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Communication

Abscisic Acid in Developing Zygotic Embryos of *Theobroma cacao*[1]

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ABSTRACT

Abscisic acid (ABA) levels were measured by enzyme-linked immunosorbent assay in developing zygotic embryos of *Theobroma cacao*. ABA was detected in all embryos tested, with a peak of ABA at levels of 1 to 3 micrograms per gram fresh weight during early maturation. This corresponded to embryos of 10 to 30% dry weight and to early stages of anthocyanin and lipid accumulation.

Cacao, an understory tree of the moist Neotropics, is cultivated as the source of chocolate. Germplasm transport and storage in this species are hindered by the fact that the seeds of cacao are recalcitrant (desiccation sensitive) as well as cold sensitive (2, 3). They cannot undergo dry or cold storage and deteriorate within a few weeks of maturity. In contrast, orthodox (desiccation tolerant) seeds undergo a natural drying to 5 to 10% moisture at maturity and can often remain viable in the dry state for years.

ABA has been implicated as one factor involved in the development of this tolerance to extreme desiccation in developing orthodox embryos. Analyses of several orthodox species have shown that ABA levels peak midway during development, preceding maturation and desiccation (13). Conversely, low ABA levels, induced by mutation or by the application of fluridone, have been correlated with the induction of vivipary and desiccation sensitivity in seeds of normally orthodox species (7, 9, 12).

Because of the association of ABA with the development of desiccation tolerance in orthodox seeds, the ABA status in developing recalcitrant embryos is of interest, since these do not develop a tolerance for drying. Such information would be useful in evaluating the relationship of ABA to the presence or absence of mechanisms adapted to surviving the stress of desiccation. The following analyses were made to determine the ABA levels in developing embryos of cacao as part of a study of the developmental physiology of tropical recalcitrant seeds.

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MATERIALS AND METHODS

Embryo Harvest and Staging

Cacao (*Theobroma cacao* L.) fruit were generously provided by the Hershey Foods Corporation. Embryos of various stages were excised and stored frozen at −30°C until analyses were performed. Embryos were staged in two ways. Immature white embryos were grouped by the length of the embryo as <2 mm (approximately 1–2 mm), 2 to 4 mm, 4 to 6 mm, 6 to 8 mm, 8 to 10 mm, 10 to 12 mm, and 12 to 14 mm in length. Those of the first three groups (<2–6 mm) were analyzed as whole embryos, while larger embryos were divided into axes and cotyledons and analyzed separately.

Embryos more developmentally advanced than these do not increase appreciably in length, but rather, undergo changes characteristic of maturation, including the accumulation of anthocyanins and lipids and a decrease in the moisture content of the embryo (11). In addition, embryos within the same fruit vary somewhat as to developmental stage. For staging these later embryos, excised embryos were frozen for storage individually in numbered vials. Cotyledon pieces from 186 of these, representing six different pods of various developmental stages, were then tested individually for the percent moisture content by drying a known weight of cotyledonary tissue overnight in a drying oven at 90°C and reweighing the dry, cooled tissue. Eight to 14 embryos in each of the following groups were then pooled for ABA analyses from axes and cotyledon tissue: >90%, 88 to 90%, 84 to 88%, 80 to 84%, 70 to 79%, 60 to 69%, 50 to 59%, 37 to 49%, and 30 to 36% moisture. Axis and cotyledonary tissue from three mature, 5-d germinating seeds were also pooled and analyzed. Pooling of cotyledon tissue from the same embryos (except germinating embryos) was done for anthocyanin and lipid quantification in order to confirm the validity of using the less time-consuming method of moisture determination as a staging tool.

ABA Analysis

For ABA extraction, embryo tissues were ground with a mortar and pestle in cold 80% methanol (v/v) (20 mL/g fresh weight), incubated for 1 h, and centrifuged at 3000*g* for 10 min. The supernatant was diluted with water to 70% methanol and passed through a C_{18} PrepSep extraction column (Fisher; prewashed with 2 mL of 70% methanol), followed by

a wash with 1 mL of 70% methanol. Methanol was removed from the sample by rotoevaporation *in vacuo*, and ABA measured by ELISA using a commercial monoclonal antibody against ABA and alkaline phosphatase-ABA enzyme tracer (Idetek, San Bruno, CA). The procedures described by Idetek for the ABA ELISA were followed. Because the antibody is specific for (+)ABA and the racemic mixture of (±)ABA was used as a standard, the resulting overestimation was corrected.

Lipid and Anthocyanin Extraction

Embryo cotyledonary tissues were extracted for lipids and anthocyanins using the technique of Folch *et al.* (5). Anthocyanins were measured in the aqueous methanol phase using the technique of Swain and Hillis (17). The total lipid extracted in the organic phase was determined gravimetrically.

RESULTS AND DISCUSSION

ABA was detected by ELISA in all embryos tested, ranging from 1 to 2 mm in length (<2 mm) to mature germinating embryos, with a peak of ABA at levels of 1 to 3 μg/g fresh weight during early maturation of both axes and cotyledons (Fig. 1). Dilution curves of cotyledon and axis extracts paralleled the dilution of ABA standard in the ELISA. Embryos with elevated levels of ABA represented the early stages of moisture loss, ranging from >90% to approximately 70% moisture and were also undergoing early anthocyanin and lipid accumulation. Anthocyanin and lipid analyses of embryos staged by moisture content indicated that these factors were closely correlated (Fig. 2). This early maturation peak of ABA observed in developing zygotic embryos of cacao is similar to that reported for developing embryos of other species (13).

Evidence has accumulated that in orthodox seeds, ABA appears to be related to the development of desiccation tolerance. Seeds of an ABA-deficient, ABA-insensitive mutant of *Arabidopsis*, a normally orthodox species, have been shown

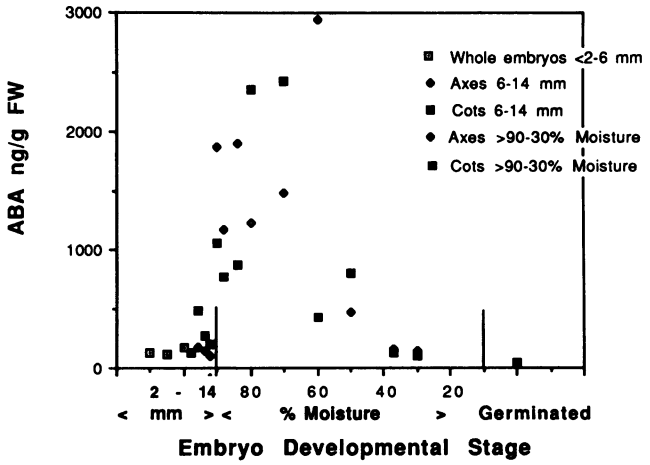

Figure 1. ABA in developing cacao embryos of various stages. Early embryos were staged by length (<2–14 mm), while older embryos were staged by percent moisture in the cotyledons. Analysis was also made of 5-d germinating embryos.

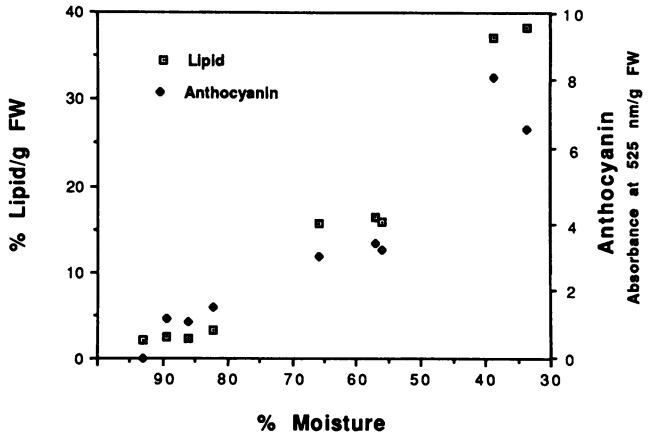

Figure 2. Anthocyanin and total lipid levels in embryos of decreasing moisture.

to be desiccation sensitive (9). Similarly, vivipary has been induced in several orthodox species either by selecting for mutants with low levels of ABA (12) or by the application of fluridone (7), an inhibitor of carotenoid, and thus, ABA synthesis. In addition, the recalcitrant, viviparous embryos of mangrove have been shown to be insensitive to ABA (16).

In cacao, however, high levels of ABA are present during development even though they do not stimulate a tolerance of desiccation in the traditional sense. Moisture levels in cacao cotyledons do not reach below approximately 30% upon maturation. This is, however, significantly lower than the moisture content of the prematuration embryo, which is greater than 90%. It is possible that some acclimation is needed for embryonic membranes to undergo even this partial drying and that ABA may be involved in adapting to this stress.

ABA has also been implicated in inhibiting precocious germination of developing embryos. Soybean and rapeseed embryos have been shown to germinate prematurely when isolated from the ovule unless they are cultured in the presence of ABA (1, 4). As development progresses in orthodox seeds, however, ABA levels generally drop, corresponding to the loss of water from the embryo. Thus, it has been postulated that in mature orthodox seeds it is the lack of water rather than the presence of ABA which inhibits germination (4, 8). In cacao, premature germination may also be inhibited by the peak of endogenous ABA which occurs in early maturation, but as ABA levels drop, factors external to the nondesiccating embryo, such as in the seed coat, appear to become important as inhibitors of germination (6).

Because significant amounts of ABA are present during cacao embryo development, such embryos may lack mechanisms for desiccation tolerance which can respond to ABA, such as membrane adaptations in lipids, sugars, antioxidants, or other factors which have been postulated for orthodox seeds (10, 14, 15). Alternatively, sensitivity to ABA may be low, as reported for the recalcitrant embryos of mangrove (16). However, exogenous ABA can stimulate maturation events in cultured immature embryos of cacao (VC Pence, manuscript in preparation), and this may be one function of

the high levels of ABA during *in vivo* development. Studies which reduce ABA levels in these embryos, either chemically or genetically, could be useful in elucidating the role of ABA in cacao embryo maturation.

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