

Pattern of Variations in Abscisic Acid Content in Suspensors, Embryos, and Integuments of Developing *Phaseolus coccineus* Seeds¹

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ABSTRACT

Free abscisic acid (ABA) content in suspensors, embryos, and integuments was determined during seed development of *Phaseolus coccineus*. A highly specific and sensitive solid-phase radioimmunoassay based on a monoclonal antibody raised against free (S)-ABA was used for ABA quantification. Very small amounts of ABA were detected in the suspensor during initial stages of development; later two peaks of ABA occurred. Levels of ABA in the embryo and integument show a coincident triphasic distribution: two maxima in ABA content occurred when the embryo was 11 to 12 and 15 to 16 millimeters in length; later, when the embryo was 19 to 20 millimeters long, a further increase was observed. The role of ABA in runner bean seeds is discussed in relation to the development of the different seed tissues.

Flowering plants are characterized by enormous variation, on a common theme, in the details of embryogenic development. The first division of the zygote forms two daughter cells with different developmental fates: the basal cell produces the suspensor while the apical cell is destined to produce the embryo proper. The suspensor cells develop rapidly by endomitosis. The embryo cells develop subsequently and more slowly by mitosis (15).

Plant species with massive suspensors have reduced endosperm and vice versa, and some interchangeability of the two structures, apparently developed for the control of early growth and differentiation of the embryo in the angiosperms, has been suggested (7). Much attention has been devoted to the embryo-suspensor system in an attempt to understand the physiological role of the suspensor. Nagl (15) considered that one function of the suspensor may be the synthesis and secretion of hormones to the growing embryo. Alpi *et al.* (2), investigating this hypothesis, found a high level of GA²-like activity in the embryo-suspensor system of *Phaseolus coccineus* at two stages of embryogenesis (heart and cotyledonary embryo stages). More recently, several GAs have been identified in both tissues (16, 17), and quantitative data show that the level of these hormones in the suspensor is almost 10 times that of the embryo. Biologically active GAs are predominant in the two tissues; in the senescing suspensor the 2 β -hydroxylated and virtually inactive GA₈ has been identified.

GA biosynthesis performed by means of a cell-free system from suspensor (5) and embryo (21) of developing seeds of *P. coccineus* showed that these tissues contain all the enzymes necessary to convert mevalonic acid to GAs.

High cytokinin activity has also been detected in developing seeds of *P. coccineus* at the same two stages of embryogenesis reported above (14). Alpi *et al.* (2) found that the methanolic extracts of suspensors and embryos showed some inhibitory activity in oat coleoptile bioassay, and suggested that these extracts may contain abscisic acid-like substances.

In this article, we report the ABA levels in embryo, suspensor, and integuments of *P. coccineus* during seed development with the aim of extending information on the hormonal relations between these seed components.

A solid-phase RIA (22) based on a monoclonal antibody against free (S)-ABA, capable of quantifying low levels of ABA in crude aqueous extracts of plants, has recently been developed. This new technique allowed analyses of ABA in suspensors and embryos in the very early developmental stages using only a very small amount of tissue. It is worth recalling that in previous experiments on GA in *P. coccineus* (16), over 10,000 and 5,000 excised suspensors and embryos, respectively, were used for analyses by physicochemical methods.

MATERIALS AND METHODS

Plant Material

Plants of *Phaseolus coccineus* of the same white-seeded variety used in our previous studies on GAs (16) were grown in the field. Growth stages of developing seeds were classified on the basis of embryo length as follows: stage A, 0.5 mm (early heart stage); stage B, 1 to 2 mm (late heart stage); stage C, 3 to 4 mm (early cotyledonary stage); stage D, 5 to 6 mm (cotyledonary stage); stages E through M, successive 2-mm increases in length (cotyledonary stages).

Seeds were collected from July to September 1989. Each stage was collected as soon as it was available on the plant throughout the entire growing season. Embryos, suspensors, and integuments were removed under a stereoscopic microscope and all seed tissue was kept in an ice bath during isolation. The collected material was then immediately weighed and frozen under liquid nitrogen.

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² Abbreviation: GA, gibberellin; RIA, radioimmunoassay.

Extraction

Isolated seed tissues (5 to 10 organs/extraction) were extracted separately in distilled water (v/w = 20:1) for 16 h at 4°C in the dark. As low as a few mg of tissue can be extracted for RIA analysis of ABA. Extracts were centrifuged (13,000g, 10 min) and the supernatants, appropriately diluted, were used for RIA analysis. Water extraction efficiency was checked and found to be over 95% (22). Extraction and analysis of ABA were repeated five times with seed tissues from collections taken at different times.

ABA Determination

The amount of ABA in the tissues was determined by means of a solid-phase RIA based on the use of a monoclonal antibody (DBPA1) raised against free (S)-ABA (22).

Validation of RIA Results

The absence of cross-reacting material other than ABA in the extracts was verified by HPLC fractionation of the crude aqueous extracts of embryos, suspensors, and integuments as described previously (23): an HPLC instrument (LDC)

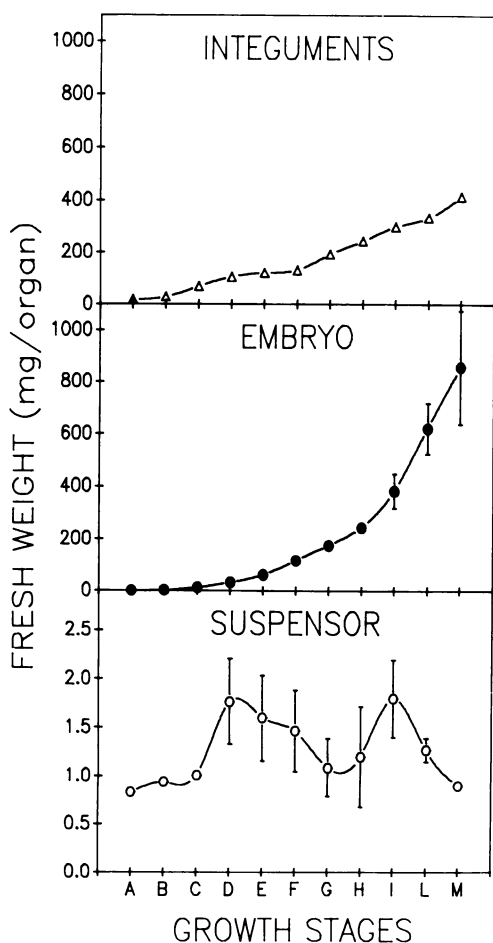


Figure 1. Growth stages of single tissues from *P. coccineus* seeds. Data ± SE (n = 10); when not shown, SE bar was smaller than the symbol.

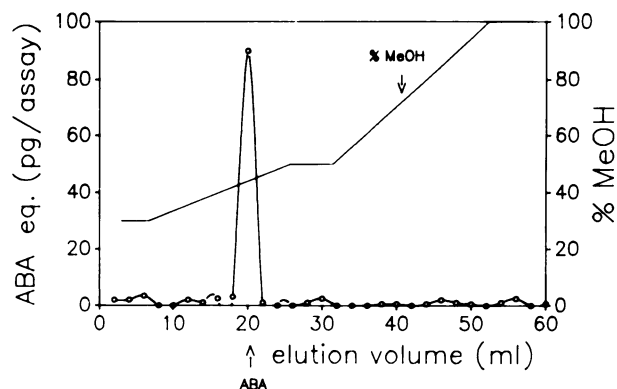


Figure 2. Distribution of immunoreactivity on HPLC fractionated crude aqueous extract of *P. coccineus* suspensors. ABA migration is indicated by the arrow.

equipped with a UV absorbance detector operating at 254 nm was used. The column (15 cm × 0.25 inch o.d., packed with Lichrosorb RP 18, 10 μm) was eluted at a flow rate of 1 mL/min using different proportions of methanol and water (added with 0.05 M acetic acid): 30% methanol for 6 min; a linear gradient 30 to 50% methanol for 20 min, 50% methanol for 6 min; and a linear gradient 50 to 100% methanol for 15 min. Two-mL fractions were collected, dried under vacuum, and resuspended in 75 mM PBS (pH 7). Each fraction was assayed in triplicate by RIA.

Noncompetitive interferences were evaluated by internal standardization experiments.

RESULTS AND DISCUSSION

Figure 1 reports the growth pattern of the single components of *P. coccineus* seeds. These results show a rapid growth of both embryos and integuments, while the weight of the suspensor remains almost the same for the whole period considered.

The small amount of material available for ABA content analysis in the early stages of *P. coccineus* seed development called for an immunological method of analysis sensitive and specific enough to allow ABA quantification in crude aqueous extracts. The HPLC experiments showed the presence of immunoreactive material only in the fraction with the same elution volume as ABA, indicating the absence of immunoreactive compounds other than ABA in crude aqueous extracts of all three tissues examined (data shown only for suspensor in Fig. 2). The absence of noncompetitive interference, checked by internal standardization experiments, was verified by plotting recovered against added ABA in the presence of constant aliquots of aqueous extracts of the tissues considered (Fig. 3). ABA content in the individual tissues is reported in Figures 4 and 5 per unit of weight and per organ, respectively.

For the first time, ABA status in the suspensor of *P. coccineus* seeds during embryogenesis is reported (Figs. 4 and 5). The suspensor ABA level was low in the first five growth stages (48 ng g⁻¹ fresh weight at stage A reaching 123 ng g⁻¹ fresh weight at stage E) and showed a biphasic distribution in

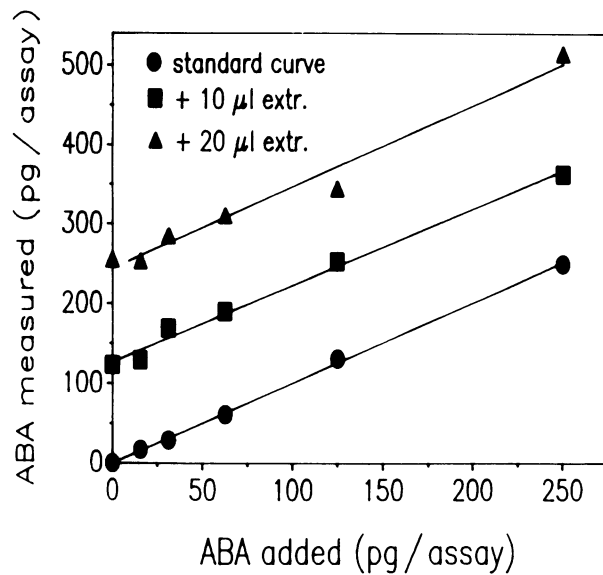


Figure 3. Internal standardization with crude aqueous extract of suspensors. Standard curve: slope = 1.007, $r = 0.999$; standard curve + 10 μl extract: slope = 0.962, $r = 0.995$; standard curve + 20 μl extract: slope = 1.028, $r = 0.987$. Experiment carried out with integument and embryo extracts gave comparable results.

the following stages. The first peak was observed at stage F (1738 ng g^{-1} fresh weight) and the second at stage I (940 ng g^{-1} fresh weight). The function assigned to the angiosperm suspensor is not purely mechanical; it may serve to transfer compounds synthesized either in the suspensor or in other parts of the seed to the developing embryo (15). *In vitro* culture of *P. coccineus* embryos has shown that the attached suspensor strongly affects embryo growth at the heart stage of development (6, 26).

The ABA level in integuments and embryos is also presented in Figures 4 and 5; both tissues show a triphasic distribution during seed growth. Considering ABA content per organ, ABA increased with the growth of the individual tissues to reach a maximum at stage M with two small peaks in both tissues at stages G and I. When ABA content was calculated per unit of weight, integuments showed a maximum at growth stage G (3426 ng g^{-1} fresh weight) coinciding with the first small ABA peak in the embryos (1494 ng g^{-1} fresh weight), while the embryos showed a maximum at growth stage I (2213 ng g^{-1} fresh weight) coinciding with the second small peak in the integuments (3021 ng g^{-1} fresh weight). A third increase was observed later, at growth stage M, in both tissues (integuments 2552 ng g^{-1} fresh weight; embryos 2156 ng g^{-1} fresh weight).

The validity of this fluctuating ABA accumulation pattern is supported by the fact that sample collection of each growth stage was made at different times of the growing season for each of the five ABA quantifications so that possible interference by insufficient sampling was minimized. Moreover, it has been reported that endogenous ABA levels show two peaks in developing embryos of pea (24) and bean (11) and three to four peaks in rapeseed (9). Findings from pea (24) have demonstrated that the pattern of ABA accumulation closely reflects the biphasic nature of seed growth.

Numerous studies have measured the level of ABA during seed development in many plants, and have shown a rise of ABA in parallel with embryo growth (13).

This pattern of rise and decline raises questions about the origin of the ABA, regulation of its level, and its role in the seed development process. In *P. coccineus*, Yeung and Sussex (26) reported that when the heart stage embryo-proper (with detached suspensor) was co-cultured with the early cotyledon stage embryo-proper, it grew much better than the embryos in the control treatment. Our results show that the ABA level in the embryo-proper at the heart stage (our stages A–B) ranges from 82 to 140 ng g^{-1} fresh weight, while at the early cotyledon stage (C–D in our stages), ABA in the embryo-proper ranges from 476 to 501 ng g^{-1} fresh weight, suggesting that the higher ABA level found in the early cotyledon stage might be responsible for stimulating growth in their experiment (26) in the heart stage embryo-proper. Yeung and Sussex (26) also report that in experiments testing the effect of different growth substances on embryo growth *in vitro*, ABA inhibited precocious germination of the embryo-proper but did not inhibit its growth expressed as increase in fresh weight. Observation on immature embryos of several species excised

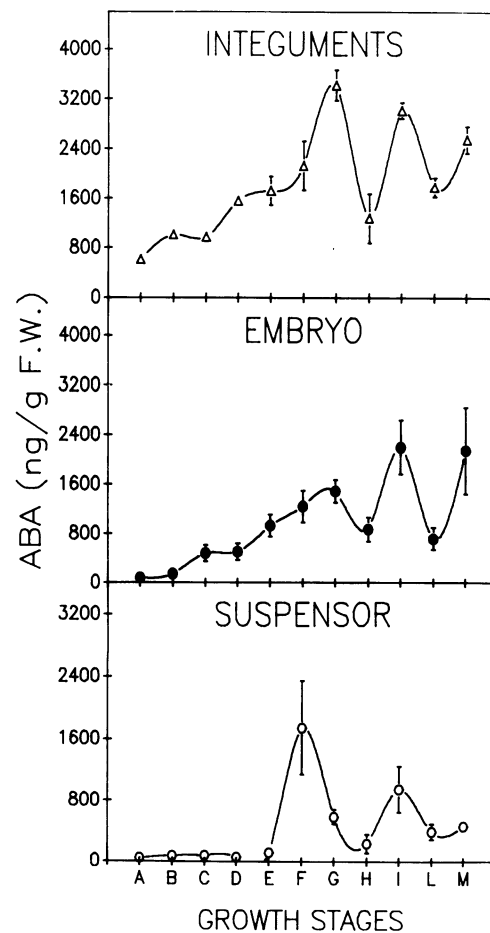


Figure 4. Pattern of endogenous ABA concentration in single tissues from *P. coccineus* seeds. Data \pm SE ($n = 5$); when not shown, SE bar was smaller than the symbol. F.W., fresh weight.

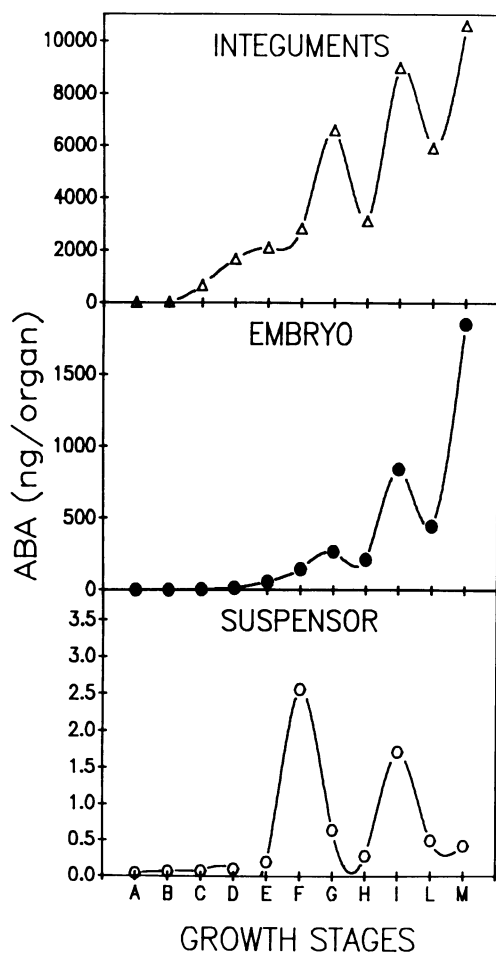


Figure 5. Total amount of ABA in integuments, embryos, and suspensors of *P. coccineus*. Data calculated on the basis of results shown in Figures 1 and 4. See these results for statistical treatment of data.

and cultured *in vitro* and on ABA-deficient mutants of various species clearly indicate that accumulation of ABA in the seed prevents precocious germination of the developing embryo (1, 12, 27).

The concentration of ABA in the integuments of *P. coccineus* seeds proved constantly higher than that in the embryo. Since it is difficult to hypothesize the same role for ABA in all seed tissues, this accumulation might indicate ABA involvement in integument phloem unloading (19, 20). Recent studies do not exclude that ABA levels in the embryos may be the result of “*in loco*” synthesis, but they also indicate that immature embryos accumulate ABA from seed coats by diffusion, a movement that seems to depend on a difference in pH between the two tissues during development (10).

It has been shown that ABA is involved in the regulation of storage protein mRNA during mid- and late embryogenesis in embryos of rapeseed (8, 9), soybean (4), and wheat (18, 25). The major storage protein families found in *P. coccineus* seed are the vicilins, legumins, and phytohemagglutinins or lectins (3). The same authors found (Bernardi R, Cecchini E, Mele T, Geri C, Durante M, unpublished data) the maximum

rate of total protein synthesis in cotyledons from 10 to 16-mm-long seeds corresponding to our growth stages F through I. They noted that the synthesis of various subunits of legumin and phytohemagglutinin can be identified from the early stages of seed maturation (6–8-mm-long seeds corresponding to our growth stages B–D); the synthesis of vicilin (the dominant storage protein in *P. coccineus*) starts late in seed growth (16–17-mm length corresponding to our growth stage I), but synthesis of its three major subunits does not occur at the same stage of development. A molecular approach could indicate whether ABA is the endogenous factor directly involved in the regulation of vicilin, legumin, and lectin gene expression in this species.

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