into kernel blocks was the same as utilized by Cobb and Hannah (4). Sections containing about two rows of six kernels each were placed in culture without removing any of the kernels. In order to culture cob sections by themselves, ears were harvested at the same DAP as other cultures, but kernels were severed from the cob well below the point of attachment, and only rectangular blocks of cob tissue, including the pith tissue, were cultured. The medium was the same as for the kernel sections.

When fluridone was used it was added to the medium prior to autoclaving, at a concentration of 100 mg $L^{-1}$. ABA, when used, was filter sterilized and added to the medium at the concentration of $10^{-4}$ M after autoclaving. Cultures were examined daily for carotenoid appearance and expression of vivipary, and contaminated cultures were discarded. Sections from fluridone containing medium and sections from standard medium were removed and transferred to medium containing ABA and fluridone on a daily basis, from 13 to 18 DAP. These sections were harvested at maturity and scored for dormancy and vivipary. Sections segregating for *w3* were harvested at various stages and frozen at −70°C for ABA analysis. Cob sections and WT kernel sections from media with and without fluridone were harvested similarly.

### ABA Analysis

The embryos (about 0.1 g) and the cob tissue (about 1 g) were weighed and homogenized in 2 mL 80:20 (v/v) MeOH:distilled $H_2O$ under low light conditions. The following steps also utilized low light and silyated glassware. Samples were shaken overnight at 150 rpm at 4°C. Extracts were then centrifuged for 15 min at 10,000 *g* and filtered. The supernatant was collected and dried by evaporation under $N_2$ at 50°C. The residue was reconstituted with 5 mL 0.1 M $NaHCO_3$ and extracted three times with 6 mL aliquots of ether in a separatory funnel. The aqueous phase was saved in each extraction then adjusted to pH 3.0 with 0.1 N HCl at the end of the third extraction and again extracted in the same manner collecting, instead, the ether phase and reextracting the aqueous phase. The collected ether fractions were dried by evaporation under $N_2$ at 55°C and stored at −20°C.

All samples were spiked with $^3$H-ABA at the beginning of the extraction procedure to estimate recovery which averaged 60%. All ABA values are corrected for percent recovery. The extracted ABA samples were reconstituted with distilled water and 100 μl aliquots were quantitated by means of an ELISA available from Idetek, Inc. (San Bruno, CA). The standard curve for the ELISA is linear when plotted as a log by logit function. The sensitivity of the assay in the linear range was $5 \times 10^{-3}$ to 1.3 ng. This monoclonal antibody (Mab) has been utilized both in direct (20) assays such as the one we utilized and indirect (17, 19, 26) assays of ABA. No, or very low (<1000-fold), cross-reactivity with other ABA related compounds was observed (15). The quantitation of ABA using this Mab has been evaluated and validated with HPLC-UV, GC-FID, as well as GC-MS (19, 20). After incubation, and 15 min after addition of the stop reagent, the plates were scanned for optical density in a Titertek Multiskan plate reader at 405 nm. Since ABA was extracted prior to quantitation we minimized other materials that may interfere with the assay although no interference has been detected with wheat extracts (26) or xylem sap (17).

## RESULTS AND DISCUSSION

### Development of WT and Mutant Caryopses

Other researchers have recommended removing all kernels except one per section in order to achieve maximum development, since cob sections containing 6 kernels resulted in 65% kernel abortion (10). Our studies, using sections containing at least 12 kernels, do not show this rate of kernel abortion. Instead, development of the kernel sections in culture resulted in two types of sections. In the first type, which comprised approximately 15% of the sections, there was virtually no development of any of the kernels after placement in culture. Kernels would continue to appear healthy and viable but showed no increase in size or mass. Nearly all of the kernels (>90%) in the remaining sections developed to maturity in a normal manner.

One possible reason for this higher rate of development is that our kernel sections were older (5 DAP) when placed into culture. Although this gives much better developmental rates, it has a disadvantage in that early events in kernel development, prior to 6 or 7 DAP, might be obscured during the acclimation period of the kernel sections to the culture system. In our investigation, this disadvantage is far offset by the ability to have 12 or more intact kernels per section. Embryo fresh weights from cultured WT kernels grown in the presence and absence of fluridone, as well as similarly treated field-grown materials, are shown in Figure 1. There is close agreement in the increase in fresh weight over time for all embryos

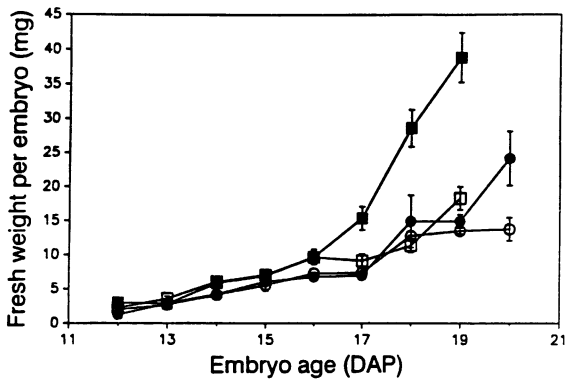


**Figure 1.** Fresh weights of embryos from TX5855 kernels. Kernels were field-grown in the presence (●) and absence (○) of fluridone (100 mg $L^{-1}$), and culture-grown in the presence (■) and absence (□) of fluridone (100 mg $L^{-1}$). Cultured kernels were placed into culture at 5 DAP.

until about 16 DAP. Fresh weight of the fluridone-treated *in vitro* cultured embryos began to increase rapidly by 17 DAP, and a similar increase in the field-grown fluridone-treated embryos was observed at 20 DAP. The main component of these fresh weight differentials appears to be due to radicle growth in the fluridone-treated kernels. Initiation of radicle elongation in fluridone treated embryos appears to have occurred at 16 DAP in cultured embryos and 19 DAP in field-grown embryos. Although radicle elongation at this age was not obvious from external examination of intact kernels, it was readily apparent when embryos were excised. Final kernel weight, size, and germinability were in close agreement for the two control groups.

Carotenoid development can normally be observed on WT ears in the field at around 12 DAP. We found that carotenoid accumulation occurred 1 to 2 d earlier in the cultured ear sections than in the field. The expression of vivipary was also detected about 2 d earlier in cultured than in field-grown kernels. These differences in developmental rates are probably due to differences in environmental conditions during kernel growth (8). The most obvious environmental differences affecting cultured kernels were that they do not experience diurnal water stress and they were grown at a constant 30°C, which is considered to be optimal (12).

In the field, since maternal plants were progeny of heterozygotes and were phenotypically normal, initial carotenoid development allowed segregating ears to be identified at about 13 DAP. Ears were sectioned and placed into culture before segregation could be observed. Therefore, we expected and found that about two-thirds of the sections from progeny of heterozygous mutant plants segregated. Homozygous *vp5* blocks phytoene desaturation and the *w3* blocks the desaturation of phytofluene (7). Segregating mutant kernels in the field and in cultured sections were white for both mutants. In the case of *vp7*, lycopene, δ- and γ-carotene accumulate (7). Thus, the cultured segregating mutants of *vp7* as well as those in the field were pink. There were no obvious differences in color between field and cultured segregating sections.

### Induction of Dormancy in Cultured Fluridone-Treated Caryopses

ABA was added to fluridone-treated, cultured kernel sections to determine if dormancy could be induced. This was done to show that the fluridone mediated induction of vivipary was due only to lack of ABA, and that ABA could be transported via the cob tissue into developing embryos. The addition of ABA into the medium of kernel sections that were older than 15 DAP had no effect in halting the onset of vivipary. The critical point for induction of dormancy is best identified by embryo stage rather than age, since embryo development is not constant in all locations. Stage 4 embryos, as defined by Abbe and Stein (1), are observed at 15 DAP in TX5855 grown in south central Texas (8). ABA addition had no noticeable effect on the external appearance of nonfluridone-treated kernel sections at any age of treatment. Addition of ABA to the medium between the ages of 13 to 15 DAP, however, had the effect of producing dormant kernels at rates of between 50 and 80% of the kernels on a section as shown in Table I. The remaining kernels still expressed vivipary.

**Table I.** *Effects of ABA on Embryo Dormancy of TX5855 Kernel Blocks Cultured with Fluridone (100 mg $L^{-1}$) and Transferred to Medium Containing Fluridone and ABA ($10^{-4}$ M) at Ages of 13 to 18 DAP*

| Age at ABA Treatment (DAP) | Total No. of Kernels | Viviparous | Dormant | Viable |
|---|---|---|---|---|
| | | | % | |
| 13 | 21 | 43 | 57 | 100 |
| 14 | 19 | 37 | 63 | 100 |
| 15 | 16 | 19 | 81 | 100 |
| 16 | 15 | 100 | 0 | NA[a] |
| 17 | 17 | 100 | 0 | NA |
| 18 | 37 | 100 | 0 | NA |

[a] Not applicable.

Dormant kernels were harvested when mature and allowed to dry for 3 months to make sure they were viable as well as dormant. Subsequent germination of these ABA-induced dormant kernels resulted in 100% germination.

ABA from whole kernels at 14 DAP that had been in media with fluridone or fluridone and ABA were assayed for ABA quantity. ABA levels in kernels developing *in vitro* with fluridone were 4.7 ± 0.7 ng ABA g fresh weight$^{-1}$ while kernels developing with fluridone and $10^{-4}$ M ABA contained 56.0 ± 4.5 ng ABA g fresh weight$^{-1}$. Since the kernels were not in contact directly with the medium, they must receive all of the nutrients from the medium through the supporting cob tissue. This provides evidence for the transport of ABA through the cob tissue into developing maize embryos. It also indicates that ABA is required for embryo dormancy in maize kernels, but there is no stringent requirement for ABA produced specifically by the kernel itself in dormancy induction. This is different from *Arabidopsis thaliana* where dual sources of ABA have also been reported (14). In addition, the ABA deficiency that characterizes many of the viviparous mutants may be satisfied by the addition of exogenous ABA if it is supplied during a critical time period.

Since placing ABA deficient kernel sections into media containing ABA after 15 DAP had no effect on the induction of embryo dormancy, it seems that there is a developmental period beyond which the embryos are insensitive to ABA or may no longer be able to take it up through the cob tissue. The response to ABA may also be concentration dependent. The level of ABA supplied to the media was high and it is possible that internal ABA concentrations in the dormant embryos were higher than normal physiological levels at the 15 DAP time period. However, the levels of ABA in the 14 DAP kernels cultured with fluridone and ABA are consistent with values shown in Table II from WT kernels grown *in vitro*. There is some evidence to suggest that maternal ABA is in some instances capable of dormancy induction. In the field, under conditions normally favorable for the production of large amounts of ABA by the heterozygous maternal plant, hot dry weather at about 14 to 16 DAP, we have observed that most of the viviparous lines will occasionally produce dormant homozygous mutant kernels.

**Table II.** *Embryo ABA Levels (ng $g^{-1}$ fresh weight) at 15 DAP for Field-Grown and TX5855 Kernel Blocks Cultured With and Without Fluridone (100 mg $L^{-1}$) and Segregating w3 and W3 Kernels*

| Plant Material | ABA ng $g^{-1}$ Fresh Weight | |
|---|---|---|
| | Field grown | *In vitro* cultured |
| *W3*·TX5855 | 15.3 ± 3.7 | 60.2 ± 7.1 |
| *w3*·TX5855 | 9.0 ± 2.2 | 2.9 ± 0.9 |
| TX5855 | 35.6 ± 19.3 | 31.7 ± 5.5 |
| TX5855+ fluridone | 9.3 ± 0.8 | 0.7 ± 0.2 |

### ABA Levels in Mutant and WT Caryopses

The amounts of ABA in 15 DAP *W3* and *w3* embryos from both field-grown and cultured sections are shown in Table II. ABA values for field-grown and cultured TX5855 embryos, both control and fluridone-treated, also are presented in Table II. The various combinations of genetic material with field or culture growth allowed the different sources of ABA to be dissected away. ABA levels *in vitro* are consistent with field grown material. With one exception, however, *in vitro* cultured material had the same or less ABA than its field grown counterpart. This indicates the magnitude of the possible maternal contribution. In addition, cultured *w3* mutants show a higher ABA level than WT kernels cultured with fluridone. The only component of potential ABA production that was different between *w3* mutant kernels in culture and the TX5855 kernels cultured with fluridone is the contribution of the cob tissue. This raises the possibility that the cob tissue is capable of producing ABA and transporting that ABA into the developing embryo.

### ABA Levels in Cultured Cob Sections

Jones and Brenner (13) have reported that cob tissue cultured in their system stays viable for only a few days. This is very different from cob tissue of TX5855 cultured by us. Isolated cob tissue showed no signs of necrosis or degradation through 22 DAP (in culture for 17 d). This allowed us to extract and measure ABA levels over time in isolated cultured cob tissue both in the presence and absence of fluridone. The amounts of ABA found in various ages of isolated cob tissue cultured with or without fluridone are shown in Figure 2. Since fluridone blocks carotenoid synthesis, the only source for ABA produced by the carotenoid pathway in cob tissue grown with fluridone is from preformed carotenoids, and the level of these carotenoids would be expected to decrease over time. ABA levels in cob tissue that were untreated, were significantly higher than fluridone-treated material indicating new carotenoid synthesis with subsequent production of ABA from those carotenoids. The decline in production of ABA by the untreated cob tissue after 18 DAP may be due to decreasing viability of the tissue, although we saw no external evidence of this. It is also possible that the cob tissue follows a genetically programmed ABA production schedule or that the levels of ABA that we observed were influenced by the *in vitro* system *per se*. This cannot be ruled out because the cob, unlike the kernels, is not developing as an intact organ.

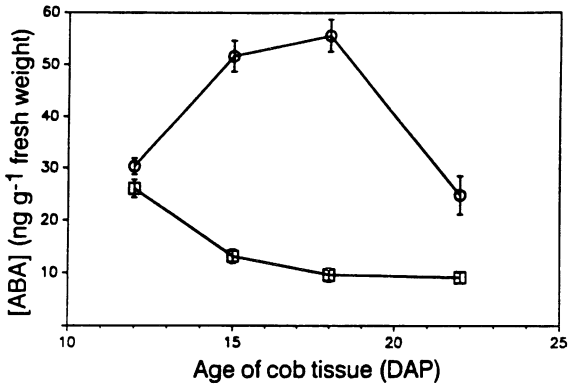


**Figure 2.** Abscisic acid levels (ng $g^{-1}$ fresh weight) of isolated TX5855 cob tissue, cultured from 5 DAP with (□) and without (○) fluridone (100 mg $L^{-1}$).

Nevertheless, the evidence suggests that the cob tissue is at least capable of producing appreciable quantities of ABA which may be translocated to the kernel.

## CONCLUSIONS

The interaction between sources of ABA found in maize embryos is complex. Nevertheless, it is possible to separate them, to some degree, using genetic mutants and chemicals which inhibit carotenoid biosynthesis, and the *in vitro* system of kernel culture. The maternal contribution of ABA to the developing embryos is not a trivial amount, but the physiological roles for this component are unknown. The maternal component includes production of ABA by the cob tissue which must be considered when investigating effects of subphysiological levels of ABA on embryo development.

By culturing normal kernel blocks with fluridone, we were able to produce embryos with very low levels of ABA (0.72 ng $g^{-1}$ fresh weight), and these kernels were 100% viviparous. However, we were able to induce dormancy in such kernels by transferring these kernel blocks to medium containing fluridone and ABA ($10^{-4}$ M) prior to 16 DAP. These carotenoid-deficient, dormant kernels matured normally, and subsequent germination was 100%. This was an effective reversal of the viviparous effects of the ABA deficiency.

Since ABA in the medium is analogous to maternal ABA, it appears that the maternal ABA component in normal, field-grown embryos may have a function in the induction of embryo dormancy.

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